NIEHS Report on the In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol (CASRN 375-82-6) in Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats (Gavage Studies)

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In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

Foreword

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About This Report

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This report was modeled after the NTP Research Report on In Vivo Repeat Dose Biological Potency Study of Triphenyl Phosphate (CAS No. 115-86-6) in Male Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats (Gavage Studies) (https://doi.org/10.22427/NTP-RR-8), which was reviewed internally at the National Institute of Environmental Health Sciences and peer reviewed by external experts. Importantly, these reports employ mathematical model-based approaches to identify and report potency of dose-responsive effects and do not attempt more subjective interpretation (i.e., make calls or reach conclusions on hazard). The peer reviewers of the initial 5-day research report determined that the study design, analysis methods, and results presentation were appropriate. The study design, analysis methods, and results presentation employed for this study are identical to those previously reviewed, approved, and reported; therefore, following internal review, the NIEHS Report on the In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol (CASRN 375-82-6) in Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats (Gavage Studies) was not subjected to further external peer review.
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In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

Abstract

**Background:** 6:1 Fluorotelomer alcohol (6:1 FTOH) is a member of the per- and polyfluoroalkyl class of compounds to which humans are widely exposed. Toxicological information on this class of chemicals is sparse. A short-term, in vivo transcriptomic study was used to assess the biological potency of 6:1 FTOH.

**Methods:** A short-term in vivo biological potency study on 6:1 FTOH in adult male and female Sprague Dawley (Hsd:Sprague Dawley® SD®) rats was conducted. 6:1 FTOH was formulated in corn oil and administered once daily for 5 consecutive days by gavage (study days 0–4). 6:1 FTOH was administered at 10 doses (0, 0.15, 0.5, 1.4, 4, 12, 37, 111, 333, and 1,000 mg/kg body weight [mg/kg]). Blood was collected from animals dedicated for internal dose assessment in the 4 and 37 mg/kg groups. On study day 5, the day after the final dose was administered, animals were euthanized, standard toxicological measures were assessed, and the liver and kidney were assayed in gene expression studies using the TempO-Seq assay. Modeling was conducted to identify the benchmark doses (BMDs) associated with apical toxicological endpoints and transcriptional changes in the liver and kidney. A benchmark response of one standard deviation was used to model all endpoints.

**Results:** Several clinical pathology and organ weight measurements showed dose-related changes from which BMD values were calculated. In male rats, the effects included significantly decreased total thyroxine concentration, increased relative liver weight, increased albumin concentration, increased relative left kidney weight, increased aspartate aminotransferase activity, increased absolute liver weight, increased alanine aminotransferase activity, increased alkaline phosphatase activity, and increased creatinine concentration. The BMDs and benchmark dose lower confidence limits (BMDLs) were 3.19 (1.774), 12.122 (9.527), 13.365 (4.084), 20.907 (4.272), 28.117 (19.352), 28.507 (15.286), 36.116 (21.468), 89.383 (74.114), and 97.38 (32.365) mg/kg, respectively. In female rats, the effects included significantly decreased reticulocyte count, increased large unstained cell count, decreased total triiodothyronine concentration, increased monocyte count, increased thyroid stimulating hormone concentration, and increased aspartate aminotransferase activity. The BMDs (BMDLs) were 15.578 (3.622), 54.339 (15.759), 161.48 (122.215), 257.111 (160.613), 356.61 (268.917), and 497.046 (340.458) mg/kg, respectively. Average 6:1 FTOH plasma concentrations at 2 hours postdose were lower in female rats than in male rats. At 24 hours postdose, the concentration fell below the limit of detection of the analytical method in both male and female rats, suggesting short plasma half-lives of 6:1 FTOH in rats.

In the liver of male and female rats, no Gene Ontology biological process or individual genes had BMD median values below the lower limit of extrapolation (<0.050 mg/kg). The most sensitive gene sets in male rats for which a reliable estimate of the BMD could be made were cellular response to epidermal growth factor stimulus and response to epidermal growth factor with median BMDs of 0.368 and 0.690 mg/kg and median BMDLs of 0.103 and 0.456 mg/kg, respectively. The most sensitive gene sets in female rats for which a reliable estimate of the BMD could be made were positive regulation of phagocytosis and regulation of phagocytosis with median BMDs of 44.730 and 48.555 mg/kg and median BMDLs of 22.260 and 27.154 mg/kg, respectively. The most sensitive upregulated genes in male rats with reliable BMD estimates included *Acot2*, *Eci1*, *Loc100911558/Spink1l*, *Spink1*, *Ehhadh*, *Crot*, *Acaa1a*, and *Acaa1b* with BMDs (BMDLs) of 1.012 (0.809), 1.013 (0.769), 1.270 (0.542), 1.270 (0.542),
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1.280 (1.047), 1.411 (1.092), 1.874 (1.524), and 1.874 (1.524) mg/kg, respectively. The most sensitive downregulated genes in male rats with reliable BMD estimates were Myc and Zfp36 with BMDs (BMDLs) of 0.186 (0.103) and 0.368 (0.097) mg/kg, respectively. In female rats, the top 10 most sensitive individual genes were upregulated. These genes were Gdf15, Igfbp1, Eci1, Etfdh, Cyp2b1, Loc108348266/Cyp2b1, Dhrs7, Dhrs7l1, Slc27a2, and Vnn1 with BMDs (BMDLs) of 17.724 (8.696), 18.792 (7.230), 32.546 (27.162), 34.846 (26.297), 35.483 (29.479), 35.483 (29.479), 35.986 (10.630), 35.986 (10.630), 36.103 (26.571), and 37.026 (30.688) mg/kg, respectively.

In the kidney of male rats, two Gene Ontology biological processes had BMD median values <0.050 mg/kg, which relate to astrocyte activation and negative regulation of response to biotic stimulus. The most sensitive gene sets in male rats for which a reliable estimate of the BMD could be made were acetyl-CoA metabolic process and acyl-CoA metabolic process with median BMDs of 1.346 and 1.928 mg/kg and median BMDLs of 0.541 and 1.305 mg/kg, respectively. No gene sets in the kidney of female rats had estimated BMD median values <0.050 mg/kg. The most sensitive gene sets in female rats for which a reliable estimate of the BMD could be made were fatty acid beta-oxidation and fatty acid oxidation with median BMDs of 21.079 and 27.058 mg/kg and median BMDLs of 13.312 and 13.877 mg/kg, respectively. No individual kidney genes in male rats had median BMD values <0.050 mg/kg. The most sensitive upregulated genes in male rats with reliable BMD estimates included Decr1, Vnn1, Hmgcs2, Ehhadh, Eci2, Acaa2, Acot1, Cyp4a1, and Ech1 with BMDs (BMDLs) of 0.680 (0.505), 0.705 (0.488), 0.804 (0.541), 0.953 (0.671), 0.989 (0.643), 1.346 (0.539), 1.363 (0.938), 1.593 (1.021), and 2.055 (1.124) mg/kg, respectively. One gene, Acmsd, was downregulated with a BMD (BMDL) of 0.775 (0.183) mg/kg. In female rats, the top 10 most sensitive individual genes were upregulated. One individual gene, Plod3, had a BMD value <0.050 mg/kg. The next most sensitive upregulated genes with reliable BMD estimates included Eci1, Vnn1, Hmgcs2, Ehhadh, Eci2, Acaa1a, Acaa1b, Ech1, and Acaa2 with BMDs (BMDLs) of 9.486 (7.353), 10.025 (7.993), 11.644 (9.266), 12.212 (9.437), 12.789 (9.488), 13.850 (11.009), 13.850 (11.009), 19.820 (14.141), and 22.339 (13.614) mg/kg, respectively.

Summary: Taken together, in male rats, the most sensitive gene set BMD (BMDL) median, individual gene BMD (BMDL), and apical endpoint BMD (BMDL) values that could be reliably determined occurred at 0.368 (0.103), 0.186 (0.103), and 3.19 (1.774) mg/kg, respectively. The BMDs (BMDLs) could not be determined for two gene sets and were estimated to be <0.050 mg/kg. In female rats, the most sensitive gene set BMD (BMDL) median, individual gene BMD (BMDL), and apical endpoint BMD (BMDL) values that could be reliably determined occurred at 21.079 (13.312), 9.486 (7.353), and 15.578 (3.622) mg/kg, respectively. The BMD (BMDL) could not be determined for one individual gene and was estimated to be <0.050 mg/kg. Future studies investigating lower doses would be helpful to obtain more accurate estimates of BMD values for the most sensitive gene sets.
Background

6:1 Fluorotelomer alcohol (6:1 FTOH) (CASRN: 375-82-6, U.S. Environmental Protection Agency [EPA] Chemical Dashboard: DTXSID00190950,1 PubChem CID: 550386,2 European Committee Number: 206-796-83) is a member of the per- and polyfluoroalkyl class of compounds that are associated with numerous toxicological effects.4 There is widespread human exposure to this class of compounds.5 6 The predicted upper 95th percentile human exposure to 6:1 FTOH is 0.0000806 mg/kg body weight/day.7 A review of the existing literature failed to identify any in vivo toxicological information on 6:1 FTOH, and according to the EPA Chemical Dashboard, no quantitative risk assessment values or quantitative hazard values exist for this test article.8 Publicly available information on 6:1 FTOH can be found in PubChem2 and the EPA Chemical Dashboard.1

Recent studies have demonstrated that short-term in vivo gavage studies coupled with transcriptomics on select target organs can be used to estimate a biological potency that provides a reasonable approximation of toxicological potency in long-term guideline toxicological assessments.9 To estimate biological potency and gain insight into the nature of biological changes elicited by 6:1 FTOH, the National Institute of Environmental Health Sciences performed a short-term in vivo biological potency study of male and female Sprague Dawley (Hsd:Sprague Dawley® SD®) rats. The results of this study are presented in this report.
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Materials and Methods

Study Design

Male and female Sprague Dawley (Hsd:Sprague Dawley® SD®) rats were obtained from Envigo (Haslett, MI). On receipt, the rats were 6–7 weeks of age. Animals were quarantined for a minimum of 10 days and then randomly assigned to 1 of 10 dose groups. The rats in each dose group were then administered 6:1 fluorotelomer alcohol (6:1 FTOH) in corn oil by gavage for 5 consecutive days (study days 0–4) at a dose level of 0, 0.15, 0.5, 1.4, 4, 12, 37, 111, 333, or 1,000 mg/kg body weight (mg/kg). There were 5 rats per sex in each dosed group and 10 per sex in the vehicle control group; an additional 3 rats per sex were added to the 4 and 37 mg/kg groups for internal dose assessment. Dosage volume was 5 mL/kg body weight and was based on each animal’s most recent body weight. Euthanasia, blood/serum collection, and tissue sample collection were completed on study day 5, the day following the final administration of the test article. Blood was also collected from animals dedicated for internal dose assessment at 2 and 24 hours following the last dose administered on study day 4. Animal identification numbers and FASTQ data file names for each animal are presented in Appendix B.

Dose Selection Rationale

Dose selection was informed by a median lethal dose (LD50) prediction from the OPEn structure-activity/property Relationship App (OPERA),10,11 which estimated 460 mg/kg/day with an uncertainty range of 230–918 mg/kg/day. Further, an estimated point of departure of 85 mg/kg/day with an uncertainty range of 0.6–637 mg/kg/day was provided by the U.S. Environmental Protection Agency (EPA).12 To be certain that a 5-day maximum tolerated dose was achieved, in addition to identifying a minimum biological effect level dose, a top dose of 1,000 mg/kg was chosen, and approximately half-log dose spacing of nine lower dose levels, including a vehicle control, was selected to carry out the study.

Chemistry

6:1 FTOH was obtained in one lot from Apollo Scientific, Ltd. (Stockport, UK; lot AS489852). The identity and purity (>99%) of the chemical were confirmed by gas chromatography-mass spectrometry (GC/MS). Bulk chemical was stored refrigerated under inert headspace. Using the same GC/MS system and authentic standards, perfluorooctanoic acid was not detected, whereas a small percentage (approximately 0.002%) of perfluorooctanesulfonic acid was identified in lot AS489852.

Dose formulations were prepared in corn oil at 0 (vehicle control), 0.03, 0.10, 0.28, 0.80, 2.4, 7.4, 22.2, 66.6, and 200 mg/mL. The preadministration concentration of test article in the vehicle was analyzed using a qualified GC/MS method. The 0.28, 7.4, 22.2, and 66.6 mg/mL concentrations were 22.5%, 10.3%, 11.6%, and 10.2% below their target concentrations, respectively. All other formulations were within 10% of the target concentration. Formulation stability was confirmed in a 0.03 mg/mL formulation for up to 22 days at refrigerated (5°C) and ambient temperatures while protected from light. All chemistry activities were conducted by MRIGlobal (Kansas City, MO).
Clinical Examinations and Sample Collection

Clinical Observations

All rats were observed twice daily for signs of mortality or moribundity. Formal (out of cage) clinical observations were performed daily.

Body and Organ Weights

Animals were weighed during quarantine for randomization on the first day of dosing (study day 0) and on the day of necropsy (study day 5). A gross necropsy was performed on all rats that died spontaneously or were humanely euthanized due to moribund condition. During necropsy for all animals, the heart, liver, and kidneys were removed, and organ weights were recorded; bilateral organs were weighed separately.

Clinical Pathology

Animals were euthanized in random order by CO₂/O₂ (70%/30%) anesthesia 1 day after the final day of dosing. Blood samples were collected from each sex within a 1-hour window and were taken via vena cava or aorta. Blood was collected into tubes containing K₃ EDTA (tripotassium ethylenediaminetetraacetic acid) for hematology analysis and into tubes void of anticoagulant for serum chemistry and thyroid hormone measurements. The following hematology parameters were measured on an Advia® 120 Hematology Analyzer (Siemens Medical Solutions USA, Inc., Malvern, PA): erythrocyte count, hemoglobin, hematocrit, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, white blood cell count and differential, reticulocyte count, platelet count, and nucleated erythrocyte count. Manual hematocrit was determined using a microcentrifuge and capillary reader. Blood smears were prepared, and qualitative evaluation of cellular morphology was performed per study protocol. The following clinical chemistry parameters were measured on a Roche cobas® c501 Chemistry Analyzer (Roche Diagnostics, Indianapolis, IN): alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), total bile acids, total bilirubin, direct bilirubin, cholesterol, creatine kinase, creatinine, glucose, sorbitol dehydrogenase (SDH), total protein, triglycerides, and urea nitrogen. Globulin, albumin/globulin (A/G) ratio, and indirect bilirubin were calculated based on direct measurements (e.g., indirect bilirubin = total bilirubin − direct bilirubin). Serum concentrations for thyroid stimulating hormone (TSH) and free thyroxine (fT4) were determined by immunoassay using commercially available immunoassay kits from EMD Millipore Corporation (Billerica, MA) for TSH and Biomatik Corporation (Kitchener, Ontario, Canada) for fT4. Serum concentrations of total thyroxine (total T4) and total triiodothyronine (total T3) were determined using a validated method as described elsewhere. Individual animal and summary clinical chemistry, hematology, and hormonal data are available in Appendix F.

Internal Dose Assessment

A screening level assessment of the internal dose was performed to determine whether the test chemical had bioaccumulative properties (i.e., if the half-life was >24 hours). Blood was collected from animals dedicated for internal dose assessment in the 4 and 37 mg/kg groups at 2 and 24 hours following the last dose administered on study day 4. At 2 hours postdose, blood was collected from the jugular vein of unanesthetized animals. At 24 hours postdose (study
day 5), blood was collected from all study animals and dedicated internal dose assessment animals from the vena cava or abdominal aorta while animals were anesthetized with CO₂/O₂ (70%/30%). Blood was collected into tubes containing K₃ EDTA and kept on wet ice until plasma isolation, within 2 hours of collection. Samples were stored frozen (−85°C to −60°C) until analysis as described in Appendix A.

Transcriptomics

Sample Collection for Transcriptomics

Within 5 minutes of euthanasia, samples from the left liver lobe and right kidney were collected from all study animals for transcriptomics analysis. Half of the left liver lobe and half of the right kidney were processed for RNA isolation. Approximately 250 mg of each tissue was cut into small pieces (approximately 5 mm³) and placed into cryotubes containing RNAlater™. The tissue samples were stored at 2°C to 8°C overnight. The RNAlater™ was then removed and the samples were stored in a −85°C to −60°C freezer until processed for RNA isolation.

RNA Isolation, Library Creation, and Sequencing

RNA isolation was performed on tissue samples preserved in RNAlater™. Tissues were homogenized in QIAzol buffer (Qiagen Inc., Valencia, CA) using the TissueLyser II bead-beating system followed by RNA extraction using the Rneasy 96 QIAcube HT kits (Cat# 74171, Qiagen Inc., Valencia, CA) with a DNA digestion step. The concentration and purity of all isolated samples were determined from absorbency readings taken at 260 and 280 nm using a NanoDrop ND-8000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The readings accurately determined the concentration of each sample while ensuring that an acceptable purity (A₂₆₀/A₂₈₀ ratio) between 1.80 and 2.20 was achieved. After quantification, RNA was stored at −70°C ± 10°C until further processing.

One microliter of each RNA sample (500–660 ng/µL) was hybridized with the S1500+ beta detector oligo pool mix (2 µL per sample) using the following thermocycler settings: 10 minutes at 70°C, followed by a gradual decrease to 45°C over 49 minutes, and ending with a 45°C hold for 1 minute. Hybridization was followed by nuclease digestion (24 µL nuclease mix addition followed by 90 minutes at 37°C), ligation (24 µL ligation mix addition followed by 60 minutes at 37°C), and heat denaturation (at 80°C for 15 minutes). Ten microliters of each ligation product were then transferred to a 96-well polymerase chain reaction (PCR) amplification microplate with 10 µL of PCR mix per well. Through 25 cycles of amplification, well-specific “barcoded” primer pairs were introduced to templates. Five microliters of the PCR amplification products from each well were then pooled into a single sequencing library. The TempO-Seq library was then processed with a PCR clean-up kit (Machery-Nagel, Mountain View, CA) prior to sequencing. Sequencing was performed using a 50-cycle single-end read flow cell on a HiSeq 2500 Sequencing System (Illumina, San Diego, CA). Processing of sequencing data was conducted using Illumina’s BCL2FASTQ software employing default parameter settings.

Sequence Data Processing

FASTQ files of TempO-Seq reads were aligned to the probe sequences from the target platform using Bowtie version 1.2.2⁴ with the following parameters: -v 3 -k 1 -m 1 --best --strata. This configuration allows up to three mismatches and reports the single best alignment. After
alignment, the total sequenced reads, the percentage of reads aligning to the platform manifest, the alignment rate, and the percentage of expressed probes (≥5 reads per probe) were calculated for each sample.

Sequencing Quality Checks and Outlier Removal

Samples were flagged for values below the following thresholds: sequencing depth <300 K, total alignment rate <40%, unique alignment rate <30%, number of aligned reads <300 K, or percentage of probes with at least five reads <50%. Filtering on the percentage of expressed probes eliminates biased samples for which the sequenced reads only reflect a small portion of the measured transcriptome. In addition, FastQC was run on all samples to ensure adequate per base quality and per base N content, where N represents bases that could not be identified. This procedure resulted in one kidney 6:1 FTOH sample on one plate being flagged and removed (unique alignment rate of 1.86%).

Principal component (PCA), hierarchical cluster, and inter-replicate correlation analyses were performed. These analyses highlighted three additional outlier liver samples, which were removed before downstream analysis.

The processing of samples from the study of 6:1 FTOH was done in parallel with three other chemicals that were studied under a similar protocol, therefore allowing for a more powerful collective assessment of the data. Specifically, the samples from all four studies were distributed over twelve 96-well plates (i.e., one plate per chemical per tissue and four additional plates with overflow samples for three of the chemicals, with nine doses plus vehicle control). For kidney samples, average read depth per chemical varied across plates. Kidney samples on one of the overflow plates also clustered separately (in the PCA and hierarchical cluster analysis) from the other kidney samples for a given chemical. Therefore, kidney samples on that overflow plate were removed, resulting in one plate of data per chemical for the downstream analysis of kidney samples. The exclusion of these data had limited impact on the analysis as the samples from each dose group were randomly sorted into the overflow plates. The final sample counts that were used for benchmark dose (BMD) analysis of the transcriptomics data are shown in Table 1.

Table 1. Final Sample Counts for Benchmark Dose Analysis of the Transcriptomics Data

<table>
<thead>
<tr>
<th></th>
<th>0 mg/kg</th>
<th>0.15 mg/kg</th>
<th>0.5 mg/kg</th>
<th>1.4 mg/kg</th>
<th>4 mg/kg</th>
<th>12 mg/kg</th>
<th>37 mg/kg</th>
<th>111 mg/kg</th>
<th>333 mg/kg</th>
<th>1,000 mg/kg</th>
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<tbody>
<tr>
<td><strong>Male</strong></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Kidney</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>5</td>
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<tr>
<td>Kidney</td>
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<td>5</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Data Normalization

The aligned read counts for attenuated probes were properly readjusted to calculate unattenuated equivalent counts using the attenuation factors provided in the platform manifest. To account for between-sample sequencing depth variation, unattenuated read counts were normalized at the probe level by applying reads per million normalization. A pseudo-read-count of 1.0 was added
to each normalized expression value, and then the values were log2 transformed to complete the normalization. Principal component-based visualizations of the final expression data set used from modeling are available in Appendix C.

Data Analysis

Statistical Analysis of Body Weights, Organ Weights, and Clinical Pathology

Two approaches were employed to assess the significance of pairwise comparisons between dosed and vehicle control groups in the analysis of continuous variables. Organ and body weight data, which have approximately normal distributions, were analyzed using the parametric multiple comparison procedures of Williams15,16 and Dunnett.17 Clinical pathology data, which typically have skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley18 and Dunn.19 The Jonckheere test20 was used to assess the significance of dose-response trends and to determine whether a trend-sensitive test (Williams or Shirley test) was more appropriate for pairwise comparisons than a test that assumes no monotonic dose response (Dunnett or Dunn test). Trend-sensitive tests were used when the Jonckheere test was significant at p ≤ 0.01.

Prior to analysis, values identified by the outlier test of Dixon and Massey21 were examined by National Institute of Environmental Health Sciences (NIEHS) staff. Values from animals suspected of illness due to causes other than experimental exposure and values that the laboratory indicated as inadequate due to measurement problems were eliminated from the analysis.

A no-observed-effect level (NOEL) was identified as the highest dose not showing a significant (p ≤ 0.05) pairwise difference relative to the vehicle control group. A lowest-observed-effect level (LOEL) was identified as the lowest dose demonstrating a significant (p ≤ 0.05) pairwise difference relative to the vehicle control group. Throughout the results section for apical endpoints, interpretation of BMDs is made in relationship to NOEL and LOEL values for specific endpoints, as defined here, and are not meant to reflect an overall study NOEL or LOEL.

Benchmark Dose Analysis of Body Weights, Organ Weights, and Clinical Pathology

Clinical pathology, body weight, and organ weight endpoints that exhibited a significant trend and pairwise test were submitted in batch for automated BMD modeling analysis. For body weight, the BMD and benchmark dose lower confidence limit (BMDL) were presented as not determined when there were no significant results. BMD modeling and analysis was conducted using a modification of Benchmark Dose Modeling Software (BMDS) version 2.7.0. Data sets were executed using the Python BMDS interface (https://pypi.python.org/pypi/bmds; version 0.11), which allows for batch processing of multiple data sets. Data for all endpoints submitted were continuous. A default benchmark response (BMR) of one standard deviation (relative to control) was used for all data sets. The following BMDS 2.7.0 models were used to model the means of the data sets:

- Linear
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- Polynomial $2^\circ, 3^\circ, 4^\circ, 5^\circ, 6^\circ, 7^\circ, 8^\circ$
- Power
- Hill
- Exponential M2, M3, M4, M5

Multiple versions of the polynomial model were executed, from a polynomial of degree 2 to a polynomial of degree equal to the number of dose groups minus 1 (e.g., if a data set had five dose groups, a $2^\circ, 3^\circ$, and $4^\circ$ polynomial model would be executed). Models were initialized using BMDS 2.7.0 model defaults, including restricting the power parameter of the power model and n-parameter of the Hill model to $>1$ and the beta parameters of the polynomial model to positive or negative, depending on the mean response direction of the data set. For all models, either a constant or nonconstant variance model was selected as outlined in the EPA BMD technical guidance and was implemented in the BMDS 2.7.0 software.

After model execution, BMDs were selected using the model recommendation procedures generally described in the EPA BMD technical guidance and the automated decision logic described in Wignall et al. and summarized in Appendix D, Table D-1. Models were placed into one of four possible bins, depending on the results and the bin recommendation logic:

1. **Failure**: model did not successfully complete
2. **Nonviable model (NVM)**: model successfully completed but failed acceptability criteria
3. **Not reportable (NR)**: model is identified and meets all acceptability criteria with the exception of the estimated BMD being below the lower limit of extrapolation ($<1/3$ the lowest nonzero dose tested); BMD reported as $<1/3$ the lowest nonzero dose tested and BMDL is not reportable
4. **Viable model**: candidate for recommended model without warning

If only one model was in the viable model bin, it was selected as the best-fitting model. If the viable bin had more than one model, consistent with EPA guidance, either the model with the lowest Akaike information criterion (AIC) or lowest BMDL was selected. If the range of BMDL values was sufficiently close (less than threefold difference), the AIC value was used; otherwise, the BMDL value was used. If no model was recommended, no BMD was presented in the results.

Details on the analysis criteria and decision tree are provided in Table D-1 and Figure D-1, respectively. To avoid effects of model extrapolation, BMD values derived from viable models that were threefold lower than the lowest nonzero dose tested were reported as $<1/3$ the lowest nonzero dose tested, and corresponding BMDL values were not reported. Finally, all modeling results from apical data yielding a BMD were reviewed by a subject matter expert to determine the validity of the modeling results and potency estimates.

**Benchmark Dose Analysis of Transcriptomics Data**

The BMD analysis of the transcriptomic data was performed in accordance with the National Toxicology Program (NTP) best practices for genomic dose-response modeling as reviewed by an independent panel of experts in October 2017. These recommendations are described in the 2018 publication, *National Toxicology Program Approach to Genomic Dose Response Modeling.*

7
Dose-response analyses of normalized gene expression data were performed using BMDEexpress 2.30.0507 BETA (https://github.com/auerbachs/BMDEexpress-2/releases). A trend test (the Williams trend test\textsuperscript{15, 16} p ≤ 0.05, 10,000 permutations) and fold change filter (1.5-fold change up or down relative to the vehicle control group for probe sets) were applied to the data set to remove probe sets demonstrating no response to chemical exposure from subsequent analysis. These filter criteria were empirically determined with the goal of balancing false discovery with reproducibility. The criteria are consistent with the MicroArray Quality Control recommendations to combine the nominal p value threshold with a fold change filter to maximize replicability of transcriptomic findings across labs.\textsuperscript{25} The following dose-response models were fit to the probe sets that passed the trend test and fold change filter:

- Hill
- Power
- Linear
- Polynomial 2°
- Exponential M2, M3, M4, M5

All gene expression data analyzed in BMDEexpress were log2 transformed, and thus nearly all probes (also known as detection oligos or DO) were assumed to exhibit constant variance across the doses. For this reason and for efficiency purposes, each model was run assuming constant variance. Lacking any broadly applicable guidance regarding the level of change in gene expression considered biologically significant, a BMR of one standard deviation (relative to the fit at control) was used in this study. This approach enables standardization of the BMR between apical endpoints and transcriptomic endpoints and provides a standard for use across multiple chemicals tested in this rapid screening paradigm. The expression direction (upregulated or downregulated) for each probe was determined by a trend test intrinsic to the model executables (provided by EPA) contained in BMDEexpress.

To identify the best-fit model for each fitted probe, the AIC values for each fitted model were compared and the model with the lowest AIC was selected. The best model for each probe was used to calculate the BMD, BMD\textsubscript{L}, and BMD upper confidence limit (BMD\textsubscript{U}). The specific parameter settings, selected from the BMDEexpress software when performing probe-level BMD analysis, were as follows: maximum iterations – 250, confidence level – 0.95, BMR factor – 1 (the multiplier of the standard deviation that defined the BMD), restrict power – no restriction, and constant variance – selected. The specific model selection setting in the BMDEexpress software when performing probe set-level BMD analysis was as follows: best poly model test – lowest AIC, flag Hill model with “k” parameters – <1/3 the lowest nonzero dose tested, and best model selection with flagged Hill model – include flagged Hill model. The inclusion of the flagged models is a deviation from EPA BMD analysis guidance.\textsuperscript{22} The justification for this deviation relates to subsequent use of the data in which the probe BMD values are grouped into gene sets from which a median BMD is derived. If the probes were removed from the analysis or forced to another model, the probe might not be counted in the gene set analysis and could lead to loss of “active” gene sets. Importantly, most of the probes that produce flagged Hill models show highly potent responses and should therefore be counted in the analysis.

To perform Gene Ontology (GO; annotation accession date: 07/15/2020) gene set analysis, only GO terms with ≥10 and ≤250 annotated genes measured on the gene expression platform were
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considered. Before sorting genes into the GO terms, the best-fit model for each probe was subjected to a filtering process to remove those probes (1) with a BMD greater than the highest dose tested, (2) that mapped to more than one gene, (3) that had a global goodness-of-fit p value ≤0.1, and (4) with a BMDU/BMDL ratio >40. GO terms that were at least 5% populated and contained three genes that passed the criteria mentioned above were considered “active” (i.e., responsive to chemical exposure). For this report, GO terms populated with identical sets of differentially expressed genes were filtered to limit redundancy in reporting based on the following selection criteria: (1) highest percentage populated and (2) most specific/highest GO level. Redundant GO terms failing to differentiate on the basis of these criteria were retained and reported. A complete list of “active” GO terms can be found in Appendix F. To avoid effects of model extrapolation, GO terms exhibiting BMD values below the lower limit of extrapolation (<1/3 the lowest nonzero dose tested) were reported as <1/3 the lowest nonzero dose tested and corresponding BMDL and BMDU values were not reported.

To perform Individual Gene Analysis, the best-fit model for each probe was subjected to a filtering process to remove those probes (1) with a BMD greater than the highest dose tested, (2) that mapped to more than one gene, (3) that had a global goodness-of-fit p value ≤0.1, or (4) with a BMDU/BMDL ratio >40. For genes that had more than one probe represented on the platform and passed this filtering process, a median BMD was used to estimate the BMD, BMDL, and BMDU values. To ensure only genes with a robust response were assessed for potency, genes with probes that had a median fold change <|2| were removed prior to reporting. A complete list of genes and their corresponding metrics can be found in Appendix F. To avoid effects of model extrapolation, genes exhibiting BMD values below the lower limit of extrapolation (<1/3 the lowest nonzero dose tested) were reported as <1/3 the lowest nonzero dose tested and corresponding BMDL and BMDU values were not reported.

A summary of the BMDExpress gene expression analysis pipeline used in this study is shown in Figure D-2.

Empirical False Discovery Rate Determination for Genomic Dose-response Modeling

The genomic dose-response analysis pipeline is a complex multistep process with multiple modeling steps and parameter variables. Because of this complexity, traditional statistical models for determining false discovery rates for the genes and pathways are not straightforward to apply. To overcome this issue, an empirical false discovery rate was determined on the basis of the totality of the analysis pipeline. This was done through the evaluation of synthetic null data sets derived from vehicle control data from four short-term repeat dose toxicogenomic studies including 6:1 FTOH (each with 10 vehicle control samples). The other toxicogenomic studies, which are reported in separate NIEHS reports, are of perfluorohexanesulfonamide,26 1,1,2,2-tetrahydroperfluoro-1-dodecanol,27 and 2,3-benzofluorene.28 Samples from all four studies were processed as a group and subjected to sequencing at the same time and were visually inspected to ensure there was no batch effect between the different studies.

To create synthetic null data for a given group (tissue per sex combination), up to 40 vehicle control samples from the original studies (10 replicates × 4 chemicals) were used to generate the data sets, with outliers excluded from the analysis. Each computationally generated sample was created by mixing two randomly selected vehicle control samples via a weighted average.
approach through which weights were obtained from random uniform (0,1) distribution. A total of 55 samples (10 vehicle control samples + 45 dosed samples [9 doses × 5 replicates]) were computationally generated per data set and assigned doses spaced by approximately half-log. A total of 20 data sets were generated per group (i.e., 20 data sets each for female kidney, male kidney, female liver, and male liver) and analyzed using both the individual gene-level and GO biological process (gene set) analysis pipeline employed to analyze the data from each study. The median empirical false discovery rates across the 20 null sets in each group for gene-level analysis across each group were 0.037%, 0.037%, 0%, and 0% (female kidney, male kidney, female liver, and male liver, respectively). The median empirical false discovery rate for each of the 20 null data sets in each group using the GO biological process (gene set) level analysis was 0%. Details of the empirical false discovery rate analysis are available in Appendix C. The associated bm2 analysis file that is the basis of the empirical false discovery rate can be found in Appendix F.

Data Accessibility

Primary and analyzed data used in this study are available to the public at https://doi.org/10.22427/NIEHS-DATA-NIEHS-07.29
Results

Animal Condition, Body Weights, and Organ Weights

Male and female rats administered 333 or 1,000 mg/kg body weight (mg/kg) of 6:1 fluorotelomer alcohol (6:1 FTOH) began exhibiting signs of overt toxicity on study days 1–2, which included red discharge from eyes, slow breathing, ruffled or unkempt coat, cold to touch, soft feces, hunched posture, prone positioning, wet urogenital area, and lethargy (Appendix F). The 1,000 mg/kg male rats exhibited high mortality, with three rats found dead on study day 4 and one male rat moribund on study day 1 and euthanized at that time due to severe toxicity. No significant changes in terminal body weight for male or female rats occurred with exposure to 6:1 FTOH (Table 2).

In male rats at study termination, a significant increase in absolute and relative liver weights occurred in dose groups ≥37 mg/kg; both endpoints had positive trends (Table 3). The benchmark doses (benchmark dose lower confidence limits)—BMDs (BMDLs)—for increased absolute and relative liver weights were 28.507 (15.286) and 12.122 (9.527) mg/kg, respectively. Relative left kidney weight showed a significant pairwise increase at 333 mg/kg with a positive trend; the BMD (BMDL) was 20.907 (4.272) mg/kg. Relative right kidney weight had significant trend and pairwise comparisons. Although a BMD was estimated for relative right kidney weight, its value was much lower (approximately 25- to 80-fold) than would be expected given the endpoint-specific no-observed-effect level (NOEL) and lowest-observed-effect level (LOEL) values, suggesting that the BMD estimate did not accurately reflect the true potency of the effect and was likely an anomalous product of the BMD modeling approach. The BMDs for all organ weights were reviewed by a subject matter expert for anomalous modeling results (i.e., when the traditional statistics are notably different from the estimated BMD values). Significant trend and pairwise comparisons were not observed in absolute left or right kidney weights or absolute or relative heart weights (Appendix F).

In female rats at study termination, a significant increase in absolute and relative liver weights occurred in the 333 and 1,000 mg/kg groups and both endpoints had positive trends (Table 3); BMDs (BMDLs) for increased absolute and relative liver weights were not determined because no viable models were available. Significant trend and pairwise comparisons were not observed in absolute or relative heart, right kidney, or left kidney weights (Appendix F).
### Table 2. Summary of Body Weights of Male and Female Rats Administered 6:1 Fluorotelomer Alcohol for Five Days

<table>
<thead>
<tr>
<th>Study Day</th>
<th>0 mg/kg</th>
<th>0.15 mg/kg</th>
<th>0.5 mg/kg</th>
<th>1.4 mg/kg</th>
<th>4 mg/kg</th>
<th>12 mg/kg</th>
<th>37 mg/kg</th>
<th>111 mg/kg</th>
<th>333 mg/kg</th>
<th>1,000 mg/kg</th>
<th>BMD_{1Std} (mg/kg)</th>
<th>BMD_{L1Std} (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>5</td>
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<td>5</td>
<td>5</td>
<td>5</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>0</td>
<td>299.9 ± 6.3</td>
<td>307.1 ± 5.6</td>
<td>300.7 ± 4.3</td>
<td>307.3 ± 8.3</td>
<td>295.0 ± 10.2</td>
<td>304.3 ± 2.6</td>
<td>300.7 ± 11.9</td>
<td>303.8 ± 9.1</td>
<td>306.4 ± 3.4</td>
<td>298.7 ± 7.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>315.0 ± 6.8</td>
<td>320.7 ± 5.3</td>
<td>310.7 ± 5.1</td>
<td>324.0 ± 10.6</td>
<td>310.8 ± 9.7</td>
<td>317.0 ± 2.4</td>
<td>316.2 ± 12.6</td>
<td>319.4 ± 10.3</td>
<td>304.4 ± 4.9</td>
<td>295.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0</td>
<td>213.2 ± 6.4</td>
<td>216.5 ± 4.8</td>
<td>213.1 ± 6.6</td>
<td>215.7 ± 4.6</td>
<td>209.1 ± 6.4</td>
<td>209.8 ± 2.8</td>
<td>218.0 ± 5.3</td>
<td>211.5 ± 4.1</td>
<td>210.5 ± 4.8</td>
<td>215.1 ± 2.8</td>
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<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>218.8 ± 7.0</td>
<td>224.9 ± 8.6</td>
<td>218.9 ± 6.0</td>
<td>219.1 ± 6.6</td>
<td>213.9 ± 6.1</td>
<td>219.3 ± 2.9</td>
<td>222.0 ± 5.5</td>
<td>216.2 ± 3.9</td>
<td>219.7 ± 5.7</td>
<td>222.8 ± 3.0</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

BMD_{1Std} = benchmark dose corresponding to a benchmark response set to one standard deviation from the mean; BMD_{L1Std} = benchmark dose lower confidence limit corresponding to a benchmark response set to one standard deviation from the mean; NA = not applicable; ND = not determined.

<sup>a</sup>Data are displayed as mean ± standard error of the mean; body weight data are presented in grams.

<sup>b</sup>Statistical analysis performed by the Jonckheere (trend) and Williams or Dunnett (pairwise) tests.

<sup>c</sup>One male rat was moribund and euthanized on study day 1 and three male rats were found dead on study day 4. Body weight data from the remaining male rat were excluded from statistical analysis and BMD calculations.
In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

Table 3. Summary of Select Organ Weights of Male and Female Rats Administered 6:1 Fluorotelomer Alcohol for Five Days

<table>
<thead>
<tr>
<th>Endpoint&lt;sup&gt;a,b,c&lt;/sup&gt;</th>
<th>0 mg/kg</th>
<th>0.15 mg/kg</th>
<th>0.5 mg/kg</th>
<th>1.4 mg/kg</th>
<th>4 mg/kg</th>
<th>12 mg/kg</th>
<th>37 mg/kg</th>
<th>111 mg/kg</th>
<th>333 mg/kg</th>
<th>1,000 mg/kg</th>
<th>BMD&lt;sub&gt;L1Std&lt;/sub&gt; (mg/kg)</th>
<th>BMD&lt;sub&gt;L1Std&lt;/sub&gt; (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
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<td>5</td>
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<td>5</td>
<td>5</td>
<td>5</td>
<td>1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Terminal Body Wt. (g)</td>
<td>315.0 ± 6.8</td>
<td>320.7 ± 5.3</td>
<td>310.7 ± 5.1</td>
<td>324.0 ± 10.6</td>
<td>310.8 ± 9.7</td>
<td>317.0 ± 2.4</td>
<td>316.2 ± 12.6</td>
<td>319.4 ± 10.3</td>
<td>304.4 ± 4.9</td>
<td>295.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Right Kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Relative (mg/g)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.10 ± 0.04**</td>
<td>3.16 ± 0.04</td>
<td>3.16 ± 0.13</td>
<td>3.19 ± 0.12</td>
<td>3.43 ± 0.08</td>
<td>3.20 ± 0.06</td>
<td>3.50 ± 0.04**</td>
<td>3.41 ± 0.10**</td>
<td>3.50 ± 0.15**</td>
<td>3.45</td>
<td>0.464&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.082&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>Left Kidney</td>
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<tr>
<td>Relative (mg/g)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.10 ± 0.06**</td>
<td>3.21 ± 0.03</td>
<td>3.15 ± 0.08</td>
<td>3.11 ± 0.07</td>
<td>3.24 ± 0.07</td>
<td>3.20 ± 0.05</td>
<td>3.37 ± 0.05</td>
<td>3.19 ± 0.09</td>
<td>3.44 ± 0.14**</td>
<td>3.28</td>
<td>20.907</td>
<td>4.272</td>
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<tr>
<td>Liver</td>
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<tr>
<td>Absolute (g)</td>
<td>11.78 ± 0.39**</td>
<td>12.05 ± 0.23</td>
<td>11.83 ± 0.52</td>
<td>12.37 ± 0.65</td>
<td>12.29 ± 0.59</td>
<td>12.12 ± 0.34</td>
<td>13.47 ± 0.55**</td>
<td>16.63 ± 0.55**</td>
<td>18.04 ± 0.20**</td>
<td>17.87</td>
<td>28.507</td>
<td>15.286</td>
</tr>
<tr>
<td>Relative (mg/g)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>37.37 ± 0.80**</td>
<td>37.58 ± 0.63</td>
<td>38.03 ± 1.21</td>
<td>38.11 ± 1.05</td>
<td>39.50 ± 1.06</td>
<td>38.26 ± 1.06</td>
<td>42.60 ± 0.63**</td>
<td>52.12 ± 1.06**</td>
<td>59.30 ± 0.73**</td>
<td>60.41</td>
<td>12.122</td>
<td>9.527</td>
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<tr>
<td>Female</td>
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<td>NA</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Terminal Body Wt. (g)</td>
<td>218.8 ± 7.0</td>
<td>224.9 ± 8.6</td>
<td>218.9 ± 6.0</td>
<td>219.1 ± 6.6</td>
<td>213.9 ± 6.1</td>
<td>219.3 ± 2.9</td>
<td>222.0 ± 5.5</td>
<td>216.2 ± 3.9</td>
<td>219.7 ± 5.7</td>
<td>222.8 ± 3.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Liver</td>
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<tr>
<td>Absolute (g)</td>
<td>7.92 ± 0.41**</td>
<td>8.47 ± 0.61</td>
<td>8.21 ± 0.22</td>
<td>7.80 ± 0.38</td>
<td>7.87 ± 0.46</td>
<td>8.15 ± 0.21</td>
<td>8.16 ± 0.24</td>
<td>7.87 ± 0.13</td>
<td>9.22 ± 0.18**</td>
<td>12.02 ± 0.17**</td>
<td>NVM</td>
<td>NVM</td>
</tr>
<tr>
<td>Relative (mg/g)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>36.00 ± 0.72**</td>
<td>37.50 ± 1.29</td>
<td>37.54 ± 0.39</td>
<td>35.52 ± 0.64</td>
<td>36.67 ± 1.13</td>
<td>37.15 ± 0.72</td>
<td>36.76 ± 0.46</td>
<td>36.40 ± 0.34</td>
<td>42.01 ± 0.50**</td>
<td>53.98 ± 0.92**</td>
<td>NVM</td>
<td>NVM</td>
</tr>
</tbody>
</table>

Statistical significance for a dosed group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.

**Statistically significant at \( p \leq 0.01 \).

BMD<sub>L1Std</sub> = benchmark dose corresponding to a benchmark response set to one standard deviation from the mean; BMD<sub>L1Std</sub> = benchmark dose lower confidence limit corresponding to a benchmark response set to one standard deviation from the mean; NA = not applicable; ND = not determined; NVM = nonviable model.

<sup>a</sup>Descriptions of organ weight endpoints and changes are provided in Appendix E.

<sup>b</sup>Data are displayed as mean ± standard error of the mean.

<sup>c</sup>Statistical analysis performed by the Jonckheere (trend) and Williams or Dunnett (pairwise) tests.

<sup>d</sup>One male rat was moribund and euthanized on study day 1 and three male rats were found dead on study day 4. Body weight and organ weight data from the remaining male rat were excluded from statistical analysis and BMD calculations.

<sup>e</sup>Relative organ weights (organ weight-to-body weight ratios) are given as mg organ weight/g body weight.

<sup>f</sup>BMD values are much lower than would be expected given the lowest-observed-effect level and no-observed-effect level values, suggesting that the BMD estimates do not accurately reflect the true potency of the effect of these endpoints and are likely an anomalous product of the BMD modeling approach.
Clinical Pathology

In male rats, triglyceride and cholesterol concentrations were significantly decreased in dose groups ≥12 mg/kg and ≥37 mg/kg, respectively; BMDs (BMDLs) were not determined for these two endpoints because no viable models were available (Table 4). Creatinine concentration was significantly increased in the 333 mg/kg male rats with a BMD (BMDL) of 97.38 (32.365) mg/kg. Albumin concentration was significantly increased in the ≥37 mg/kg male rats with a BMD (BMDL) of 13.365 (4.084) mg/kg. Alanine aminotransferase activity was significantly increased in male rats in the ≥37 mg/kg groups and aspartate aminotransferase (AST) activity was significantly increased in the ≥111 mg/kg groups with BMDs (BMDLs) of 36.116 (21.468) and 28.117 (19.352) mg/kg, respectively. Alkaline phosphatase activity was significantly increased in the 333 mg/kg male rat group with a BMD (BMDL) of 89.383 (74.114) mg/kg. In female rats, AST activity was significantly increased in the 1,000 mg/kg group with a BMD (BMDL) of 497.046 (340.458) mg/kg. Globulin concentration in male rats, albumin/globulin (A/G) ratio in male rats, and cholesterol concentration in female rats had significant trend and pairwise comparisons. Although a BMD was estimated for each of these endpoints, these values were much lower (approximately 10- to 35-fold, 10- to 25-fold, and 70- to 210-fold for globulin concentration in male rats, A/G ratio in male rats, and cholesterol concentration in female rats, respectively) than would be expected given the endpoint-specific NOEL and LOEL values, suggesting that the BMD estimate did not accurately reflect the true potency of the effect and was likely an anomalous product of the BMD modeling approach. The BMDs for all clinical pathology endpoints were reviewed by a subject matter expert for anomalous modeling results (i.e., when the traditional statistics are notably different from the estimated BMD values).

The reticulocyte count was significantly decreased in the ≥37 mg/kg female groups with a BMD (BMDL) of 15.578 (3.622) mg/kg (Table 5). In addition, in female rats, the monocyte and large unstained cell counts were significantly increased in dose groups ≥111 mg/kg and ≥37 mg/kg with BMDs (BMDLs) of 257.111 (160.613) mg/kg and 54.339 (15.759) mg/kg, respectively. The reticulocyte count in male rats had significant trend and pairwise comparisons. Although a BMD was estimated, its value was much lower (approximately 120- to 360-fold) than would be expected given the endpoint-specific NOEL and LOEL values, suggesting that the BMD estimate did not accurately reflect the true potency of the effect and was likely an anomalous product of the BMD modeling approach.

In male rats, total thyroxine (total T4) concentration was significantly decreased in the ≥4 mg/kg groups with a BMD (BMDL) of 3.19 (1.774) mg/kg (Table 6). In addition, free thyroxine (fT4) concentration was significantly decreased in the 333 mg/kg male rats; a BMD (BMDL) was not determined because no viable model was available. In female rats, the total triiodothyronine (total T3) and total T4 concentrations were significantly decreased in the ≥333 mg/kg groups. The BMD (BMDL) for decreased total T3 was 161.48 (122.215) mg/kg. A BMD (BMDL) was not determined for decreased total T4 because no viable model was available. Additionally, thyroid stimulating hormone (TSH) concentration was significantly increased in female rats in the 1,000 mg/kg group with a BMD (BMDL) of 356.61 (268.917) mg/kg. Total T3 concentration in male rats had significant trend and pairwise comparisons. Although a BMD was estimated, its value was much lower (approximately 10- to 25-fold) than would be expected given the endpoint-specific NOEL and LOEL values, suggesting that the BMD estimate did not accurately
reflect the true potency of the effect and was likely an anomalous product of the BMD modeling approach.
Table 4. Summary of Select Clinical Chemistry Data for Male and Female Rats Administered 6:1 Fluorotelomer Alcohol for Five Days

<table>
<thead>
<tr>
<th>Endpointa,b</th>
<th>0 mg/kg</th>
<th>0.15 mg/kg</th>
<th>0.5 mg/kg</th>
<th>1.4 mg/kg</th>
<th>4 mg/kg</th>
<th>12 mg/kg</th>
<th>37 mg/kg</th>
<th>111 mg/kg</th>
<th>333 mg/kg</th>
<th>1,000 mg/kg</th>
<th>BMD1Std</th>
<th>BMDL1Std</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
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<td>5</td>
<td>14</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.41 ± 0.01**</td>
<td>0.42 ± 0.02</td>
<td>0.42 ± 0.02</td>
<td>0.42 ± 0.02</td>
<td>0.43 ± 0.03</td>
<td>0.38 ± 0.02</td>
<td>0.44 ± 0.02</td>
<td>0.46 ± 0.02</td>
<td>0.50 ± 0.03**</td>
<td>0.50</td>
<td>97.38</td>
<td>32.365</td>
</tr>
<tr>
<td>Globulin (g/dL)</td>
<td>1.95 ± 0.03**</td>
<td>1.96 ± 0.05</td>
<td>2.09 ± 0.06</td>
<td>2.28 ± 0.16</td>
<td>2.03 ± 0.09</td>
<td>1.70 ± 0.03*</td>
<td>1.76 ± 0.05*</td>
<td>1.40 ± 0.10**</td>
<td>1.34 ± 0.14**</td>
<td>1.20</td>
<td>0.322</td>
<td>0.188</td>
</tr>
<tr>
<td>A/G Ratio</td>
<td>2.29 ± 0.06**</td>
<td>2.28 ± 0.06</td>
<td>2.13 ± 0.04</td>
<td>2.03 ± 0.13</td>
<td>2.27 ± 0.08</td>
<td>2.69 ± 0.07*</td>
<td>2.71 ± 0.06*</td>
<td>3.50 ± 0.31**</td>
<td>3.75 ± 0.31**</td>
<td>3.92</td>
<td>0.456</td>
<td>0.237</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.46 ± 0.06**</td>
<td>4.46 ± 0.07</td>
<td>4.46 ± 0.08</td>
<td>4.54 ± 0.06</td>
<td>4.58 ± 0.05</td>
<td>4.56 ± 0.05</td>
<td>4.76 ± 0.07**</td>
<td>4.78 ± 0.07**</td>
<td>4.86 ± 0.11**</td>
<td>4.70</td>
<td>13.365</td>
<td>4.084</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>106.6 ± 3.8**</td>
<td>107.6 ± 1.8</td>
<td>103.2 ± 2.2</td>
<td>114.6 ± 15.8</td>
<td>92.8 ± 5.4</td>
<td>89.6 ± 6.9</td>
<td>84.8 ± 4.2**</td>
<td>75.6 ± 2.8**</td>
<td>69.2 ± 4.9**</td>
<td>50.0</td>
<td>NVM</td>
<td>NVM</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>99.9 ± 9.9**</td>
<td>85.6 ± 9.9</td>
<td>107.4 ± 19.0</td>
<td>118.4 ± 35.2</td>
<td>82.0 ± 3.0</td>
<td>48.8 ± 8.3**</td>
<td>66.8 ± 14.0*</td>
<td>53.0 ± 2.9**</td>
<td>58.6 ± 14.8*</td>
<td>111.0</td>
<td>NVM</td>
<td>NVM</td>
</tr>
<tr>
<td>Aspartate Aminotransferase (IU/L)</td>
<td>61.2 ± 3.6**</td>
<td>64.0 ± 5.2</td>
<td>55.0 ± 2.6</td>
<td>58.8 ± 3.2</td>
<td>70.5 ± 4.3</td>
<td>57.4 ± 3.7</td>
<td>72.2 ± 3.1*</td>
<td>110.2 ± 19.6*</td>
<td>209.2 ± 67.2**</td>
<td>134.0</td>
<td>36.116</td>
<td>21.468</td>
</tr>
<tr>
<td>Alkaline Phosphatase (IU/L)</td>
<td>302.2 ± 19.9**</td>
<td>315.8 ± 18.4</td>
<td>277.2 ± 11.1</td>
<td>322.8 ± 18.7</td>
<td>346.0 ± 15.5</td>
<td>325.0 ± 16.9</td>
<td>335.8 ± 23.5</td>
<td>357.2 ± 18.3</td>
<td>527.8 ± 34.1**</td>
<td>466.0</td>
<td>89.383</td>
<td>74.114</td>
</tr>
<tr>
<td>Female</td>
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<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>95.6 ± 4.9**</td>
<td>100.6 ± 7.7</td>
<td>104.8 ± 8.0</td>
<td>93.6 ± 11.9</td>
<td>82.2 ± 3.8</td>
<td>84.8 ± 2.2</td>
<td>90.4 ± 2.7</td>
<td>92.8 ± 3.1</td>
<td>38.0 ± 4.5**</td>
<td>54.0 ± 6.8**</td>
<td>1.591</td>
<td>0.58</td>
</tr>
<tr>
<td>Aspartate Aminotransferase (IU/L)</td>
<td>72.80 ± 1.78**</td>
<td>73.60 ± 2.44</td>
<td>71.80 ± 2.03</td>
<td>76.40 ± 3.53</td>
<td>77.00 ± 2.59</td>
<td>75.60 ± 2.48</td>
<td>79.20 ± 2.97</td>
<td>74.00 ± 3.70</td>
<td>76.00 ± 2.74</td>
<td>103.60 ± 19.79</td>
<td>497.046</td>
<td>340.458</td>
</tr>
</tbody>
</table>

Statistical significance for a dosed group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.

*Statistically significant at \( p \leq 0.05; **p \leq 0.01.

BMD1Std = benchmark dose corresponding to a benchmark response set to one standard deviation from the mean; BMDL1Std = benchmark dose lower confidence limit corresponding to a benchmark response set to one standard deviation from the mean; NA = not applicable; A/G Ratio = ratio of albumin to globulin; NVM = nonviable model.

aData are displayed as mean ± standard error of the mean.

bStatistical analysis performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.

cOne sample in the indicated dose group was not received.

dOne male rat was moribund and euthanized on study day 1 and three male rats were found dead on study day 4. Clinical chemistry data from the remaining male rat were excluded from statistical analysis and BMD calculations.

eBMD values are much lower than would be expected given the lowest-observed-effect level and no-observed-effect level values, suggesting that the BMD estimates do not accurately reflect the true potency of the effect of these endpoints and are likely an anomalous product of the BMD modeling approach.

fOne value for alkaline phosphatase in the vehicle control group and the 0.15 mg/kg group were excluded due to sample and/or analysis concerns.
In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

Table 5. Summary of Select Hematology Data for Male and Female Rats Administered 6:1 Fluorotelomer Alcohol for Five Days

<table>
<thead>
<tr>
<th>Endpointa,b</th>
<th>0 mg/kg</th>
<th>0.15 mg/kg</th>
<th>0.5 mg/kg</th>
<th>1.4 mg/kg</th>
<th>4 mg/kg</th>
<th>12 mg/kg</th>
<th>37 mg/kg</th>
<th>111 mg/kg</th>
<th>333 mg/kg</th>
<th>1,000 mg/kg</th>
<th>BMD_{1Std} (mg/kg)</th>
<th>BMD_{L1Std} (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
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<tr>
<td>n</td>
<td>9c</td>
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<td>5</td>
<td>4c</td>
<td>4c</td>
<td>1d</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>240.2 ± 8.5**</td>
<td>237.2 ± 4.3</td>
<td>207.4 ± 13.6</td>
<td>228.4 ± 14.9</td>
<td>222.2 ± 6.6</td>
<td>211.9 ± 8.6</td>
<td>235.4 ± 9.9</td>
<td>194.9 ± 12.1*</td>
<td>89.9 ± 9.8**</td>
<td>88.4</td>
<td>0.308e</td>
<td>0.112e</td>
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<td>(10^3/μL)</td>
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<td>Female</td>
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<tr>
<td>Reticulocytes</td>
<td>224.6 ± 11.5**</td>
<td>235.1 ± 17.6</td>
<td>205.3 ± 17.2</td>
<td>206.6 ± 7.6</td>
<td>210.7 ± 11.9</td>
<td>200.6 ± 14.0</td>
<td>161.9 ± 6.5**</td>
<td>199.4 ± 20.2*</td>
<td>126.4 ± 9.4**</td>
<td>112.3 ± 12.7**</td>
<td>15.578</td>
<td>3.622</td>
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<td>(10^3/μL)</td>
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<tr>
<td>Monocytes</td>
<td>0.19 ± 0.04**</td>
<td>0.31 ± 0.08</td>
<td>0.28 ± 0.03</td>
<td>0.29 ± 0.04</td>
<td>0.28 ± 0.05</td>
<td>0.22 ± 0.04</td>
<td>0.28 ± 0.03</td>
<td>0.37 ± 0.05*</td>
<td>0.40 ± 0.06**</td>
<td>0.66 ± 0.18**</td>
<td>257.111</td>
<td>160.613</td>
</tr>
<tr>
<td>(10^3/μL)</td>
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<tr>
<td>Large Unstained cells</td>
<td>0.04 ± 0.01**</td>
<td>0.06 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.06 ± 0.00**</td>
<td>0.07 ± 0.01**</td>
<td>0.07 ± 0.01**</td>
<td>0.07 ± 0.02*</td>
<td>54.339</td>
<td>15.759</td>
</tr>
</tbody>
</table>

Statistical significance for a dosed group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test. 
*Statistically significant at p ≤ 0.05; **p ≤ 0.01.
BMD_{1Std} = benchmark dose corresponding to a benchmark response set to one standard deviation from the mean; BMD_{L1Std} = benchmark dose lower confidence limit corresponding to a benchmark response set to one standard deviation from the mean; NA = not applicable.

a Data are displayed as mean ± standard error of the mean.
b Statistical analysis performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
c One sample from each of the indicated dose groups had a clot present and was not analyzed.
d One male rat was moribund and euthanized on study day 1 and three male rats were found dead on study day 4. Hematology data from the remaining male rat were excluded from statistical analysis and BMD calculations.
e BMD values are much lower than would be expected given the lowest-observed-effect level and no-observed-effect level values, suggesting that the BMD estimates do not accurately reflect the true potency of the effect of these endpoints and are likely an anomalous product of the BMD modeling approach.
Table 6. Summary of Select Hormone Data for Male and Female Rats Administered 6:1 Fluorotelomer Alcohol for Five Days

| Endpoint| 0 mg/kg| 0.15 mg/kg| 0.5 mg/kg| 1.4 mg/kg| 4 mg/kg| 12 mg/kg| 37 mg/kg| 111 mg/kg| 333 mg/kg| 1,000 mg/kg| BMD$_{1\text{Std}}$ (mg/kg)| BMD$_{L1\text{Std}}$ (mg/kg) |
|---------|-------|-----------|---------|---------|-------|-------|-------|---------|---------|---------|---------|---------|---------|
| Male    |       |           |         |         |       |       |       |         |         |         |         |         |         |
| n       | 9$^c$ | 5         | 5       | 4$^c$   | 4$^c$ | 5     | 5     | 4$^c$   | 4$^c$   | 5       | 5       | 1$^d$   | NA     | NA     |
| Total T3 (ng/dL) | 49.24 ± 2.130** | 51.72 ± 1.832 | 56.58 ± 4.871 | 47.65 ± 0.999 | 44.97 ± 1.527 | 34.92 ± 2.801** | 31.96 ± 2.646** | 33.02 ± 0.999** | 33.15 ± 4.547** | 27.600 | 0.462$^e$ | 0.245$^e$ |
| fT4 (ng/dL) | 6.76 ± 0.817** | 5.86 ± 0.686 | 6.68 ± 0.316 | 5.75 ± 0.496 | 6.41 ± 0.393 | 4.98 ± 0.435 | 5.72 ± 0.661 | 5.11 ± 0.301 | 4.36 ± 0.424** | 3.780 | NVM     | NVM     |
| Total T4 (μg/dL) | 2.71 ± 0.16** | 2.47 ± 0.26 | 2.57 ± 0.30 | 2.52 ± 0.14 | 1.58 ± 0.16** | 1.01 ± 0.07** | 0.93 ± 0.10** | 0.66 ± 0.04** | 0.50 ± 0.02** | 0.66 | 3.19    | 1.774   |
| Female  |       |           |         |         |       |       |       |         |         |         |         |         |         |
| n       | 9$^c$ | 4$^c$     | 3$^f$   | 4$^c$   | 5     | 5     | 4$^c$ | 5       | 5       | 1$^d$   | NA     | NA     |
| TSH (ng/mL) | 3.12 ± 0.371** | 3.40 ± 1.013 | 2.80 ± 0.551 | 2.42 ± 0.375 | 3.02 ± 0.475 | 3.40 ± 0.826 | 2.54 ± 0.367 | 4.80 ± 0.388 | 3.77 ± 1.150 | 6.90 ± 0.933** | 356.61  | 268.917 |
| Total T3 (ng/dL) | 66.00 ± 3.455** | 58.82 ± 1.727 | 66.90 ± 4.891 | 59.82 ± 4.950 | 61.54 ± 4.315 | 62.70 ± 4.457 | 63.74 ± 2.761 | 59.04 ± 2.090 | 42.42 ± 2.062** | 31.06 ± 1.857** | 161.48  | 122.215 |
| Total T4 (μg/dL) | 3.12 ± 0.29** | 2.83 ± 0.43 | 2.31 ± 0.08 | 2.64 ± 0.43 | 2.76 ± 0.11 | 2.79 ± 0.16 | 2.31 ± 0.22 | 2.64 ± 0.26 | 1.20 ± 0.18** | 0.86 ± 0.14** | NVM     | NVM     |

Statistical significance for a dosed group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.

**Statistically significant at p \(\leq 0.01\).

BMD$_{1\text{Std}}$ = benchmark dose corresponding to a benchmark response set to one standard deviation from the mean; BMD$_{L1\text{Std}}$ = benchmark dose lower confidence limit corresponding to a benchmark response set to one standard deviation from the mean; NA = not applicable; total T3 = total triiodothyronine; fT4 = free thyroxine; NVM = nonviable model; total T4 = total thyroxine; TSH = thyroid stimulating hormone.

$^a$Data are displayed as mean ± standard error of the mean.

$^b$Statistical analysis performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.

$^c$One sample in the indicated dose groups did not have sufficient specimen volume available for analysis.

$^d$One male rat was moribund and euthanized on study day 1 and three male rats were found dead on study day 4. Hormone data from the remaining male rat were excluded from statistical analysis and BMD calculations.

$^e$BMD values are much lower than would be expected given the lowest-observed-effect level and no-observed-effect level values, suggesting that the BMD estimates do not accurately reflect the true potency of the effect of these endpoints and are likely an anomalous product of the BMD modeling approach.

$^f$Two samples in the indicated dose group did not have sufficient specimen volume available for analysis.
Internal Dose Assessment

For the 4 and 37 mg/kg groups, 6:1 FTOH plasma concentrations were determined at 2 and 24 hours following the last dose administered on study day 4 to male and female rats. Average 6:1 FTOH concentrations are given in Table 7. In the 4 mg/kg male rats at 2 hours following administration, the average concentration was slightly above the limit of detection (LOD = 2.9 ng/mL) of the analytical method. As the administered dose increased from 4 to 37 mg/kg (a ninefold increase), there was a more-than-proportional increase (38-fold) in the average 6:1 FTOH plasma concentration, suggesting changes in the absorption, distribution, metabolism, and excretion processes (e.g., saturation of clearance pathways) as the dose increased. In female rats at 2 hours postdose, the 6:1 FTOH plasma concentration was below the LOD of the analytical method in the 4 mg/kg group, and at 37 mg/kg, the average group concentration was lower than that observed in male rats (female, 115 ng/mL; male, 148 ng/mL), demonstrating some sex differences. At 24 hours postdose, the concentration fell below the LOD for both male and female rats suggesting short plasma half-lives of 6:1 FTOH in rats.

Table 7. Summary of Plasma Concentration Data for Male and Female Rats Administered 6:1 Fluorotelomer Alcohol for Five Days\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>4 mg/kg</th>
<th>37 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Male 2 Hours Postdose (ng/mL)</td>
<td>3.86 ± 0.164</td>
<td>148 ± 29.0</td>
</tr>
<tr>
<td>24 Hours Postdose (ng/mL)</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td>Female 2 Hours Postdose (ng/mL)</td>
<td>BD(^b)</td>
<td>115 ± 44.9</td>
</tr>
<tr>
<td>24 Hours Postdose (ng/mL)</td>
<td>BD</td>
<td>BD</td>
</tr>
</tbody>
</table>

If over 20% of the animals in a group are above the limit of detection, then half the limit of detection value is substituted for values that are below it.

BD = below detection; group did not have over 20% of its values above the limit of detection so mean and standard error were not calculated.

\(^a\)Data are displayed as mean ± standard error of the mean.

\(^b\)Only two samples were received for the 4 mg/kg female rats at 2 hours postdose.

Apical Endpoint Benchmark Dose Summary

A summary of the calculated BMDs for each toxicological endpoint is provided in Table 8. The endpoint-specific LOEL and NOEL are included and could be informative for endpoints that lack a calculated BMD either because no viable model was available or because the estimated BMD was below the lower limit of extrapolation (<0.050 mg/kg).
In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

Table 8. BMD, BMD\textsubscript{L1Std}, LOEL, and NOEL Summary for Apical Endpoints, Sorted by BMD or LOEL from Low to High

| Endpoint                              | BMD\textsubscript{1Std} (mg/kg) | BMD\textsubscript{L1Std} (mg/kg) | LOEL (mg/kg)
|---------------------------------------|---------------------------------|----------------------------------|--------------
| Total Thyroxine                       | 3.19                            | 1.774                            | 4            | 1.4          | DOWN         |
| Relative Liver Weight                 | 12.122                          | 9.527                            | 37           | 12           | UP           |
| Albumin                               | 13.365                          | 4.084                            | 37           | 12           | UP           |
| Relative Left Kidney Weight           | 20.907                          | 4.272                            | 333          | 111          | UP           |
| Aspartate Aminotransferase            | 28.117                          | 19.352                           | 111          | 37           | UP           |
| Absolute Liver Weight                 | 28.507                          | 15.286                           | 37           | 12           | UP           |
| Alanine Aminotransferase              | 36.116                          | 21.468                           | 37           | 12           | UP           |
| Alkaline Phosphatase                  | 89.383                          | 74.114                           | 333          | 111          | UP           |
| Creatinine                            | 97.38                           | 32.365                           | 333          | 111          | UP           |
| A/G Ratio                             | UREP\textsuperscript{b}         | UREP\textsuperscript{b}          | 12           | 4            | –            |
| Globulin                              | UREP                            | UREP                             | 12           | 4            | –            |
| Total Triiodothyronine                | UREP                            | UREP                             | 12           | 4            | –            |
| Triglycerides                         | NVM                             | NVM                              | 12           | 4            | DOWN         |
| Cholesterol                           | NVM                             | NVM                              | 37           | 12           | DOWN         |
| Relative Right Kidney Weight          | UREP                            | UREP                             | 37           | 12           | –            |
| Reticulocytes                         | UREP                            | UREP                             | 111          | 37           | –            |
| Free Thyroxine                        | NVM                             | NVM                              | 333          | 111          | DOWN         |

**Male**

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>BMD\textsubscript{1Std} (mg/kg)</th>
<th>BMD\textsubscript{L1Std} (mg/kg)</th>
<th>LOEL (mg/kg)</th>
<th>NOEL (mg/kg)</th>
<th>Direction of Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reticulocytes</td>
<td>15.578</td>
<td>3.622</td>
<td>37</td>
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<tr>
<td>Large Unstained Cells</td>
<td>54.339</td>
<td>15.759</td>
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<td>12</td>
<td>UP</td>
</tr>
<tr>
<td>Total Triiodothyronine</td>
<td>161.48</td>
<td>122.215</td>
<td>333</td>
<td>111</td>
<td>DOWN</td>
</tr>
<tr>
<td>Monocytes</td>
<td>257.111</td>
<td>160.613</td>
<td>111</td>
<td>37</td>
<td>UP</td>
</tr>
<tr>
<td>Thyroid Stimulating Hormone</td>
<td>356.61</td>
<td>268.917</td>
<td>1,000</td>
<td>333</td>
<td>UP</td>
</tr>
<tr>
<td>Aspartate Aminotransferase</td>
<td>497.046</td>
<td>340.458</td>
<td>1,000</td>
<td>333</td>
<td>UP</td>
</tr>
<tr>
<td>Absolute Liver Weight</td>
<td>NVM</td>
<td>NVM</td>
<td>333</td>
<td>111</td>
<td>UP</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>UREP</td>
<td>UREP</td>
<td>333</td>
<td>111</td>
<td>–</td>
</tr>
<tr>
<td>Relative Liver Weight</td>
<td>NVM</td>
<td>NVM</td>
<td>333</td>
<td>111</td>
<td>UP</td>
</tr>
<tr>
<td>Total Thyroxine</td>
<td>NVM</td>
<td>NVM</td>
<td>333</td>
<td>111</td>
<td>DOWN</td>
</tr>
</tbody>
</table>

**Female**

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>BMD\textsubscript{1Std} (mg/kg)</th>
<th>BMD\textsubscript{L1Std} (mg/kg)</th>
<th>LOEL (mg/kg)</th>
<th>NOEL (mg/kg)</th>
<th>Direction of Change</th>
</tr>
</thead>
</table>

BMD\textsubscript{1Std} = benchmark dose corresponding to a benchmark response set to one standard deviation from the mean; BMD\textsubscript{L1Std} = benchmark dose lower confidence limit corresponding to a benchmark response set to one standard deviation from the mean; LOEL = lowest-observed-effect level; NOEL = no-observed-effect level; A/G Ratio = ratio of albumin to globulin; UREP = unreliable estimate of potency is a label based on review by a subject matter expert and rejection of BMD modeling results; NVM = nonviable model, defined as a modeling result that does not meet prespecified fit criteria and hence is deemed unreliable.  

\textsuperscript{a}Values in bold text indicate the LOEL of endpoints for which a BMD could not be calculated.  

\textsuperscript{b}BMD values are much lower than would be expected given the end-point specific LOEL and NOEL values, suggesting that the BMD estimates do not accurately reflect the true potency of the effect of these endpoints and are likely an anomalous product of the BMD modeling approach.
Gene Set Benchmark Dose Analysis

Chemical-induced alterations in liver and kidney gene transcript expression were examined to determine those gene sets most sensitive to 6:1 FTOH exposure. To that end, BMD analysis of transcripts and gene sets (Gene Ontology [GO] biological process) was conducted to determine the potency of the chemical to elicit gene expression changes in the liver and kidney. This analysis used transcript-level BMD data to assess an aggregate score of gene set potency (median transcript BMD) and enrichment.

The “active” gene sets in the liver and kidney with the lowest BMD median values are shown in Table 9 and Table 10, respectively. The gene sets in Table 9 and Table 10 should be interpreted with caution from the standpoint of the underlying biological mechanism and any relationship to toxicity or toxic agents referenced in the GO term definitions. The data primarily should be considered a metric of potency for chemical-induced transcriptional changes (i.e., a concerted biological change) that could serve as a surrogate of estimated biological potency and, by extension, toxicological potency when more definitive toxicological data are unavailable.

No gene sets in the liver of male or female rats had estimated BMD median values <0.050 mg/kg. In male rats, the most sensitive GO biological processes for which a BMD value could be reliably calculated were cellular response to epidermal growth factor stimulus (GO:0071364) and response to epidermal growth factor (GO:0070849) with median BMDs (BMDLs) of 0.368 (0.103) and 0.690 (0.456) mg/kg, respectively. In female rats, the most sensitive GO biological processes for which a BMD value could be reliably calculated were positive regulation of phagocytosis (GO:0050766) and regulation of phagocytosis (GO:0050764) with median BMDs (BMDLs) of 44.730 (22.260) and 48.555 (27.154) mg/kg, respectively.

Two gene sets in the kidney of male rats had estimated BMD median values <0.050 mg/kg, which were related to astrocyte activation (GO:0048143) and negative regulation of response to biotic stimulus (GO:002832). The most sensitive GO biological processes for which a BMD value could be reliably calculated were acetyl-CoA metabolic process (GO:0006084) and acyl-CoA metabolic process (GO:0006637) with median BMDs (BMDLs) of 1.346 (0.541) and 1.928 (1.305) mg/kg, respectively. No gene sets in the kidney of female rats had estimated BMD median values <0.050 mg/kg. The most sensitive GO biological processes for which a BMD value could be reliably calculated were fatty acid beta-oxidation (GO:0006635) and fatty acid oxidation (GO:0019395) with median BMDs (BMDLs) of 21.079 (13.312) and 27.058 (13.877) mg/kg, respectively. The full list of affected gene sets in the liver and kidney of male and female rats can be found in Appendix F.
<table>
<thead>
<tr>
<th>Category Name</th>
<th>No. of Active Genes/Platform Genes in Gene Set</th>
<th>% Gene Set Coverage</th>
<th>Active Genes</th>
<th>BMD_{1std} Median of Gene Set Transcripts (mg/kg)</th>
<th>Median BMD_{115std} – BMD_{115std} (mg/kg)</th>
<th>Genes with Changed Direction Up</th>
<th>Genes with Changed Direction Down</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0071364</td>
<td>cellular response to epidermal growth factor stimulus</td>
<td>3/20</td>
<td>15%</td>
<td>Zfp36; Ncl; Myc</td>
<td>0.368</td>
<td>0.103 – 1.817</td>
<td>1</td>
</tr>
<tr>
<td>GO:0070849</td>
<td>response to epidermal growth factor</td>
<td>4/26</td>
<td>15%</td>
<td>Zfp36; Ncl; Myc; Acot2</td>
<td>0.690</td>
<td>0.456 – 1.597</td>
<td>2</td>
</tr>
<tr>
<td>GO:0006635</td>
<td>fatty acid beta-oxidation</td>
<td>10/33</td>
<td>30%</td>
<td>Slec27a2; Gcdh; Etfih; Ehhadh; Eei1; Ech1; Crot; Cpt1b; Acox1; Acot2; Acadsb</td>
<td>2.017</td>
<td>1.401 – 3.184</td>
<td>9</td>
</tr>
<tr>
<td>GO:0009062</td>
<td>fatty acid catabolic process</td>
<td>12/42</td>
<td>29%</td>
<td>Slec27a2; Hacl1; Gcdh; Etfih; Ehhadh; Eei1; Ech1; Crot; Cpt1b; Acox1; Acot2; Acadsb</td>
<td>2.017</td>
<td>1.288 – 3.184</td>
<td>11</td>
</tr>
<tr>
<td>GO:0072329</td>
<td>monocarboxylic acid catabolic process</td>
<td>15/48</td>
<td>31%</td>
<td>Slec27a2; Hacl1; Gcdh; Etfih; Ehhadh; Eei1; Ech1; Cyp26b1; Crot; Cpt1b; Agxt2; Acox1; Acot2; Acadsb; Abat</td>
<td>2.238</td>
<td>1.439 – 3.909</td>
<td>12</td>
</tr>
<tr>
<td>GO:0070371</td>
<td>ERK1 and ERK2 cascade</td>
<td>3/14</td>
<td>21%</td>
<td>Tf; Myc; Apoa1</td>
<td>2.377</td>
<td>1.208 – 5.187</td>
<td>0</td>
</tr>
</tbody>
</table>
In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

<table>
<thead>
<tr>
<th>Category Name</th>
<th>No. of Active Genes/Platform Genes in Gene Set</th>
<th>% Gene Set Coverage</th>
<th>Active Genes</th>
<th>BMD_{1std} Median of Gene Set Transcripts (mg/kg)</th>
<th>Median BMD_{1std}–BMD_{U1std} (mg/kg)</th>
<th>Genes with Changed Direction Up</th>
<th>Genes with Changed Direction Down</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0000038 very long-chain fatty acid metabolic process</td>
<td>5/12</td>
<td>42%</td>
<td>Slc27a2; Acox1; Acot4; Acot2; Acot1</td>
<td>2.721</td>
<td>1.888–4.505</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>GO:0046395 carboxylic acid catabolic process</td>
<td>21/87</td>
<td>24%</td>
<td>Cemip2; Slc27a2; Kynu; Kmo; Hac1l; Gcdh; Etfdh; Ehhadh; Eci1; Ech1; Cyp26b1; Crot; Cpt1b; Cdo1; Blnh; Agxt2; Acox1; Acot4; Acot2; Acadsb; Abat</td>
<td>3.806</td>
<td>1.610–6.038</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>GO:0006637 acyl-CoA metabolic process</td>
<td>9/31</td>
<td>29%</td>
<td>Mpc2; Kynu; Gcdh; Acss2; Acot4; Acot2; Acot12; Acot1; Acadsb</td>
<td>4.030</td>
<td>2.953–10.491</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>GO:0044282 small molecule catabolic process</td>
<td>23/120</td>
<td>19%</td>
<td>Cemip2; Sul1e1; Slc27a2; Kynu; Kmo; Inpp1; Hac1l; Gcdh; Etfdh; Ehhadh; Eci1; Ech1; Cyp26b1; Crot; Cpt1b; Cdo1; Blnh; Agxt2; Acox1; Acot4; Acot2; Acadsb</td>
<td>4.136</td>
<td>1.888–10.491</td>
<td>17</td>
<td>6</td>
</tr>
</tbody>
</table>

**Female**

<table>
<thead>
<tr>
<th>Category Name</th>
<th>No. of Active Genes/Platform Genes in Gene Set</th>
<th>% Gene Set Coverage</th>
<th>Active Genes</th>
<th>BMD_{1std} Median of Gene Set Transcripts (mg/kg)</th>
<th>Median BMD_{1std}–BMD_{U1std} (mg/kg)</th>
<th>Genes with Changed Direction Up</th>
<th>Genes with Changed Direction Down</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0050766 positive regulation of phagocytosis</td>
<td>4/35</td>
<td>11%</td>
<td>Cd36; C3; Apoa2; Ahsg</td>
<td>44.730</td>
<td>22.260–122.653</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Category Name</td>
<td>No. of Active Genes/Platform Genes in Gene Set</td>
<td>% Gene Set Coverage</td>
<td>Active Genes</td>
<td>BMD\textsubscript{1std} Median of Gene Set Transcripts (mg/kg)</td>
<td>Median BMD\textsubscript{1 std}–BMD\textsubscript{U 1std} (mg/kg)</td>
<td>Genes with Changed Direction</td>
<td>Genes with Changed Direction</td>
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<tr>
<td>---------------</td>
<td>---------------------------------------------</td>
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<td>--------------</td>
<td>-------------------------------------------------</td>
<td>-------------------------------------------------</td>
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</tr>
<tr>
<td>GO:0050764</td>
<td>5/51</td>
<td>10%</td>
<td>Cd36; C3; Apoa2; Alox15; Ahsg</td>
<td>48.555</td>
<td>27.154–159.716</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>regulation of phagocytosis</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GO:0002718</td>
<td>3/36</td>
<td>8%</td>
<td>Ticam1; Cd36; Apoa2</td>
<td>48.555</td>
<td>17.366–159.716</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>regulation of cytokine production involved in immune response</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GO:0034381</td>
<td>3/11</td>
<td>27%</td>
<td>Cd36; Apoc3; Apoa2</td>
<td>48.555</td>
<td>17.366–159.716</td>
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<td>1</td>
</tr>
<tr>
<td>plasma lipoprotein particle clearance</td>
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<tr>
<td>GO:0016051</td>
<td>3/32</td>
<td>9%</td>
<td>Gpd1; G6pd; Acadm</td>
<td>48.822</td>
<td>39.627–63.173</td>
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<td>0</td>
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<tr>
<td>carbohydrate biosynthetic process</td>
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<td>GO:0006399</td>
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<td>15%</td>
<td>Tsen2; Iars2; Hsd17b10</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0051181</td>
<td>3/10</td>
<td>30%</td>
<td>Slc27a1; Slc22a8; Abcg2</td>
<td>60.649</td>
<td>31.055–121.705</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>cofactor transport</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0044262</td>
<td>3/40</td>
<td>8%</td>
<td>Inpp1; Acadm; Abcg2</td>
<td>60.649</td>
<td>39.560–121.705</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>cellular carbohydrate metabolic process</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0045926</td>
<td>4/72</td>
<td>6%</td>
<td>Gdf15; G6pd; Cdkn1a; Ahsg</td>
<td>61.369</td>
<td>35.006–115.629</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>negative regulation of growth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

<table>
<thead>
<tr>
<th>Category Name</th>
<th>No. of Active Genes/Platform Genes in Gene Set</th>
<th>% Gene Set Coverage</th>
<th>Active Genes</th>
<th>BMD\text{1std} Median of Gene Set Transcripts (mg/kg)</th>
<th>Median BMD\text{1std–U1std} (mg/kg)</th>
<th>Genes with Changed Direction Up</th>
<th>Genes with Changed Direction Down</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006635 fatty acid beta-oxidation</td>
<td>18/33</td>
<td>55%</td>
<td>Slc27a2; Hadhb; Hadh; Gcdh; Efdh; Ehhadh; Eci1; Ech1; Decr1; Crot; Cpt2; Cpt1b; Acox1; Acadsb; Acadm; Acadl; Acaa2</td>
<td>64.027</td>
<td>46.553–95.058</td>
<td>17</td>
<td>1</td>
</tr>
</tbody>
</table>

BMD\text{1std} = benchmark dose corresponding to a benchmark response set to one standard deviation from the mean;

BMD\text{L1std} = benchmark dose lower confidence limit corresponding to a benchmark response set to one standard deviation from the mean; BMD\text{U1std} = benchmark dose upper confidence limit corresponding to a benchmark response set to one standard deviation from the mean; GO = Gene Ontology.

Definitions of GO terms were adapted from the Gene Ontology Resource.\textsuperscript{30} Official gene symbols from the Rat Genome Database\textsuperscript{31} are shown in the “Active Genes” column.

GO process description version: https://doi.org/10.22427/NTP-DATA-002-00600-0002-000-0

GO:0071364 cellular response to epidermal growth factor stimulus: Any process that results in a change in state or activity of a cell (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of an epidermal growth factor stimulus.

GO:0070849 response to epidermal growth factor: Any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of an epidermal growth factor stimulus.

GO:0006635 fatty acid beta-oxidation: A fatty acid oxidation process that results in the complete oxidation of a long-chain fatty acid. Fatty acid beta-oxidation begins with the addition of coenzyme A to a fatty acid and occurs by successive cycles of reactions during each of which the fatty acid is shortened by a two-carbon fragment removed as acetyl-coenzyme A; the cycle continues until only two or three carbons remain (as acetyl-CoA or propionyl-CoA, respectively).

GO:0009062 fatty acid catabolic process: The chemical reactions and pathways resulting in the breakdown of a fatty acid, any of the aliphatic monocarboxylic acids that can be liberated by hydrolysis from naturally occurring fats and oils. Fatty acids are predominantly straight-chain acids of 4 to 24 carbon atoms, which may be saturated or unsaturated; branched fatty acids and hydroxy fatty acids also occur, and very long-chain acids of over 30 carbons are found in waxes.

GO:0072329 monocarboxylic acid catabolic process: The chemical reactions and pathways resulting in the breakdown of monocarboxylic acids, any organic acid containing one carboxyl (\(-\text{COOH}\)) group.

GO:0070371 ERK1 and ERK2 cascade: An intracellular protein kinase cascade containing at least ERK1 or ERK2 (MAPKs), a MEK (a MAPKK) and a MAP3K. The cascade may involve four different kinases, as it can also contain an additional tier: the upstream MAP4K. The kinases in each tier phosphorylate and activate the kinase in the downstream tier to transmit a signal within a cell.

GO:0000038 very long-chain fatty acid metabolic process: The chemical reactions and pathways involving a fatty acid with a chain length greater than C22.

GO:0046395 carboxylic acid catabolic process: The chemical reactions and pathways resulting in the breakdown of carboxylic acids, which are any organic acid containing one or more carboxyl (\(-\text{COOH}\)) groups.

GO:0006637 acyl-CoA metabolic process: The chemical reactions and pathways involving acyl-CoA, which is any derivative of coenzyme A in which the sulfhydryl group is in thiolester linkage with an acyl group.

GO:0044282 small molecule catabolic process: The chemical reactions and pathways resulting in the breakdown of small molecules, which are any low molecular weight, monomeric, nonencoded molecule.

GO:0050766 positive regulation of phagocytosis: Any process that activates or increases the frequency, rate, or extent of phagocytosis.

GO:0050764 regulation of phagocytosis: Any process that modulates the frequency, rate, or extent of phagocytosis, the process in which phagocytes engulf external particulate material.

GO:0002718 regulation of cytokine production involved in immune response: Any process that modulates the frequency, rate, or extent of cytokine production that contributes to an immune response.
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GO:0034381 plasma lipoprotein particle clearance: The process in which a lipoprotein particle is removed from the blood via receptor-mediated endocytosis and its constituent parts degraded.
GO:0016051 carbohydrate biosynthetic process: The chemical reactions and pathways resulting in the formation of carbohydrates, which are any of a group of organic compounds based on the general formula C₆(H₂O)₉.
GO:0006399 tRNA metabolic process: The chemical reactions and pathways involving tRNA (transfer RNA), which is a class of relatively small RNA molecules responsible for mediating the insertion of amino acids into the sequence of nascent polypeptide chains during protein synthesis. Transfer RNA is characterized by the presence of many unusual minor bases, the function of which has not been completely established.
GO:0051181 cofactor transport: The directed movement of a cofactor into, out of, or within a cell, or between cells, by means of some agent such as a transporter or pore. A cofactor is a substance that is required for the activity of an enzyme or other protein.
GO:0044262 cellular carbohydrate metabolic process: The chemical reactions and pathways involving carbohydrates, which are any of a group of organic compounds based on the general formula C₆(H₂O)₉, as carried out by individual cells.
GO:0045926 negative regulation of growth: Any process that stops, prevents, or reduces the rate or extent of growth (the increase in size or mass of all or part of an organism).

Table 10. Top 10 Kidney Gene Ontology Biological Process Gene Sets Ranked by Potency of Perturbation, Sorted by Benchmark Dose Median

<table>
<thead>
<tr>
<th>Category Name</th>
<th>No. of Active Genes/Platform Genes in Gene Set</th>
<th>% Gene Set Coverage</th>
<th>Active Genes</th>
<th>BMD₁std Median of Gene Set Transcripts (mg/kg)</th>
<th>Median BMD₁std–BMDU₁std (mg/kg)</th>
<th>Genes with Changed Direction</th>
<th>Genes with Changed Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0048143 astrocyte activation</td>
<td>3/11</td>
<td>27%</td>
<td>Il1b; Grn; C1qa</td>
<td>&lt;0.050b</td>
<td>NR</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>GO:0002832 negative regulation of response to biotic stimulus</td>
<td>3/27</td>
<td>11%</td>
<td>Parp14; Grn; Tkfc</td>
<td>&lt;0.050</td>
<td>NR</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>GO:0006084 acetyl-CoA metabolic process</td>
<td>3/16</td>
<td>19%</td>
<td>Hmgcs2; Acot12; Acaa2</td>
<td>1.346</td>
<td>0.541–4.058</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>GO:0006637 acyl-CoA metabolic process</td>
<td>6/31</td>
<td>19%</td>
<td>Hmgcs2; Acot4; Acot2; Acot12; Acot1; Acaa2</td>
<td>1.928</td>
<td>1.305–4.031</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>GO:0010883 regulation of lipid storage</td>
<td>3/22</td>
<td>14%</td>
<td>Srebf1; Nfkbia; C3</td>
<td>2.189</td>
<td>0.888–5.495</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>GO:0030522 intracellular receptor signaling pathway</td>
<td>3/42</td>
<td>7%</td>
<td>Srebf1; Nr1d2; Nfkbia</td>
<td>2.189</td>
<td>0.888–5.495</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>GO:0006721 terpenoid metabolic process</td>
<td>3/34</td>
<td>9%</td>
<td>Pecr; Hmgcs2; Cyp2e1</td>
<td>2.994</td>
<td>0.682–13.980</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

26
<table>
<thead>
<tr>
<th>Category Name</th>
<th>No. of Active Genes/Platform Genes in Gene Set</th>
<th>% Gene Set Coverage</th>
<th>Active Genes</th>
<th>BMD$_{1std}$ Median of Gene Set Transcripts (mg/kg)</th>
<th>Median BMD$<em>{1std}$– BMD$</em>{U1std}$ (mg/kg)</th>
<th>Genes with Changed Direction Up</th>
<th>Genes with Changed Direction Down</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0002698 negative regulation of immune effector process</td>
<td>4/43</td>
<td>9%</td>
<td>Lgals3; Grn; Enpp3; Tkfc</td>
<td>3.710</td>
<td>1.436–9.709</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>GO:0009062 fatty acid catabolic process</td>
<td>12/42</td>
<td>29%</td>
<td>Hadh; Etfah; Ehhadh; Eci2; Ech1; Decr1; Cpt2; Adipoq; Acot1; Acot2; Acadm; Acadm</td>
<td>3.895</td>
<td>1.678–6.474</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>GO:0000038 very long-chain fatty acid metabolic process</td>
<td>4/12</td>
<td>33%</td>
<td>Acot1; Acot4; Acot2; Acot1</td>
<td>4.235</td>
<td>2.759–6.872</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

| Female | | | | | | | |
| GO:0006635 fatty acid beta-oxidation | 8/33 | 24% | Ppargc1a; Ehhadh; Eci2; Ech1; Acadm; Acadm; Acaa2 | 21.079 | 13.312–34.031 | 7 | 1 |
| GO:0019395 fatty acid oxidation | 10/43 | 23% | Ppargc1a; Hao2; Ehhadh; Eci2; Eci1; Ech1; Cd36; Acadm; Acadm; Acaa2 | 27.058 | 13.877–61.247 | 8 | 2 |
| GO:0009062 fatty acid catabolic process | 12/42 | 29% | Ppargc1a; Pck2; Pck1; Ehhadh; Eci2; Eci1; Ech1; Ces1d; Acot2; Acadm; Acadm; Acaa2 | 27.540 | 15.179–63.270 | 10 | 2 |
In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

<table>
<thead>
<tr>
<th>Category Name</th>
<th>No. of Active Genes/Platform Genes in Gene Set</th>
<th>% Gene Set Coverage</th>
<th>Active Genes</th>
<th>BMD\textsubscript{1\text{std}} Median of Gene Set Transcripts (mg/kg)</th>
<th>Median BMD\textsubscript{1\text{std}}–BMD\textsubscript{U\text{1\text{std}}} (mg/kg)</th>
<th>Genes with Changed Direction Up</th>
<th>Genes with Changed Direction Down</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006637 acyl-CoA metabolic process</td>
<td>7/31</td>
<td>23%</td>
<td>Hmgcs2; Acss2; Acot4; Acot2; Acot12; Acot1; Acaa2</td>
<td>38.393</td>
<td>23.116–70.139</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>GO:0000038 very long-chain fatty acid metabolic process</td>
<td>3/12</td>
<td>25%</td>
<td>Acot4; Acot2; Acot1</td>
<td>38.393</td>
<td>23.116–70.139</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>GO:0033875 ribonucleoside bisphosphate metabolic process</td>
<td>8/41</td>
<td>20%</td>
<td>Pank1; Hmgcs2; Acss2; Acot4; Acot2; Acot12; Acot1; Acaa2</td>
<td>44.906</td>
<td>31.624–70.563</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>GO:0030258 lipid modification</td>
<td>13/70</td>
<td>19%</td>
<td>Ppard; Hao2; Ephx2; Ehhadh; Eci2; Eci1; Ecl1; Cyp2e1; Cyp1a1; Cd36; Acadm; Acadl; Acaa2</td>
<td>49.703</td>
<td>38.943–83.829</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>GO:0009409 response to cold</td>
<td>3/27</td>
<td>11%</td>
<td>Cxcl10; Acadm; Acadl</td>
<td>61.062</td>
<td>46.535–88.000</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>GO:0009150 purine ribonucleotide metabolic process</td>
<td>10/80</td>
<td>13%</td>
<td>Pank1; Pde4c; Hmgcs2; Aldoc; Acss2; Acot4; Acot2; Acot12; Acot1; Acaa2</td>
<td>67.924</td>
<td>50.324–104.308</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>GO:0006732 coenzyme metabolic process</td>
<td>12/67</td>
<td>18%</td>
<td>Vnn1; Rgn; Pank1; Mthfd2; Hmgcs2; Gclc; Acss2; Acot4; Acot2; Acot12; Acot1; Acaa2</td>
<td>67.924</td>
<td>50.324–104.308</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>

\text{BMD}_{\text{1\text{std}}} = \text{benchmark dose corresponding to a benchmark response set to one standard deviation from the mean;}
\text{BMD}_{\text{U\text{1\text{std}}}} = \text{benchmark dose upper confidence limit corresponding to a benchmark response set to one standard deviation from the mean;}
BMD_{\text{L\text{1\text{std}}}} = \text{benchmark dose lower confidence limit corresponding to a benchmark response set to one standard deviation from the mean;}
\text{BMD}_{\text{U\text{1\text{std}}}} = \text{benchmark dose upper confidence limit corresponding to a benchmark response set to one standard deviation from the mean;}
\text{BMD}_{\text{L\text{1\text{std}}}} = \text{benchmark dose lower confidence limit corresponding to a benchmark response set to one standard deviation from the mean;}

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deviation from the mean; GO = Gene Ontology; NR = the BMDL1Std–BMDU1Std range is not reportable because the BMD1Std median is below the lower limit of extrapolation (<1/3 of the lowest nonzero dose tested).

*Definitions of GO terms were adapted from the Gene Ontology Resource. Official gene symbols from the Rat Genome Database are shown in the “Active Genes” column.

b<0.050 = a best-fit model was identified and a BMD1Std was estimated that was <1/3 of the lowest nonzero dose tested.

**GO process description version:** [https://doi.org/10.22427/NTP-DATA-002-00600-0002-0-0](https://doi.org/10.22427/NTP-DATA-002-00600-0002-0-0).

**Gene Benchmark Dose Analysis**

The top 10 genes based on BMD potency in the liver and kidney (fold change >|2|, significant Williams trend test, global goodness-of-fit p value >0.1, and BMDU/BMDL ≤40) are shown in Table 11 and Table 12. As with the GO analysis, the biological or toxicological significance of...
the changes in gene expression shown in Table 11 and Table 12 should be interpreted with caution. The data primarily should be considered a metric of potency for chemical-induced transcriptional changes that could serve as a conservative surrogate of estimated biological potency, and by extension toxicological potency, when more definitive toxicological data are unavailable.

No liver genes in male or female rats had estimated BMD median values <0.050 mg/kg. In male rats, the most sensitive upregulated genes with a calculated BMD were Acot2 (acyl-CoA thioesterase 2), Eci1 (enoyl-CoA delta isomerase 1), Loc100911558/Spink11 (serine peptidase inhibitor, Kazal type 1-like), Spink1 (serine peptidase inhibitor, Kazal type 1), Ehhadh (enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase), Crot (carnitine O-octanoyltransferase), Acaa1a (acetyl-CoA acyltransferase 1A), and Acaa1b (acetyl-Coenzyme A acyltransferase 1B) with BMDs (BMDLs) of 1.012 (0.809), 1.013 (0.769), 1.270 (0.542), 1.270 (0.542), 1.280 (1.047), 1.411 (1.092), 1.874 (1.524), and 1.874 (1.524) mg/kg, respectively. The most sensitive genes exhibiting a decrease in expression were Myc (MYC proto-oncogene, bHLH transcription factor) and Zfp36 (zinc-finger protein 36) with BMDs (BMDLs) of 0.186 (0.103) and 0.368 (0.097) mg/kg, respectively.

In female rats, all 10 of the most sensitive liver genes were upregulated. These genes were Gdf15 (growth differentiation factor 15), Igfbp1 (insulin-like growth factor-binding protein 1), Eci1 (enoyl-CoA delta isomerase 1), Etfdh (electron transfer flavoprotein dehydrogenase), Cyp2b1 (cytochrome P450, family 2, subfamily b, polypeptide 1), Loc108348266/Cyp2b1 (cytochrome P450 2B1), Dhrs7 (dehydrogenase/reductase 7), Dhrs7l1 (dehydrogenase/reductase [SDR family] member 7-like 1), Slc27a2 (solute carrier family 27 member 2), and Vnn1 (vanin 1) with BMDs (BMDLs) of 17.724 (8.696), 18.792 (7.230), 32.546 (27.162), 34.846 (26.297), 35.483 (29.479), 35.483 (29.479), 35.986 (10.630), 35.986 (10.630), 36.103 (26.571), and 37.026 (30.688) mg/kg, respectively.

None of the top 10 most sensitive kidney genes in male rats had estimated BMD median values <0.050 mg/kg. The most sensitive upregulated genes with a calculated BMD were Decr1 (2,4-dienoyl-CoA reductase 1), Vnn1 (vanin 1), Hmgcs2 (3-hydroxy-3-methylglutaryl-CoA synthase 2), Ehhadh (enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase), Eci2 (enoyl-CoA delta isomerase 2), Acaa2 (acetyl-CoA acyltransferase 2), Acot1 (acyl-CoA thioesterase 1), Cyp4a1 (cytochrome P450, family 4, subfamily a, polypeptide 1), and Ech1 (enoyl-CoA hydratase 1) with BMDs (BMDLs) of 0.680 (0.505), 0.705 (0.488), 0.804 (0.541), 0.953 (0.671), 0.989 (0.643), 1.346 (0.539), 1.363 (0.938), 1.593 (1.021), and 2.055 (1.124) mg/kg, respectively. One gene, Acmsd (aminocarboxymuconate semialdehyde decarboxylase), was downregulated with a BMD (BMDL) of 0.775 (0.183) mg/kg.

The most sensitive kidney gene in female rats, exhibiting an increase in expression, was Plod3 (procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3) with an estimated BMD median value <0.050 mg/kg. The most sensitive upregulated genes with a calculated BMD were Eci1 (enoyl-CoA delta isomerase 1), Vnn1 (vanin 1), Hmgcs2 (3-hydroxy-3-methylglutaryl-CoA synthase 2), Ehhadh (enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase), Eci2 (enoyl-CoA delta isomerase 2), Acaa1a (acetyl-CoA acyltransferase 1A), Acaa1b (acetyl-Coenzyme A acyltransferase 1B), Ech1 (enoyl-CoA hydratase 1), and Acaa2 (acetyl-CoA acyltransferase 2) with BMDs (BMDLs) of 9.486 (7.353), 10.025 (7.993), 11.644 (9.266), 12.212 (9.437), 12.789 (9.638), 13.082 (10.021), 13.872 (10.346), 14.362 (10.630), 14.513 (10.630), and 14.763 (10.630) mg/kg, respectively.
(9.488), 13.850 (11.009), 13.850 (11.009), 19.820 (14.141), and 22.339 (13.614) mg/kg, respectively. None of the top 10 most sensitive kidney genes in female rats were downregulated.

Table 11. Top 10 Liver Genes Ranked by Potency of Perturbation, Sorted by Benchmark Dose Median

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Entrez Gene IDs</th>
<th>Probe IDs</th>
<th>BMD\textsubscript{std} (BMD\textsubscript{U1std}–BMD\textsubscript{L1std}) in mg/kg</th>
<th>Maximum Fold Change</th>
<th>Direction of Expression Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myc</td>
<td>24577</td>
<td>MYC_9271</td>
<td>0.186 (0.103–0.478)</td>
<td>3.3</td>
<td>DOWN</td>
</tr>
<tr>
<td>Zfp36</td>
<td>79426</td>
<td>ZFP36_10204</td>
<td>0.368 (0.097–1.817)</td>
<td>2.1</td>
<td>DOWN</td>
</tr>
<tr>
<td>Acot2</td>
<td>192272</td>
<td>ACOT2_7969</td>
<td>1.012 (0.809–1.377)</td>
<td>19.9</td>
<td>UP</td>
</tr>
<tr>
<td>Eci1</td>
<td>29740</td>
<td>ECI1_8520</td>
<td>1.013 (0.769–1.465)</td>
<td>23.5</td>
<td>UP</td>
</tr>
<tr>
<td>Loc100911558/Spink1l</td>
<td>100911558</td>
<td>SPINK1_32461</td>
<td>1.270 (0.542–3.173)</td>
<td>2.0</td>
<td>UP</td>
</tr>
<tr>
<td>Spink1</td>
<td>266602</td>
<td>SPINK1_32461</td>
<td>1.270 (0.542–3.173)</td>
<td>2.0</td>
<td>UP</td>
</tr>
<tr>
<td>Ehhadh</td>
<td>171142</td>
<td>EHHAZH_8534</td>
<td>1.280 (1.047–1.879)</td>
<td>95.2</td>
<td>UP</td>
</tr>
<tr>
<td>Crot</td>
<td>83842</td>
<td>CROT_8384</td>
<td>1.411 (1.092–1.919)</td>
<td>7.5</td>
<td>UP</td>
</tr>
<tr>
<td>Acaa1a</td>
<td>24157</td>
<td>ACAIA1A_7954</td>
<td>1.874 (1.524–2.760)</td>
<td>39.0</td>
<td>UP</td>
</tr>
<tr>
<td>Acaa1b</td>
<td>501072</td>
<td>ACAIA1A_7954</td>
<td>1.874 (1.524–2.760)</td>
<td>39.0</td>
<td>UP</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gdf15</td>
<td>29455</td>
<td>GDF15_33113</td>
<td>17.724 (8.696–37.098)</td>
<td>5.8</td>
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<tr>
<td>Igfbp1</td>
<td>25685</td>
<td>IGFBP1_32306</td>
<td>18.792 (7.230–51.558)</td>
<td>4.3</td>
<td>UP</td>
</tr>
<tr>
<td>Eci1</td>
<td>29740</td>
<td>ECI1_8520</td>
<td>32.546 (27.162–40.287)</td>
<td>9.6</td>
<td>UP</td>
</tr>
<tr>
<td>Etfdh</td>
<td>295143</td>
<td>ETFDH_8575</td>
<td>34.846 (26.297–48.311)</td>
<td>2.0</td>
<td>UP</td>
</tr>
<tr>
<td>Cyp2b1</td>
<td>24300</td>
<td>CYP2B1_32451</td>
<td>35.483 (29.479–44.233)</td>
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</tr>
<tr>
<td>Loc108348266/Cyp2b1</td>
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<td>CYP2B1_32451</td>
<td>35.483 (29.479–44.233)</td>
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<td>UP</td>
</tr>
<tr>
<td>Dhrs7</td>
<td>299135</td>
<td>DHRS7_8469</td>
<td>35.986 (10.630–156.958)</td>
<td>4.4</td>
<td>UP</td>
</tr>
<tr>
<td>Dhrs7l1</td>
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<td>DHRS7L_8469</td>
<td>35.986 (10.630–156.958)</td>
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<td>UP</td>
</tr>
<tr>
<td>Slc27a2</td>
<td>65192</td>
<td>SLC27A2_9860</td>
<td>36.103 (26.571–51.373)</td>
<td>2.3</td>
<td>UP</td>
</tr>
<tr>
<td>Vnn1</td>
<td>29142</td>
<td>VNN1_10157</td>
<td>37.026 (30.688–46.324)</td>
<td>15.8</td>
<td>UP</td>
</tr>
</tbody>
</table>

BMD\textsubscript{std} = benchmark dose corresponding to a benchmark response set to one standard deviation from the mean; BMD\textsubscript{L1std} = benchmark dose lower confidence limit corresponding to a benchmark response set to one standard deviation from the mean; BMD\textsubscript{U1std} = benchmark dose upper confidence limit corresponding to a benchmark response set to one standard deviation from the mean.

aDescriptions of orthologous human genes are shown due to the increased detail available in public resources such as UniprotKB\textsuperscript{32} and Entrez Gene.\textsuperscript{33} Gene definitions adapted from Human UniprotKB were used as the primary resource due to the greater breadth of annotation and depth of functional detail provided. Gene definitions adapted from Rat UniprotKB were used as the secondary resource if the primary source did not provide a detailed description of function. Human Entrez Gene was used as the third resource. Rat Entrez Gene was used as the fourth resource.

bIn some cases, a probe may map to more than one gene, resulting in duplicate reporting of that probe mapped to different genes.

Gene definition version: https://doi.org/10.22427/NTP-DATA-002-00600-000-000-000-0

Myc: Human Uniprot function (Human MYC): Transcription factor that binds DNA in a nonspecific manner, yet also specifically recognizes the core sequence 5'-CAC[GA]TG-3'. Activates the transcription of growth-related genes. Binds to the VEGFA promoter, promoting VEGFA production and subsequent sprouting angiogenesis (PubMed24940000). Regulator of somatic reprogramming; controls self-renewal of embryonic stem cells. Functions with TAF6L to activate target gene expression through...
**Ehhadh**: Human Uniprot function (Human EHHADH): Peroxisomal trifunctional enzyme possessing 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and delta 3, delta 2-enoyl-CoA isomerase activities. Catalyzes two of the four reactions of the long straight-chain fatty acids peroxisomal beta-oxidation pathway. Optimal isomerase for 2,5 double bonds into 3,5 form isomerization in a range of enoyl-CoA species (probable). Also able to isomerize both 3-cis and 3-trans double bonds into the 2-trans form in a range of enoyl-CoA species (by similarity). With HSD17B4, catalyzes the hydration of trans-2-enoyl-CoA and the dehydrogenation of 3-hydroxyacyl-CoA, but with opposite chiral specificity (PubMed15060085). Regulates the amount of medium-chain dicarboxylic fatty acids, which are essential regulators of all fatty acid oxidation pathways (by similarity). Also involved in the degradation of long-chain dicarboxylic acids via peroxisomal beta-oxidation (PubMed15060085).

**Crot**: Human Uniprot function (Human CROT): Beta-oxidation of fatty acids. The highest activity concerns the C6 to C10 chain length substrate. Converts the end product of pristanic acid beta-oxidation, 4,8-dimethylnonanoyl-CoA, to its corresponding carnitine ester. (E00000269|PubMed10486279).

**Acaal1a**: Human Entrez Gene Summary (Human ACAAL1A): This gene encodes an enzyme operative in the beta-oxidation system of the peroxisomes. Deficiency of this enzyme leads to pseudo-Zellweger syndrome. Alternative splicing results in multiple transcript variants. (provided by RefSeq, Jul 2008)

**Acaal1b**: Human Entrez Gene Summary (Human ACAAL1B): This gene encodes an enzyme operative in the beta-oxidation system of the peroxisomes. Deficiency of this enzyme leads to pseudo-Zellweger syndrome. Alternative splicing results in multiple transcript variants. (provided by RefSeq, Jul 2008)


**Igfbp1**: Human Uniprot function (Human IGFBP1): IGF-binding proteins prolong the half-life of the IGFs and have been shown to either inhibit or stimulate the growth-promoting effects of the IGFs on cell culture. They alter the interaction of IGFs with their cell surface receptors. Promotes cell migration. (E00000269|PubMed15972819).

**Etfdh**: Human Uniprot function (Human ETFDH): Accepts electrons from ETF and reduces ubiquinone.


**Dhrs7**: Human Entrez Gene Summary (Human DHRS7): This gene encodes a member of the short-chain dehydrogenases/reductases (SDR) family, which has over 46,000 members. Members in this family are enzymes that metabolize many different compounds, such as steroid hormones, prostaglandins, retinoids, lipids, and xenobiotics. (provided by RefSeq, Apr 2016)

**Dhrs7l1**: Human Entrez Gene Summary (Human DHRS7L1): This gene encodes a member of the SDR family, which has over 46,000 members. Members in this family are enzymes that metabolize many different compounds, such as steroid hormones, prostaglandins, retinoids, lipids, and xenobiotics. (provided by RefSeq, Apr 2016)
**Slc27a2**: Human Uniprot function (Human **SLC27A2**): Acyl-CoA synthetase that activates long-chain and very long-chain fatty acids (VLCFAs) by catalyzing the formation of fatty acyl-CoA (PubMed10198260, PubMed10749848, PubMed11980911). Can also activate fatty acids such as phytanic acid and pristanic acid (PubMed10198260). Does not activate C24 bile acids, cholate, and chenodeoxycholate (PubMed11980911). In vitro, activates 3-alpha, 7-alpha, 12-alpha-trihydroxy-5-beta-cholestanate (THCA), the C27 precursor of cholic acid deriving from the de novo synthesis from cholesterol (PubMed11980911). Exhibits long-chain fatty acids (LCFAs) transport activity and plays an important role in hepatic fatty acid uptake (PubMed20530735). {ECO0000269|PubMed10198260, ECO0000269|PubMed10749848, ECO0000269|PubMed11980911, ECO0000269|PubMed20530735}. FUNCTION [Isoform 1]: Exhibits both LCFAs transport activity and acyl-CoA synthetase toward VLCFAs (PubMed21768100). Shows a preference for generating CoA derivatives of n-3 fatty acids, which are preferentially trafficked into phosphatidylinositol (PubMed21768100). {ECO0000269|PubMed21768100}. FUNCTION [Isoform 2]: Exhibits LCFAs transport activity but lacks acyl-CoA synthetase toward VLCFAs. {ECO0000269|PubMed21768100}.

**Vnn1**: Human Uniprot function (Human **VNN1**): Amidohydrolase that hydrolyzes one of the carboamide linkages specifically in D-pantetheine thus recycling pantothenic acid (vitamin B5) and releasing cysteamine. {ECO0000269|PubMed10567687, ECO0000269|PubMed11491533, ECO0000269|PubMed25478849}.

Table 12. Top 10 Kidney Genes Ranked by Potency of Perturbation, Sorted by Benchmark Dose Median

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Entrez Gene IDs</th>
<th>Probe IDs</th>
<th>BMD_{\text{Lstd}} \text{ (BMD}<em>{\text{Lstd}}-\text{BMD}</em>{\text{Ustd}}) \text{ in mg/kg}</th>
<th>Maximum Fold Change</th>
<th>Direction of Expression Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decr1</td>
<td>117543</td>
<td>DECR1_8458</td>
<td>0.680 (0.505–1.264)</td>
<td>3.1</td>
<td>UP</td>
</tr>
<tr>
<td>Vnn1</td>
<td>29142</td>
<td>VNN1_10157</td>
<td>0.705 (0.488–1.353)</td>
<td>6.0</td>
<td>UP</td>
</tr>
<tr>
<td>Acmrd</td>
<td>171385</td>
<td>ACMSD_32377</td>
<td>0.775 (0.183–4.177)</td>
<td>2.3</td>
<td>DOWN</td>
</tr>
<tr>
<td>Hmgs2</td>
<td>24450</td>
<td>HMGS2_8812</td>
<td>0.804 (0.541–1.442)</td>
<td>11.8</td>
<td>UP</td>
</tr>
<tr>
<td>Ehhadh</td>
<td>171142</td>
<td>EHHADH_8534</td>
<td>0.953 (0.671–1.585)</td>
<td>8.7</td>
<td>UP</td>
</tr>
<tr>
<td>Eci2</td>
<td>291075</td>
<td>ECI2_8521</td>
<td>0.989 (0.643–1.915)</td>
<td>2.1</td>
<td>UP</td>
</tr>
<tr>
<td>Acaa2</td>
<td>170465</td>
<td>ACAA2_7955</td>
<td>1.346 (0.539–4.058)</td>
<td>2.8</td>
<td>UP</td>
</tr>
<tr>
<td>Acot1</td>
<td>50559</td>
<td>ACOT1_7968</td>
<td>1.363 (0.938–2.405)</td>
<td>2.2</td>
<td>UP</td>
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<tr>
<td>Cyp4a1</td>
<td>50549</td>
<td>CYP4A1_3311</td>
<td>1.593 (1.021–3.028)</td>
<td>4.5</td>
<td>UP</td>
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<tr>
<td>Ech1</td>
<td>64526</td>
<td>ECH1_8516</td>
<td>2.055 (1.124–4.620)</td>
<td>2.2</td>
<td>UP</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Plod3</td>
<td>288583</td>
<td>PLOD3_9507</td>
<td>&lt;0.050*(NR)</td>
<td>2.1</td>
<td>UP</td>
</tr>
<tr>
<td>Eci1</td>
<td>29740</td>
<td>ECI1_8520</td>
<td>9.486 (7.353–12.810)</td>
<td>4.4</td>
<td>UP</td>
</tr>
<tr>
<td>Vnn1</td>
<td>29142</td>
<td>VNN1_10157</td>
<td>10.025 (7.993–13.110)</td>
<td>7.8</td>
<td>UP</td>
</tr>
<tr>
<td>Hmgs2</td>
<td>24450</td>
<td>HMGS2_8812</td>
<td>11.644 (9.266–15.267)</td>
<td>37.7</td>
<td>UP</td>
</tr>
<tr>
<td>Ehhadh</td>
<td>171142</td>
<td>EHHADH_8534</td>
<td>12.212 (9.437–16.563)</td>
<td>6.7</td>
<td>UP</td>
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<tr>
<td>Eci2</td>
<td>291075</td>
<td>ECI2_8521</td>
<td>12.789 (9.488–18.156)</td>
<td>2.0</td>
<td>UP</td>
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<tr>
<td>Acaa1a</td>
<td>24157</td>
<td>ACAA1A_7954</td>
<td>13.850 (11.009–18.172)</td>
<td>5.2</td>
<td>UP</td>
</tr>
<tr>
<td>Acaa1b</td>
<td>501072</td>
<td>ACAA1A_7954</td>
<td>13.850 (11.009–18.172)</td>
<td>5.2</td>
<td>UP</td>
</tr>
<tr>
<td>Ech1</td>
<td>64526</td>
<td>ECH1_8516</td>
<td>19.820 (14.141–29.396)</td>
<td>2.1</td>
<td>UP</td>
</tr>
<tr>
<td>Acaa2</td>
<td>170465</td>
<td>ACAA2_7955</td>
<td>22.339 (13.614–38.665)</td>
<td>2.3</td>
<td>UP</td>
</tr>
</tbody>
</table>

*BMD_{\text{Lstd}}* = benchmark dose corresponding to a benchmark response set to one standard deviation from the mean; 
*BMD_{\text{Ustd}}* = benchmark dose lower confidence limit corresponding to a benchmark response set to one standard deviation from the mean; 
*BMD_{\text{Ustd}}* = benchmark dose upper confidence limit corresponding to a benchmark response set to one standard deviation from the mean.
deviation from the mean; NR = the BMDL1STD–BMDU1STD range is not reportable because the BMD1STD median is below the lower limit of extrapolation (<1/3 of the lowest nonzero dose tested).

4 Descriptions of orthologous human genes are shown due to the increased detail available in public resources such as UniprotKB3 and Entrez Gene.3 Gene definitions adapted from Human UniprotKB were used as the primary resource due to the greater breadth of annotation and depth of functional detail provided. Gene definitions adapted from Rat UniprotKB were used as the secondary resource if the primary source did not provide a detailed description of function. Human Entrez Gene was used as the third resource. Rat Entrez Gene was used as the fourth resource.

In some cases, a probe may map to more than one gene, resulting in duplicate reporting of that probe mapped to different genes. c<0.050 = a best-fit model was identified and a BMD1STD was estimated that was <1/3 of the lowest nonzero dose tested.

**Decr1**: Human Uniprot function (Human DECR1): Auxiliary enzyme of beta-oxidation. It participates in the metabolism of unsaturated fatty enoyl-CoA esters having double bonds in both even- and odd-numbered positions in mitochondria. Catalyzes the NADP-dependent reduction of 2,4-dienoyl-CoA to yield trans-3-enoyl-CoA. [ECO0000269|PubMed15531764].

**Vnn1**: Human Uniprot function (Human VNN1): Amidohydrolase that hydrolyzes one of the carboxamide linkages specifically in D-pantetheine thus recycling pantothenic acid (vitamin B5) and releasing cysteamine. [ECO0000269|PubMed10567687, ECO0000269|PubMed25478849].

**Acmsd**: Human Uniprot function (Human ACMSD): Converts alpha-amino-beta-carboxymuconate-episilon-semialdehyde (ACMS) to alpha-aminomuconate semialdehyde (AMS). ACMS can be converted nonenzymatically to quinolate, a key precursor of NAD, and a potent endogenous excitotoxin of neuronal cells, which is implicated in the pathogenesis of various neurodegenerative disorders. In the presence of ACMS, ACS is converted to AMS, a benign catabolite. ACMSD ultimately controls the metabolic fate of tryptophan catabolism along the kynurenine pathway. [ECO0000269|PubMed19843166].

**Hmgcs2**: Human Uniprot function (Human HMGCS2): Catalyzes the first irreversible step in ketogenesis, condensing acetyl-CoA to acetoacetate-CoA to form HMG-CoA, which is converted by HMG-CoA reductase (HMGCR) into mevalonate. [ECO0000269|PubMed11228257, ECO0000269|PubMed23751782, ECO0000269|PubMed29597274].

**Ehhadi**: Human Uniprot function (Human EHHADH): Peroxisomal trifunctional enzyme possessing 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and delta 3, delta 5 isomerase activities. Catalyzes two of the four reactions of the long straight-chain fatty acids peroxisomal beta-oxidation pathway. Optimal isomerase for 2,5 double bonds into 3,5 form isomerization in a range of enoyl-CoA species (probable). Also able to isomerize both 3-cis and 3-trans double bonds into the 2-trans form in a range of enoyl-CoA species (by similarity). With HSD17B4, catalyzes the hydration of trans-2-enoyl-CoA and the dehydrogenation of 3-hydroxyacyl-CoA, but with opposite chiral specificity (PubMed15060085). Regulates the amount of medium-chain dicarboxylic fatty acids, which are essential regulators of all fatty acid oxidation pathways (by similarity). Also involved in the degradation of long-chain dicarboxylic acids through peroxisomal beta-oxidation (PubMed15060085).

{ECO0000250|UniProtKB:P07896, ECO0000250|UniProtKB:Q9DBM2, ECO0000269|PubMed15060085, ECO0000305|PubMed15060085}.

**Eci2**: Human Uniprot function (Human EC12): Able to isomerize both 3-cis and 3-trans double bonds into the 2-trans form in a range of enoyl-CoA species. Has a preference for 3-trans substrates. [ECO0000269|PubMed10419495].

**Acau2**: Human Uniprot function (Human ACAU2): In the production of energy from fats, this is one of the enzymes that catalyzes the last step of the mitochondrial beta-oxidation pathway, an aerobic process breaking down fatty acids into acetyl-CoA (probable). Using free coenzyme A/CoA, catalyzes the thiolytic cleavage of medium- to long-chain unbranched 3-oxoacyl-CoAs into acetoacetyl-CoA and a fatty acyl-CoA shortened by two carbon atoms (probable). Also catalyzes the condensation of two acetyl-CoA molecules into acetoacetyl-CoA and could be involved in the production of ketone bodies (probable). Also displays hydrolase activity on various fatty acyl-CoAs (PubMed25478839), therefore, could be responsible for the production of acetate in a side reaction to beta-oxidation (probable). Abolishes BNIP3-mediated apoptosis and mitochondrial damage (PubMed18371312). [ECO0000269|PubMed18371312, ECO0000269|PubMed25478839, ECO0000305|PubMed25478839].

**Acot1**: Human Uniprot function (Human ACOT1): Acyl-CoA thioesterases are a group of enzymes that catalyze the hydrolysis of acyl-CoAs into free fatty acids and coenzyme A (CoASH), regulating intracellular levels of acyl-CoAs, free fatty acids, and CoASH. More active toward saturated and unsaturated long-chain fatty acyl-CoAs (C12-C20) [ECO0000269|PubMed16940157].

**Cyp4a**: Human Uniprot function (Human CYP4A2): Catalyzes the omega- and (omega-1)-hydroxylation of various fatty acids such as laurate and palmitate. Shows no activity toward arachidonic acid and prostaglandin A1. Lacks functional activity in the kidney and does not contribute to renal 20-hydroxyecosatetraenoic acid (20-HETE) biosynthesis [ECO0000269|PubMed10860550, ECO0000269|PubMed15611369].

**Ech1**: Human Uniprot function (Human ECH1): Isomerization of 3-trans,5-cis-dienoyl-CoA to 2-trans,4-trans-dienoyl-CoA. [ECO0000250|UniProtKB:Q62651].

In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats


Eci1: Human Uniprot function (Human ECII): Able to isomerize both 3-cis and 3-trans double bonds into the 2-trans form in a range of enoyl-CoA species. [ECO0000269|PubMed7818490].

Acaa1a: Human Entrez Gene Summary (Human ACA1): This gene encodes an enzyme operative in the beta-oxidation system of the peroxisomes. Deficiency of this enzyme leads to pseudo-Zellweger syndrome. Alternative splicing results in multiple transcript variants. (provided by RefSeq, Jul 2008)

Acaa1b: Human Entrez Gene Summary (Human ACA1): This gene encodes an enzyme operative in the beta-oxidation system of the peroxisomes. Deficiency of this enzyme leads to pseudo-Zellweger syndrome. Alternative splicing results in multiple transcript variants. (provided by RefSeq, Jul 2008)
In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

Summary

6:1 Fluorotelomer alcohol (6:1 FTOH) is a member of the per- and polyfluoroalkyl class of compounds to which humans are widely exposed. A review of the literature did not identify toxicological data for estimating the potential adverse health effects of 6:1 FTOH. This study used a transcriptomic approach and standard toxicological endpoints to estimate the in vivo biological potency of 6:1 FTOH.

A subset of standard toxicological endpoints (albumin/globulin ratio, globulin concentration, total triiodothyronine concentration, relative right kidney weight, and reticulocyte count in male rats; cholesterol concentration in female rats) exhibited benchmark dose (BMD) values much lower than would be expected given the endpoint-specific no-observed-effect level and lowest-observed-effect level values. Expert review of the data suggests that the BMD estimates do not accurately reflect the true potency of the effect of these endpoints and were likely an anomalous product of the BMD modeling approach.

Taking this into account, the most sensitive apical endpoint in male rats was a decrease in total thyroxine concentration with an estimated BMD and benchmark dose lower confidence limit (BMDL) of 3.19 (1.774) mg/kg. Increases in relative liver weight and albumin concentration were the next most sensitive apical endpoint changes observed in male rats with BMDs (BMDLs) of 12.122 (9.527) and 13.365 (4.084) mg/kg, respectively. In female rats, the most sensitive apical endpoint was a decrease in reticulocyte count with a BMD (BMDL) of 15.578 (3.622) mg/kg. The next most sensitive apical endpoints observed were an increase in large unstained cell count and a decrease in total triiodothyronine concentration with BMDs (BMDLs) of 54.339 (15.759) and 161.48 (122.215) mg/kg, respectively.

Gene set-level transcriptional changes in the liver following 6:1 FTOH exposure were estimated to occur at a BMD (BMDL) as low as 0.368 (0.103) mg/kg in male rats, corresponding to cellular response to epidermal growth factor stimulus (GO:0071364), and as low as 44.730 (22.260) mg/kg in female rats, corresponding to positive regulation of phagocytosis (GO:0050766). The most sensitive liver gene for which a reliable BMD could be determined was Myc, with a BMD (BMDL) of 0.186 (0.103) mg/kg, in male rats and Gdf15, with a BMD (BMDL) of 17.724 (8.696) mg/kg, in female rats.

Gene set-level transcriptional changes in the kidney were estimated to occur at a BMD (BMDL) as low as 1.346 (0.541) mg/kg in male rats, corresponding to acetyl-CoA metabolic process (GO:0006084), and as low as 21.079 (13.312) mg/kg in female rats, corresponding to fatty acid beta-oxidation (GO:0006635). Two kidney gene sets in male rats had BMD estimates below the lower limit of extrapolation (<0.050 mg/kg). The most sensitive kidney gene in male rats for which a reliable BMD could be determined was Decr1 with a BMD (BMDL) of 0.680 (0.505) mg/kg. In female rats, one kidney gene exhibited changes in expression at dose levels below which a reliable estimate of potency could be achieved (<0.050 mg/kg). The most sensitive gene in female rats for which a reliable BMD could be determined was Eci1 with a BMD (BMDL) of 9.486 (7.353) mg/kg.

Under the conditions of this short-duration transcriptomic study in Sprague Dawley (Hsd:Sprague Dawley® SD®) rats, the most sensitive point of departure with a reliable estimate in male rats was a transcriptional change in a gene, Myc, with a BMD (BMDL) of 0.186
(0.103) mg/kg. Gene set transcriptional changes provided potency estimates slightly higher than Myc, while apical endpoints provided potency estimates higher than Myc. In female rats, the most sensitive point of departure with a reliable estimate was a transcriptional change in a gene, Eci1, with a BMD (BMDL) of 9.486 (7.353) mg/kg. Gene set transcriptional changes and apical endpoints provided potency estimates slightly higher than Eci1. Follow-up studies that investigate transcriptional changes at lower doses will be a useful future direction to determine the biological potency of 6:1 FTOH more accurately.
In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

References


In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats


Appendix A. Internal Dose Assessment

Table of Contents

A.1. Quantitation of 6:1 Fluorotelomer Alcohol in Plasma.............................................................. A-2
A.1. Quantitation of 6:1 Fluorotelomer Alcohol in Plasma

Quantification of 6:1 fluorotelomer alcohol (6:1 FTOH) in plasma samples was completed by MRIGlobal (Kansas City, MO). A high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed to determine 6:1 FTOH concentrations in rat plasma. A six-point matrix calibration curve, in the range of 10–160 ng/mL, was prepared by adding 10 μL of an appropriate spiking solution of 6:1 FTOH in methanol to 50 μL of control matrix (adult male Sprague Dawley rat plasma). Quality control (QC) samples were prepared similarly at a target concentration of 50 ng/mL in plasma. Blanks and study samples were prepared like standards, except 10 μL of methanol was used in place of spiking solution. To each sample, 100 μL of prechilled solution containing 250 ng 2-perfluorohexyl-[1,1-2H2]-[1,2-13C2]-ethanol/mL (internal standard) in methanol was added, mixed for 1 minute, and allowed to stand at 4°C for 10 minutes. All samples were centrifuged at approximately 18,000 × g for 10 minutes and supernatants were collected for analysis.

All samples were analyzed using a Sciex Exion AC LC coupled to an API 4000 MS/MS (Framingham, MA). An Agilent Zorbax Eclipse Plus C18 column (2.1 × 150 mm, 5 µm) was used with mobile phases A (water) and B (methanol). A flow rate of 0.3 mL/min was run with a linear gradient of 80%–100% B in 4 minutes and held for 4 minutes. The electrospray ion source was operated in negative ion mode with a source temperature of 400°C and an ion spray voltage of ~4,500 V. Transition ranges monitored were m/z 349 to 169 (quantitation ion) and 349 to 309 (confirmation ion) for 6:1 FTOH, and m/z 367 to 306 for the internal standard.

A linear regression with 1/X weighting was used to relate peak area ratio of analyte to internal standard and analyte concentration. Calibration curves were linear (r > 0.99). The limit of detection (LOD; 2.9 ng/mL) was estimated as three times the standard deviation of the lower limit of quantitation (LOQ; 10.0 ng/mL), expressed as concentration. For QC samples, the accuracy measured as percent relative error was within ±27.8% of the nominal concentration with relative standard deviations ≤4.7%. The concentrations (ng/mL) of 6:1 FTOH in study samples were calculated using peak area ratios and the regression equation. All values above LOD were reported.
Appendix B. Animal Identifiers

Tables
Table B-1. Animal Numbers and FASTQ Data File Names .......................................................B-2
## Table B-1. Animal Numbers and FASTQ Data File Names

<table>
<thead>
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### In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

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### In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

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</tr>
<tr>
<td>414</td>
<td>Male</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>333.0</td>
<td>Yes</td>
<td>Kidney</td>
<td>Plate6-414</td>
</tr>
<tr>
<td>414</td>
<td>Male</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>333.0</td>
<td>Yes</td>
<td>Liver</td>
<td>Plate4-414</td>
</tr>
<tr>
<td>415</td>
<td>Male</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>333.0</td>
<td>Yes</td>
<td>Kidney</td>
<td>Plate6-415</td>
</tr>
</tbody>
</table>
# In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Sex</th>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Survived to Study Termination</th>
<th>Tissue</th>
<th>FASTQ File ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>415</td>
<td>Male</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>333.0</td>
<td>Yes</td>
<td>Liver</td>
<td>Plate4-415</td>
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<tr>
<td>416</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>333.0</td>
<td>Yes</td>
<td>Kidney</td>
<td>Plate6-416</td>
</tr>
<tr>
<td>416</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>333.0</td>
<td>Yes</td>
<td>Liver</td>
<td>Plate2-416</td>
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<td>417</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>333.0</td>
<td>Yes</td>
<td>Kidney</td>
<td>Plate6-417</td>
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<tr>
<td>417</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>333.0</td>
<td>Yes</td>
<td>Liver</td>
<td>Plate2-417</td>
</tr>
<tr>
<td>418</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>333.0</td>
<td>Yes</td>
<td>Kidney</td>
<td>Plate8-418&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>418</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>333.0</td>
<td>Yes</td>
<td>Liver</td>
<td>Plate2-418</td>
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<tr>
<td>419</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>333.0</td>
<td>Yes</td>
<td>Kidney</td>
<td>Plate8-419&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>419</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>333.0</td>
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<td>Liver</td>
<td>Plate2-419</td>
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<tr>
<td>420</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>333.0</td>
<td>Yes</td>
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<tr>
<td>421</td>
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<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>Yes</td>
<td>Kidney</td>
<td>Plate8-421&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>421</td>
<td>Male</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>Yes</td>
<td>Liver</td>
<td>Plate2-421&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>422</td>
<td>Male</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>No</td>
<td>None</td>
<td>NA</td>
</tr>
<tr>
<td>423</td>
<td>Male</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>No</td>
<td>None</td>
<td>NA</td>
</tr>
<tr>
<td>424</td>
<td>Male</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>No</td>
<td>None</td>
<td>NA</td>
</tr>
<tr>
<td>425</td>
<td>Male</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>No</td>
<td>None</td>
<td>NA</td>
</tr>
<tr>
<td>426</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>Yes</td>
<td>Kidney</td>
<td>Plate6-426</td>
</tr>
<tr>
<td>426</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>Yes</td>
<td>Liver</td>
<td>Plate2-426</td>
</tr>
<tr>
<td>427</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>Yes</td>
<td>Kidney</td>
<td>Plate6-427</td>
</tr>
<tr>
<td>427</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>Yes</td>
<td>Liver</td>
<td>Plate2-427</td>
</tr>
<tr>
<td>428</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>Yes</td>
<td>Kidney</td>
<td>Plate6-428</td>
</tr>
<tr>
<td>428</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>Yes</td>
<td>Liver</td>
<td>Plate2-428</td>
</tr>
<tr>
<td>429</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>Yes</td>
<td>Kidney</td>
<td>Plate6-429</td>
</tr>
<tr>
<td>429</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>Yes</td>
<td>Liver</td>
<td>Plate4-429</td>
</tr>
<tr>
<td>430</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>Yes</td>
<td>Kidney</td>
<td>Plate6-430</td>
</tr>
<tr>
<td>430</td>
<td>Female</td>
<td>6:1 Fluorotelomer alcohol</td>
<td>1,000.0</td>
<td>Yes</td>
<td>Liver</td>
<td>Plate2-430</td>
</tr>
</tbody>
</table>

**NA** = no transcriptomics data collected for selected animal.

<sup>a</sup> Removed due to plate/batch effect.

<sup>b</sup> Removed due to principal component analysis/hierarchical cluster analysis outlier.

<sup>c</sup> Removed due to quality control fail.
In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

Appendix C. Transcriptomic Quality Control and Empirical False Discovery Rate

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C.1. Gene Expression Quality Control

A principal component analysis (PCA) plot enables visualization of global transcriptional changes in two dimensions, with each plot showing a different angle on the basis of the principal components plotted. Global transcript data are shown for individual animals (dots) within each dose group (designated by color). Dots that are spatially closer to each other indicate more similarity in global expression profiles; dots that are farther apart indicate dissimilarity in global expression profiles for those animals. The data represented in the plot are those employed in dose response modeling (i.e., if outliers were identified in the quality control process, they were removed from the data set and are not present in the plot). Visual inspection does not suggest subgrouping of the data other than dose-related changes, which indicates any technical batch-related effects are minimal.

Figure C-1. A Principal Component Analysis of the Normalized Data from the Liver of Male Rats
A principal component analysis (PCA) plot enables visualization of global transcriptional changes in two dimensions, with each plot showing a different angle on the basis of the principal components plotted. Global transcript data are shown for individual animals (dots) within each dose group (designated by color). Dots that are spatially closer to each other indicate more similarity in global expression profiles; dots that are farther apart indicate dissimilarity in global expression profiles for those animals. The data represented in the plot are those employed in dose response modeling (i.e., if outliers were identified in the quality control process, they were removed from the data set and are not present in the plot). Visual inspection does not suggest subgrouping of the data other than dose-related changes, which indicates any technical batch-related effects are minimal.

Figure C-2. A Principal Component Analysis of the Normalized Data from the Liver of Female Rats
A principal component analysis (PCA) plot enables visualization of global transcriptional changes in two dimensions, with each plot showing a different angle on the basis of the principal components plotted. Global transcript data are shown for individual animals (dots) within each dose group (designated by color). Dots that are spatially closer to each other indicate more similarity in global expression profiles; dots that are farther apart indicate dissimilarity in global expression profiles for those animals. The data represented in the plot are those employed in dose response modeling (i.e., if outliers were identified in the quality control process, they were removed from the data set and are not present in the plot). Visual inspection does not suggest subgrouping of the data other than dose-related changes, which indicates any technical batch-related effects are minimal.
In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

Figure C-4. A Principal Component Analysis of the Normalized Data from the Kidney of Female Rats

A principal component analysis (PCA) plot enables visualization of global transcriptional changes in two dimensions, with each plot showing a different angle on the basis of the principal components plotted. Global transcript data are shown for individual animals (dots) within each dose group (designated by color). Dots that are spatially closer to each other indicate more similarity in global expression profiles; dots that are farther apart indicate dissimilarity in global expression profiles for those animals. The data represented in the plot are those employed in dose response modeling (i.e., if outliers were identified in the quality control process, they were removed from the data set and are not present in the plot). Visual inspection does not suggest subgrouping of the data other than dose-related changes, which indicates any technical batch-related effects are minimal.
C.2. Empirical False Discovery Rate

C.2.1. Methods

Empirical false discovery assessment was performed to evaluate the performance of the benchmark dose (BMD) analysis technique and underlying probe/pathway filtering criteria. Toward this goal, 20 computationally generated data sets were used with this study design (each data set containing 10 vehicle control replicates and 5 replicates per dose), and equivalent BMD analysis was performed using the same parameter configurations. The 20 data sets were generated from the original 6:1 fluorotelomer alcohol (6:1 FTOH) study data, along with data from three other chemicals that were studied in parallel under a similar protocol.26-28

For a given group (tissue per sex combination), up to 40 vehicle control samples from the original studies (10 replicates × 4 chemicals) were used for this analysis. The previously identified outlier vehicle control samples and overflow plate control samples exhibiting a batch effect were excluded from this analysis.

Each computationally generated sample was created by randomly mixing the normalized expression signal from two randomly selected vehicle control samples using a weighted average approach. The weights utilized during per-probe mixing were randomly simulated from uniform (0,1) distribution. A total of 55 samples (10 vehicle control samples + 45 dosed samples [9 doses × 5 replicates]) were computationally generated per data set and assigned to either vehicle control or 1 of the 9 dosed groups that were separated by approximately half-log spacing, consistent with the dose spacing used in the original studies. For each group, 20 such data sets were generated. Because each of the 20 generated data sets used in the empirical false discovery analysis was derived from actual vehicle control samples, none of the data sets should have any true dose-responsive genes.

Each data set was then analyzed using the same parameter settings and significance criteria that were implemented in the original study. At the gene level, genes that passed the following criteria were considered false positive discoveries: fold change ≥2, Williams’s trend p value ≤0.05, global goodness-of-fit p value >0.1, BMD upper confidence limit/BMD lower confidence limit (BMDU/BMDL) ≤40, and BMD < highest dose tested. Categorical analysis on Gene Ontology (GO) gene sets was performed using the genes that passed the gene-level criteria with maximum absolute fold change ≥1.5. At the gene set GO level, GO biological processes that passed the following criteria were considered false positive discoveries: ≥3 genes that pass all filters, totaling at least 5% of the genes in a gene set.

False positive discovery rates were assessed for each computationally generated data set using the following equations:

\[
\text{False Positive Gene Rate} = \frac{\text{# False Positive Entrez Gene IDs}}{2,680} \times 100
\]

\[
\text{False Positive GO Biological Process Rate} = \frac{\text{# False Positive GO Biological Processes}}{5,667} \times 100
\]

where 2,680 is the number of unique Entrez Gene IDs on the rat S1500+ platform and 5,667 is the number of GO biological processes that have at least three genes in rat S1500+. 
Mean and median false discovery rates across all 20 computationally generated data sets were calculated for each tissue per sex in the study.

C.2.2. Results

The number of false positives for genes and GO biological processes are given in Table C-1. Mean and median false positive rates were <0.1% for genes and <0.5% for GO biological processes for all tissue per sex group (Figure C-5 and Figure C-6). The maximum false positive rates for any of the 80 computationally generated control data sets were 0.3% (gene) and 4.4% (GO biological process).

Figure C-5. Boxplots of the False Positive Gene Rate for Each Tissue per Sex Combination

Each boxplot displays the distribution of the false positive rates for 20 computationally generated data sets.
Figure C-6. Boxplots of the False Positive Gene Ontology Biological Process Rate for Each Tissue per Sex Combination

Each boxplot displays the distribution of the false positive rates for 20 computationally generated data sets.

Table C-1. Number of False Positives

<table>
<thead>
<tr>
<th>Generated Data Set</th>
<th># False Positive Genes</th>
<th># False Positive GO Biological Process</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kidney Female</td>
<td>Kidney Male</td>
</tr>
<tr>
<td>01</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>02</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>03</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>04</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>05</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>06</td>
<td>1</td>
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<td>4</td>
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</tr>
<tr>
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<td>0</td>
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</tr>
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<td>0</td>
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</tr>
<tr>
<td>18</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>19</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

GO = Gene Ontology.
Appendix D. Benchmark Dose Model Recommendation and Selection Methodologies

Tables

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Table D-1. Benchmark Dose Model Recommendation/Selection Rules for Apical Endpoints

<table>
<thead>
<tr>
<th>Rule</th>
<th>Criteria for “Viable”</th>
<th>Numerical Threshold</th>
<th>Bin Placement for Rule Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD Existence</td>
<td>A BMD exists.</td>
<td>NA</td>
<td>Failure</td>
</tr>
<tr>
<td>BMD(\L) Existence</td>
<td>A BMD(\L) exists.</td>
<td>NA</td>
<td>Failure</td>
</tr>
<tr>
<td>AIC Existence</td>
<td>An AIC exists.</td>
<td>NA</td>
<td>Failure</td>
</tr>
<tr>
<td>Residual of Interest Existence</td>
<td>The residual at the dose group closest to the BMD (i.e., the residual of interest) exists.</td>
<td>NA</td>
<td>Failure</td>
</tr>
<tr>
<td>Variance Model Fit</td>
<td>The variance model used fits the data.</td>
<td>NA</td>
<td>Nonviable</td>
</tr>
<tr>
<td>Variance Model Selection</td>
<td>The variance model is appropriate.</td>
<td>NA</td>
<td>Nonviable</td>
</tr>
<tr>
<td>Global Goodness of Fit</td>
<td>The mean model fits the data means sufficiently well (BMDS 2.7.0 Test 4 p value &gt;N).</td>
<td>0.1</td>
<td>Nonviable</td>
</tr>
<tr>
<td>Degrees of Freedom</td>
<td>There is at least 1 degree of freedom (i.e., more dose groups than model parameters).</td>
<td>NA</td>
<td>Nonviable</td>
</tr>
<tr>
<td>BMD-to-BMD(\L) Ratio</td>
<td>The ratio of BMD to BMD(\L) is not large (BMD/BMD(\L) &lt;N).</td>
<td>20</td>
<td>Viable</td>
</tr>
<tr>
<td>High BMD(\L)</td>
<td>The BMD(\L) is &lt;N times higher than the maximum dose.</td>
<td>1</td>
<td>Viable</td>
</tr>
<tr>
<td>High BMD</td>
<td>The BMD is &lt;N times higher than the maximum dose.</td>
<td>1</td>
<td>Viable</td>
</tr>
<tr>
<td>Low BMD</td>
<td>The BMD is &lt;N times lower than the minimum nonzero dose.</td>
<td>3</td>
<td>Nonreportable</td>
</tr>
<tr>
<td>Control Residual</td>
<td>The residual at control is small (residual &lt;N).</td>
<td>2</td>
<td>Nonviable</td>
</tr>
<tr>
<td>Control Standard Deviation</td>
<td>The modeled standard deviation is similar to the actual (&lt;N times different).</td>
<td>1.5</td>
<td>Nonviable</td>
</tr>
<tr>
<td>Residual of Interest</td>
<td>The residual at the dose group closest to the BMD (i.e., the residual of interest) is small (residual &lt;N).</td>
<td>2</td>
<td>Nonviable</td>
</tr>
<tr>
<td>No Warnings Reported</td>
<td>No warnings in the BMD model system were reported.</td>
<td>NA</td>
<td>Viable</td>
</tr>
</tbody>
</table>

BMD = benchmark dose; NA = not applicable; BMD\(\L\) = benchmark dose lower confidence limit; AIC = Akaike information criterion; BMDS = Benchmark Dose Software; N = numerical threshold.
In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

**Figure D-1. Benchmark Dose Model Recommendation/Selection Methodology for Automated Benchmark Dose Execution of Apical Endpoints**

Source: Figure adapted from Wignall et al. (2014)²³

BMD = benchmark dose; BMDL = benchmark dose lower confidence limit; AIC = Akaike information criterion.
In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

Figure D-2. Benchmark Dose Model Recommendation/Selection Methodology for Benchmark Dose Execution of Gene Sets with Expression Changes Enacted by Chemical Exposure

Adapted from Thomas et al. (2007)34
Exp = exponential; Poly = polynomial; BMD = benchmark dose; BMDL = benchmark dose lower confidence limit; BMDU = benchmark dose upper confidence limit; AIC = Akaike information criterion; GGOF = global goodness of fit; GO = Gene Ontology.
Appendix E. Organ Weight Descriptions

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In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

E.1. Organ Weight Descriptions

Liver: The liver carries out biotransformation and excretion of endogenous and xenobiotic substances, regulation of blood sugar, enzymatic transformation of essential nutrients, generation of blood proteins involved in fluid balance and clotting, and bile production for digestion and absorption of fats. Liver weight changes can be an indication of chemical-induced stress. Specifically, in subacute studies, increases in liver weight in response to low doses of toxicants typically stem from increases in xenobiotic metabolizing enzymes and associated hepatocyte hypertrophy or peroxisome proliferation. Increased liver weight, particularly when accompanied by evidence of leakage of liver-specific enzymes into blood, likely reflects hemodynamic changes related to severe hepatotoxicity. Higher liver weight relative to body weight may also occur at any dose level that causes a slowed rate of body growth and does not necessarily indicate liver toxicity. Decreased liver weight in subacute studies is typically of unknown toxicological significance but in rare cases may be related to glycogen depletion.

Kidney: The kidneys remove waste products and xenobiotics from the body, balance blood electrolytes, regulate blood pressure through the release of hormones, synthesize the active form of vitamin D, and control the production of erythropoiesis. In subacute studies, changes in kidney weight may reflect renal toxicity (particularly if accompanied by increases in other markers of kidney toxicity, e.g., increased Kim-1) and/or tubular hypertrophy. Decreased kidney weights in subacute studies are typically of unknown toxicological significance.

Heart: The heart drives the circulatory system, supplying oxygen and essential macro- and micronutrients to the tissues. Increased heart weight in subacute studies would indicate severe cardiotoxicity, compensatory myocardial hypertrophy, and/or pulmonary injury. Decreased heart weight in subacute studies is often of unknown toxicological significance; however, it may be caused by decreased load on the heart from dehydration or modulation of contractility.
In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

Appendix F. Supplemental Data

The following supplemental files are available at https://doi.org/10.22427/NIEHS-DATA-NIEHS-07.

F.1. Apical Benchmark Dose Analysis

Mean Body Weight Summary
C20027-01_Mean_Body_Weight_Summary.docx

Organ Weights Summary
C20027-01_Organ_Weights_Summary.docx

Clinical Chemistry Summary
C20027-01_Clinical_Chemistry_Summary.docx

Hematology Summary
C20027-01_Hematology_Data_Summary.docx

Hormone and Enzymes Summary
C20027-01_Hormone_Summary.docx

BMD, NOEL and LOEL Summary for Apical Endpoints
C20027-01_BMD_BMDL_LOEL_and_NOEL_Summary_for_Apical_Endpoints_Sorted_by_BMD_LOEL_from_Low_to_High.docx

Male BMD Apical Endpoints Model Fits
C20027-01_Appendix_Male_07282021.docx

Female BMD Apical Endpoints Model Fits
C20027-01_Appendix_Female_07282021.docx

BMD Model Recommendation Selection Rules
C20027-01_Benchmark_Dose_Model_Recommendation_Selection_Rules_for_Apical_Endpoints.docx

Read Me
C20027-01_ReadME.docx

Male Model Parameters
C20027-01_Parameter_Male_07282021.xlsx

Female Model Parameters
C20027-01_Parameter_Female_07282021.xlsx

BMDs code package
C20027-01_bmds.zip
F.2. Genomic Benchmark Dose Analysis

**BMDExpress Project File (bm2 format)**
C20027-01_EPA_PFAS_Kidney_Overflow_plate_removed_S1500_Plus_Analysis_Traditional.bm2

**Top 10 Genes Ranked by Potency of Perturbation_Kidney**
C20027-01_Kidney_Top_10_Genes_Ranked_by_Potency_of_Perturbation_Sorted_by_BMD_Median.docx

**Top 10 GO Biological Process Gene Sets_Kidney**

**Top 10 Genes Ranked by Potency of Perturbation_Liver**
C20027-01_Liver_Top_10_Genes_Ranked_by_Potency_of_Perturbation_Sorted_by_BMD_Median.docx

**Top 10 GO Biological Process Gene Sets_Liver**

**BMDExpress Expression Data_Kidney_Female**
C20027-01_Kidney_6-1_FTOH_Female.txt

**BMDExpress Expression Data_Kidney_Male**
C20027-01_Kidney_6-1_FTOH_Male.txt

**BMDExpress Expression Data_Liver_Female**
C20027-01_Liver_6-1_FTOH_Female.txt

**BMDExpress Expression Data_Liver_Male**
C20027-01_Liver_6-1_FTOH_Male.txt

**BMDExpress Individual Gene BMD Results_Kidney_Male**
C20027-01_Kidney_6-1_FTOH_Male_williams_0.05_NOMTC_foldfilter1.5_BMD_S1500_Plus_Rat_GENE_true_true_pval0.1_ratio40_foldchange2_conf0.5.txt

**BMDExpress GO Biological Process Deduplicated BMD Results_Kidney_Male**
C20027-01_Kidney_6-1_FTOH_Male_williams_0.05_NOMTC_foldfilter1.5_BMD_S1500_Plus_Rat_GO_BP_true_true_pval0.1_ratio40_conf0.5_deduplicate.txt
In Vivo Repeat Dose Biological Potency Study of 6:1 Fluorotelomer Alcohol in Sprague Dawley Rats

**BMDExpress Individual Gene BMD Results_Kidney_Female**
C20027-01_Kidney_6-
1_FTOH_Female_williams_0.05_NOMTC_foldfilter1.5_BMD_S1500_Plus_Rat_GENE_true_true_pval0.1_ratio40_foldchange2_conf0.5.txt

**BMDExpress GO Biological Process Deduplicated BMD Results_Kidney_Female**
C20027-01_Kidney_6-
1_FTOH_Female_williams_0.05_NOMTC_foldfilter1.5_BMD_S1500_Plus_Rat_GO_BP_true_true_pval0.1_ratio40_conf0.5_deduplicate.txt

**BMDExpress Individual Gene BMD Results_Liver_Male**
C20027-01_Liver_6-
1_FTOH_Male_williams_0.05_NOMTC_foldfilter1.5_BMD_S1500_Plus_Rat_GENE_true_true_pval0.1_ratio40_conf0.5_deduplicate.txt

**BMDExpress GO Biological Process Deduplicated BMD Results_Liver_Male**
C20027-01_Liver_6-
1_FTOH_Male_williams_0.05_NOMTC_foldfilter1.5_BMD_S1500_Plus_Rat_GO_BP_true_true_pval0.1_ratio40_conf0.5_deduplicate.txt

**BMDExpress Individual Gene BMD Results_Liver_Female**
C20027-01_Liver_6-
1_FTOH_Female_williams_0.05_NOMTC_foldfilter1.5_BMD_S1500_Plus_Rat_GENE_true_true_pval0.1_ratio40_conf0.5_deduplicate.txt

**BMDExpress GO Biological Process Deduplicated BMD Results_Liver_Female**
C20027-01_Liver_6-
1_FTOH_Female_williams_0.05_NOMTC_foldfilter1.5_BMD_S1500_Plus_Rat_GO_BP_true_true_pval0.1_ratio40_conf0.5_deduplicate.txt

**BMDExpress Prefilter Results_Kidney_Female**
C20027-01_BMDExpress_Prefilter_Results_Kidney_Female.txt

**BMDExpress Prefilter Results_Kidney_Male**
C20027-01_BMDExpress_Prefilter_Results_Kidney_Male.txt

**BMDExpress Prefilter Results_Liver_Female**
C20027-01_BMDExpress_Prefilter_Results_Liver_Female.txt

**BMDExpress Prefilter Results_Liver_Male**
C20027-01_BMDExpress_Prefilter_Results_Liver_Male.txt

**Animal and Fastaq Metadata**
C20027-01_Animal_and_FASTQ_Metadata.zip

**Kidney Principal Components Analysis Files**
C20027-01_Kidney_PCA.zip

**Liver Principal Components Analysis Files**
C20027-01_Liver_PCA.zip
Individual Gene BMD Analysis Results File
C20027-01_Individual_Gene_DEFINED_Category_Files_for_Gene_Level_BMD_Analysis_of_Array_Platform_GPL1355.zip

BMDExpress Software
C20027-01_Software.zip

BMDExpress Project File (JSON format)
C20027-01_EPA_PFAS_Kidney_Overflow_plate_removed_S1500_Plus_Analysis_Traditional_JSON.zip

GO Biological Process BMD Analysis Results
C20027-01_Functional_Classification_Annotation_Files_for_GO_Biological_Process_Analysis_of_Array_Platform_GPL1355.zip

F.3. Study Tables

I04 – Mean Body Weight Summary
C20027-01_I04_-_Mean_Body_Weight_Summary.pdf

I05 – Clinical Observations Summary
C20027-01_I05_-_Clinical_Observations_Summary.pdf

PA06 – Organ Weights Summary
C20027-01_PA06_-_Organ_Weights_Summary.pdf

PA41 – Clinical Chemistry Summary
C20027-01_PA41_-_Clinical_Chemistry_Summary.pdf

PA43 – Hematology Summary
C20027-01_PA43_-_Hematology_Summary.pdf

PA48 – Summary of Tissue Concentration
C20027-01_PA48_-_Summary_of_Tissue_Concentration.pdf

R07 – Hormone Summary
C20027-01_R07_-_Hormone_Summary.pdf

F.4. Individual Animal Data

Individual Animal Body Weight Data
C20027-01_Individual_Animal_Body_Weight_Data.xlsx

Individual Animal Clinical Chemistry Data
C20027-01_Individual_Animal_Clinical_Chemistry_Data.xlsx
Individual Animal Clinical Observations Data
C20027-01_Individual_Animal_Clinical_Observations_Data.xlsx

Individual Animal Hormone Data
C20027-01_Individual_Animal_Hormone_Data.xlsx

Individual Animal Organ Weight Data
C20027-01_Individual_Animal_Organ_Weight_Data.xlsx

Individual Animal Hematology Data
C20027-01_Individual_Animal_Hematology_Data.xlsx

Individual Animal Tissue Concentration Data
C20027-01_Individual_Animal_Tissue_Concentration_Data.xlsx