National Institute of
Environmental Health Sciences

NIEHS Report on the In Vivo Repeat Dose Biological Potency Study of Isopropylated Phenol Phosphate (CASRN 68937-41-7) in Male Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats (Gavage Studies)

NIEHS 05

October 2022

NIEHS Report on the In Vivo Repeat Dose Biological Potency Study of Isopropylated Phenol Phosphate (CASRN 68937-41-7) in Male Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats (Gavage Studies)

NIEHS Report 05

October 2022

National Institute of Environmental Health Sciences
Public Health Service
U.S. Department of Health and Human Services
ISSN: 2768-5632

Research Triangle Park, North Carolina, USA

Foreword

The National Institute of Environmental Health Sciences (NIEHS) is one of 27 institutes and centers of the National Institutes of Health, which is part of the U.S. Department of Health and Human Services. The NIEHS mission is to discover how the environment affects people in order to promote healthier lives. NIEHS works to accomplish its mission by conducting and funding research on human health effects of environmental exposures; developing the next generation of environmental health scientists; and providing critical research, knowledge, and information to citizens and policymakers who are working to prevent hazardous exposures and reduce the risk of disease and disorders connected to the environment. NIEHS is a foundational leader in environmental health sciences and committed to ensuring that its research is directed toward a healthier environment and healthier lives for all people.

The NIEHS Report series began in 2022. The environmental health sciences research described in this series is conducted primarily by the Division of Translational Toxicology (DTT) at NIEHS. NIEHS/DTT scientists conduct innovative toxicology research that aligns with real-world public health needs and translates scientific evidence into knowledge that can inform individual and public health decision-making.

NIEHS reports are available free of charge on the NIEHS/DTT website and cataloged in PubMed, a free resource developed and maintained by the National Library of Medicine (part of the National Institutes of Health).

Table of Contents

Tables

About This Report

Authors

Scott S. Auerbach[1], Mamta V. Behl [1], Bradley J. Collins[1], Michelle C. Cora[1], Jennifer M. Fostel[1], Brandall L. Ingle[2], Ying F. Liu[3], Jeanne Luh[2], Georgia K. Roberts[1], Kelly A. Shipkowski[1], Suramya Waidyanatha[1], AtLee T.D. Watson[1]

[1]Division of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA

[2]ICF, Fairfax, Virginia, USA

[3]ASRC Federal, Research Triangle Park, North Carolina, USA

Division of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA
Evaluated and interpreted the results and reported findings; developed reporting framework
Scott S. Auerbach, Ph.D.
Mamta V. Behl, Ph.D.
Michelle C. Cora, D.V.M.
Georgia K. Roberts, Ph.D.
Kelly A. Shipkowski, Ph.D.
AtLee T.D. Watson, Ph.D.

Coordinated data integration
Jennifer M. Fostel, Ph.D.

Analyzed and interpreted analytical chemistry data
Bradley J. Collins, M.S.P.H
Suramya Waidyanatha, Ph.D.

Coordinated data assembly
Georgia K. Roberts, Ph.D.

ICF, Fairfax, Virginia, USA
Contributed to technical writing and data integration and ensured report quality
Brandall L. Ingle, Ph.D.
Jeanne Luh, Ph.D.

ASRC Federal, Research Triangle Park, North Carolina, USA
Developed data tables and supplemental materials
Ying F. Liu, Ph.D.

Contributors

Division of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA
Critically reviewed report and results
John R. Bucher, Ph.D.
Fred M. Parham, Ph.D.
Nigel J. Walker, Ph.D.

Directed in-life studies
Daniel L. Morgan, Ph.D.

Contributed to development and review of reporting framework
Michael J. DeVito, Ph.D.
William M. Gwinn, Ph.D.
Scott A. Masten, Ph.D.
Matthew D. Stout, Ph.D.
Greg S. Travlos, D.V.M.
Mary S. Wolfe, Ph.D.

Developed and updated BMDS software package
Andrew J. Shapiro, M.S.P.H.

Alion, McLean, Virginia, USA
Conducted in-life studies
Dorian S. Olivera, Ph.D.

ASRC Federal, Research Triangle Park, North Carolina, USA
Developed data tables and supplemental materials
Julie Berke, B.S.
Karen Gilbert, B.S.
Shihan He, Ph.D.
Amber Macpherson, B.S.
Christina Myers, M.S.
Ashwin Raghuraman, M.S.

ICF, Fairfax, Virginia, USA
Provided contract oversight
David F. Burch, M.E.M., Principal Investigator
Jessica A. Wignall, M.S.P.H.

Prepared and edited report
Joshua Cleland, M.E.M.
Tara Hamilton, M.S.
Penelope E. Kellar, M.S.
Whitney Mitchell, B.S.
Lisa M. Prince, Ph.D.

MRIGlobal, Kansas City, Missouri, USA
Provided chemical formulations
Kristin L. Aillon, Ph.D.
Joseph W. Algaier, Ph.D.

Peer Review

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 Isopropylated Phenol Phosphate (CASRN 68937-41-7) in Male Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats (Gavage Studies)* was not subjected to further external peer review.

Publication Details

Publisher: National Institute of Environmental Health Sciences

Publishing Location: Research Triangle Park, NC

ISSN: 2768-5632

DOI: https://doi.org/10.22427/NIEHS-05

Report Series: NIEHS Report Series

Report Series Number: 05

Official citation: Auerbach SS, Behl MV, Collins BJ, Cora MC, Fostel JM, Ingle BL, Liu YF, Luh J, Roberts GK, Shipkowski KA, Waidyanatha S, Watson ATD. 2022. NIEHS report on the in vivo repeat dose biological potency study of isopropylated phenol phosphate (CASRN 68937-41-7) in male Sprague Dawley (Hsd:Sprague Dawley® SD®) rats (gavage studies). Research Triangle Park, NC: National Institute of Environmental Health Sciences. NIEHS Report 05.

Acknowledgments

This work was supported by the Intramural Research Program (ES103316, ES103318, ES103319, ES102505) at the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health and performed for NIEHS under contracts GS00Q14OADU417 (Order No. HHSN273201600015U), HHSN273201400020C, HHSN316201200054W, and HHSN291200775561C.

Abstract

Background: Isopropylated phenol phosphate (IPP) is an organophosphate flame retardant currently on the market that is used as a replacement for phased-out polybrominated diphenyl ethers. Commercially available IPP is a mixture of triphenyl phosphate and mono-, di-, and tri-isopropyl-substituted isomers. Toxicological information on this class of chemicals is sparse. A short-term, in vivo transcriptomic study was used to assess the biological potency of IPP.

Methods: Scientists at the Division of Translational Toxicology, National Institute of Environmental Health Sciences conducted this short-term in vivo biological potency study on IPP in young adult male Sprague Dawley (Hsd:Sprague Dawley® SD®) rats. IPP was formulated in corn oil and administered once daily for 4 consecutive days by gavage. IPP was tested at six doses (0, 77, 153, 306, 611, and 1,222 mg/kg body weight [mg/kg] corresponding to 0, 0.169, 0.338, 0.675, 1.35, and 2.7 mmol/kg). On study day 4, animals were euthanized, standard toxicological measures were assessed, and the liver was assayed in gene expression studies using Affymetrix microarrays. Modeling was conducted to identify the benchmark doses (BMDs) associated with apical toxicological endpoints and transcriptional changes in the liver. 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 could be obtained. The effects include decreased serum albumin/globulin ratio, increased relative liver weight, decreased serum albumin concentration, decreased creatinine level, decreased terminal body weight, and increased absolute liver weight. The BMDs and benchmark dose lower confidence limits (BMD_Ls) were 51.3 (27.0), 55.8 (33.7), 167.6 (63.2), 223.9 (158.9), 278.2 (187.3), and 400.0 (246.0) mg/kg, respectively. Although serum cholinesterase activity was significantly decreased and total cholesterol concentration was significantly increased in all dosed groups, beginning with 77 mg/kg (the lowest-observed-effect level), their BMD values were below the lower limit of extrapolation (<25.7 mg/kg). High-density lipoprotein (HDL) cholesterol concentration was also significantly increased at all doses, beginning with 77 mg/kg, but a BMD (BMD_L) was not determined because no viable model was available.

The top 10 most sensitive Gene Ontology biological processes had estimated BMD median values <25.7 mg/kg. The top 10 most sensitive individual genes also exhibited changes in expression at dose levels below which a reliable estimate of potency could be achieved (<25.7 mg/kg). Six of these genes were upregulated: *Tbx3*, *Nr1d1*, *Nr1d2*, *Sik2*, *Oaf*, and *Dbp*. Four genes, *Gck*, *Syne1*, *Cdkn1a*, and *Cldn1*, were downregulated.

Summary: Taken together, the most sensitive apical endpoint BMD (BMD_L) value that could be reliably determined occurred at 51.3 (27.0) mg/kg. The BMDs (BMD_Ls) could not be determined for the top 10 most sensitive gene sets or the top 10 most sensitive individual genes and were estimated to be <25.7 mg/kg. Serum cholinesterase inhibition and increases in serum total cholesterol were also estimated to be <25.7 mg/kg. HDL cholesterol appeared to be a sensitive endpoint at all doses, but its BMD (BMD_L) was not determined because no viable model was available. Future studies investigating lower doses would be helpful to obtain more accurate estimates of BMD values for the most sensitive gene sets, individual genes, and for serum cholinesterase inhibition and total cholesterol increases.

Background

Isopropylated phenol phosphate (IPP) is an organophosphate flame retardant (OPFR). OPFRs are organic phosphate esters used in a diverse collection of products to interrupt or hinder combustion.[1] OPFRs can leach from treated materials and persist in the environment.[2] They have been detected in indoor air, household dust, wastewater treatment plant effluent, drinking water, and wildlife samples.[3-6] The literature contains little information on the incidence and potency of health effects associated with exposure to this chemical class. For this reason, OPFRs were nominated to the National Institute of Environmental Health Sciences for toxicological characterization.

Reported here are the results of a repeat dose study of IPP performed in male rats. The goal of this study is to provide a rapid assessment of in vivo biological potency by evaluating a combination of traditional toxicological endpoints and transcriptomics analysis to broadly query biological space for any dose-related change. The justification for using this type of assessment relates to the observation that gene set benchmark dose values from short-term transcriptomic studies have been shown to approximate dose responsiveness of the most sensitive apical endpoints from resource intensive guideline toxicological assessments (e.g., carcinogenicity).[7; 8] Importantly, the study reported here is not intended to assess or identify hazards. In particular, any observations related to traditional toxicological hazards gleaned from qualitative interpretation of the transcriptomics data should be considered hypotheses requiring further evaluation.

Materials and Methods

Study Design

Young adult male Sprague Dawley (Hsd:Sprague Dawley® SD®) rats were obtained from Harlan Laboratories, Inc. (now Envigo, Indianapolis, IN). Males were selected because of the historical precedent of using males in transcriptomic studies to avoid challenges with hormonal cyclicity in female rats that can affect interpretation of gene expression data. On receipt, the rats were 7–8 weeks of age. Animals were quarantined for 7 days, and then randomly assigned to one of six dose groups, each containing five to seven rats. The rats in each dose group then were administered isopropylated phenol phosphate (IPP) by gavage in corn oil at a dose level of 0, 77, 153, 306, 611, or 1,222 mg/kg body weight. These doses correspond to molar equivalencies of 0, 0.169, 0.338, 0.675, 1.35, and 2.7 mmol/kg. Corn oil was selected as the vehicle on the basis of physical and chemical properties that indicated the test article would exhibit maximal solubility in corn oil relative to other commonly used vehicles. Dosing of the animals with the test article occurred on 4 consecutive days. Dosage volume was 5 mL/kg body weight and was based on the most recently measured body weight. Euthanasia, blood/serum collection, and tissue sample collection were completed on the day following the final administration of the test article (study day 4). Animal identification numbers and microarray data file names for each animal are presented in Appendix A.

Dose Selection Rationale

Dose selection was informed by National Toxicology Program (NTP) subchronic studies of tricresyl phosphate, a chemical structurally similar to IPP. At dose levels of approximately 1,000 mg per kg body weight per day (mg/kg/day) for 90 days, tricresyl phosphate produced significant histopathological manifestations in the liver of rats, which indicated the animals were adequately challenged. An equimolar dose of IPP was estimated to be approximately 1,222 mg/kg/day; thus, this dose was selected as the highest dose in the present study.

Chemistry

IPP (CASRN 68937-41-7; $C_{27}H_{33}O_4P$; molar mass 452.52 g/mol) was obtained from AmplaChem (Carmel, IN; lot 20111210) in two drums. The contents of the drums were filtered and blended to form a single lot (20111210, batch 04). IPP is a mixture of triphenyl phosphate (TPHP) and mono-, di-, and tri-isopropyl-substituted isomers. The identity of lot 20111210, batch 04 was confirmed using Fourier transform infrared spectroscopy, ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy, and mass spectrometry. The purity (approximately 99%) was determined by gas chromatography with flame ionization detection (GC-FID) as the sum of the peak areas of TPHP (26%) and 20 peaks of IPP isomers (73%).

Dose formulations were prepared in corn oil at target concentrations of 0 (vehicle), 0.0338, 0.0676, 0.135, 0.270, and 0.540 mmol/mL, analyzed by GC-FID, and shipped to Alion (Research Triangle Park, NC). All formulations were within ± 10% of target concentrations and no IPP was detected in any control formulation. The stability of the corn oil formulations was assessed using the 0.0338 mmol/mL concentration for up to 21 days when stored at ambient temperature in sealed glass bottles under inert gas; the measured concentration was within 10% of the nominal

concentration on day 0, demonstrating the stability during the period of use. All chemistry activities were conducted by MRIGlobal (Kansas City, MO).

Clinical Examinations and Sample Collection

Clinical Observations

Standard clinical observations were performed within 4 hours post dosing on all study days. Animals were observed for signs of cholinesterase inhibition with specific signs recorded, such as weakness, lethargy, tremors, eye-bulging, salivation, lacrimation, and diarrhea.

Body and Organ Weights

Animals were weighed on the first day of dosing and on the day of necropsy. During necropsy, the entire liver and brain were removed, and organ weights were recorded for each animal.

Clinical Pathology

Animals were terminated in random order by CO_2/O_2 (70%/30%) anesthesia one day after the final day of dosing. Blood samples were taken via cardiocentesis. Five mL of blood was collected into a tube void of anticoagulant and the serum harvested for clinical chemistry, total thyroxine (T_4), and cholinesterase measurements. The following clinical chemistry parameters were measured on an Olympus AU400e chemistry analyzer (Olympus America, Inc., Irvin, TX) using reagents obtained from Beckman Coulter (Brea, CA) or Diazyme (Poway, CA): urea nitrogen, creatinine, total protein, albumin, sorbitol dehydrogenase, alanine aminotransferase, aspartate aminotransferase, bile acids, cholesterol, triglycerides, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and cholinesterase. Total T_4 was measured using an MP Biomedical T4 radioimmunoassay kit with an Apex automatic gamma counter (ICN Micromedic Systems, Inc., Huntsville, AL). Toxicological study data tables are presented in Appendix B.

Transcriptomics

Sample Collection for Transcriptomics

Liver transcriptomics were performed on samples taken from three animals per dose group (randomly selected). Half the left liver lobe was processed for RNA isolation. Specifically, three pieces (3-mm cubes) were dissected and transferred to a weigh boat containing liquid nitrogen. Once flash frozen, the liver tissue for each animal was placed into a single, prechilled 2-mL cryotube and stored at or below −70°C. Frozen liver samples were shipped to the Battelle Biomedical Research Center (West Jefferson, OH) on dry ice.

RNA Isolation and cDNA Synthesis

The frozen liver tissues were submerged in 10 volumes of prechilled RNA*later*®-ICE (Life Technologies, Carlsbad, CA) and stored at −20°C ± 10°C for a minimum of 16 hours. The tissues were removed from the RNA*later*®-ICE and weighed. Each liver tissue sample, weighing between 21 and 30 mg, was added to lysis buffer and homogenized using plastic disposable pestles (Fisher Scientific, Pittsburgh, PA). Following homogenization, samples were stored at

−70°C ± 10°C until RNA was isolated. Samples were thawed and centrifuged. RNA was extracted from the supernatant, subjected to DNase digestion, and isolated using the Qiagen RNeasy Mini Kit (Cat #: 74104; Qiagen, Valencia, CA). Each RNA sample was analyzed for quantity and purity by UV analysis using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). All samples were evaluated for RNA integrity using an RNA 6000 Nano Kit with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and then stored at −70°C ± 10°C until further processing.

Total RNA (100 ng), isolated from each liver sample, was used to synthesize single-stranded DNA, which was subsequently converted into a double-stranded complementary DNA (cDNA) template for transcription. An in vitro transcription (IVT) reaction, which incorporates biotinylated ribonucleotide analogs, then was used to create labeled amplified RNA (aRNA). This RNA target preparation was performed using the Affymetrix GeneChip® 3' IVT Express Kit (Cat #: 901228; Affymetrix Inc., Santa Clara, CA) and an Eppendorf Mastercycler® thermal cycler (Eppendorf, Hamburg, Germany).

Labeled aRNA was fragmented and subsequently hybridized to the Affymetrix Rat Genome 230 2.0 Array (Cat #: 900505; 31,099 probe sets) using an Affymetrix GeneChip® Hybridization Oven 645. The arrays were washed and stained using the Affymetrix GeneChip® Hybridization Wash and Stain kit (Cat #: 900720) and a Fluidics Station 450 according to the Affymetrix-recommended protocol (FS450_0001). After washing and staining, arrays were scanned using an Affymetrix GeneChip® Scanner 3000 7G, and the raw microarray data (.CEL files) were acquired using Affymetrix GeneChip® Command Console® Software. The Rat Genome 230 2.0 Array provides coverage of more than 30,000 known transcripts; although the array provides comprehensive coverage of global transcript expression, of note is that it does not cover the entirety of the rat transcriptome.

Analysis of GeneChip Data Quality

Quality control measurements were evaluated to determine if the data generated from each Affymetrix GeneChip® array were of sufficient quality. Affymetrix-recommended guidelines for evaluating quality were used to evaluate the output files for each GeneChip® array using the R/Bioconductor package, Simpleaffy.[9] The following quality control parameters were evaluated for each array: average background, scale factor, percentage of genes scored as present, 3' to 5' ratios for the internal control genes beta-actin and glyceraldehyde-3-phosphate dehydrogenase, values for hybridization control transcripts, and values for poly (A) controls.

For samples that failed to pass quality control evaluation due to insufficient data quality, an additional round of RNA isolation and cDNA synthesis was performed and additional GeneChip® arrays were run, which were designated with –R after each sample number.

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 Williams[10; 11] and Dunnett.[12] Hormone data and clinical chemistry, which typically have skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley[13] and Dunn.[14] The Jonckheere test[15] 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 $p \leq 0.01$.

Prior to analysis, values identified by the outlier test of Dixon and Massey[16] were examined by National Institute of Environmental Health Sciences 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 ($p \leq 0.05$) pairwise difference relative to the vehicle control group. A lowest-observed-effect level (LOEL) was identified as the lowest dose demonstrating a significant ($p \leq 0.05$) pairwise difference relative to the vehicle control group.

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 benchmark dose (BMD) modeling analysis. For body weight, the BMD and benchmark dose lower confidence limit (BMD_L) were reported 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 1 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
- Polynomial 2°, 3°, 4°, 5°
- 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°, 3°, and 4° 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 U.S. Environmental Protection Agency (EPA) BMD technical guidance[17] and was implemented in the BMDS 2.7.0 software.

After model execution, BMDs were selected using the model recommendation procedures generally described,[17] and the automated decision logic described in Wignall et al.[18] 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 with serious issues
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,[17] either the model with the lowest Akaike information criterion (AIC) or lowest BMD_L was selected. If the range of BMD_L values was sufficiently close (less than threefold different), the AIC value was used; otherwise, the BMD_L 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 Appendix D, 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 BMD_L values were not reported.

Benchmark Dose Analysis of Transcriptomics Data

The BMD analysis of the transcriptomic data was performed in accordance with 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*.[19]

Probe set intensities from raw microarray data (.CEL files from Affymetrix Rat Genome 230 2.0 Arrays) were normalized by applying the Robust Multi-array Average (RMA) algorithm from the genomics analysis tool, GeneSpring GX 12.6 (Agilent Technology, Foster City, CA). The microarray studies of multiple organophosphate phosphates (data to be reported elsewhere) were performed at the same time such that .CEL files from those related studies were normalized together with the data sets collected in this study. Principal component analysis (PCA) of the primary RMA-normalized data indicated a batch effect; due to randomization of the samples in the processing and detailed metadata capture, the source of the batch effect could be identified as the hybridization date. To correct the batch effect, the primary normalized data were loaded into Partek Genomic Suite version 6.16.0812 (St. Louis, MO) and annotated with chemical exposure/dose group and hybridization date annotations. The ANOVA-based remove batch effect function in Partek Genomic Suite then was used to remove quantitative impacts from the hybridization date batch effect. Quality control of the batch-corrected, normalized data was performed by visual inspection, using a PCA plot and normalized intensity histograms (Appendix C).

Dose-response analyses of RMA-normalized, batch-corrected probe set intensities from the IPP study samples were performed using BMDExpress 2.20.0148 beta[20]

(https://github.com/auerbachs/BMDExpress-2/releases), an updated version of BMDExpress 1.41 that uses an updated modeling approach. First, control genes (AFFX-) were removed from each data set. A trend test (the Williams trend test,[10; 11] $p \leq 0.05$) and fold change filter (1.5-fold change up or down relative to the vehicle control group for probe sets) was 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. 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 BMDExpress were log2 transformed, and thus nearly all probe sets 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 to be biologically significant, a BMR of 1 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 set was determined by a trend test intrinsic to the model executables (provided by EPA) contained in BMDExpress.

To identify the best-fit model for each fitted probe set, the AIC values for each fitted model were compared and the model with the lowest AIC selected. The best model for each probe set was used to calculate the BMD, BMD_L, and BMD upper confidence limit (BMD_U). The specific parameter settings, selected from the BMDExpress software when performing probe set-level BMD analysis, were as follows: maximum iterations – 250, confidence level – 0.95, BMR factor – 1 (the multiplier of the SD that defined the BMD), restrict power – no restriction, and constant variance – selected. The specific model selection setting in the BMDExpress 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 guidance. The justification for this deviation relates to subsequent use of the data in which the probe set BMD values are grouped into gene sets from which a median BMD is derived. If the probe sets were removed from the analysis or forced to another model, the probe set might not be counted in the gene set analysis and could lead to loss of "active" gene sets. Importantly, most of the probe sets 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: 03/09/18) gene set analysis, only GO terms with ≥10 and ≤250 annotated genes measured on the gene expression platform were considered. Before sorting genes into the GO terms, the best-fit model for each probe set was

subjected to a filtering process to remove those probe sets (1) with a BMD >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 BMD_U/BMD_L ratio >40. GO terms that were at least 5% populated and contained three genes that passed the above criteria 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 BMD_L and BMD_U values were not reported.

To perform Individual Gene Analysis, a Defined Category Analysis in BMDExpress was performed that mapped probe sets to genes using a probe-to-gene annotation file. In short, the best-fit model for each probe set was subjected to a filtering process to remove those probe sets (1) with a BMD >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 BMD_U/BMD_L ratio >40. For genes that had more than one probe set represented on the microarray and passed the above filtering, a median BMD was used to estimate the BMD, BMD_L, and BMD_U values. To ensure only genes with a robust response were assessed for potency, genes with probe sets 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 BMD_L and BMD_U values were not reported.

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

Data Accessibility

Primary and analyzed data used in this study are available to the public at https://doi.org/10.22427/NIEHS-DATA-NIEHS-05.[21]

Results

Animal Condition, Body Weights, and Organ Weights

Animals in the highest dose group (1,222 mg/kg) began exhibiting signs of cholinesterase inhibition 1–2 days post exposure, including red discharge from eyes and nose, lethargy, and diarrhea, as well as a 13%–25% decrease in body weight (data not shown). One animal in this dose group was found dead 2 days post exposure, and the remaining six were moribund and euthanized at that time due to severe toxicity. Animals in the 0, 77, 153, 306, and 611 mg/kg groups did not exhibit signs of overt cholinesterase inhibition toxicity, and all survived to the end of the study. A negative trend was found in terminal body weight, along with a significant decrease in the second highest dose group (611 mg/kg) as compared to the vehicle control group (Table 1). The benchmark dose (benchmark dose lower confidence limit)—BMD (BMD_L)—for terminal body weight at study day 4 was 278.2 (187.3) mg/kg.

Table 1. Body Weight Summary

Study Day	0 mg/kg[a,b] n = 5	77 mg/kg n = 5	153 mg/kg n = 5	306 mg/kg n = 5	611 mg/kg n = 5	1,222 mg/kg n = 7	BMD_{1Std} (mg/kg)	BMD_{L1Std} (mg/kg)
0	225.8 ± 3.7	227.7 ± 2.1	226.6 ± 3.6	224.0 ± 2.8	225.4 ± 1.9	226.4 ± 2.2	ND	ND
4	247.2 ± 3.8**	251.2 ± 3.5	245.8 ± 5.3	239.4 ± 4.2	230.6 ± 4.5*	N/A	278.2	187.3

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 $p \leq 0.05$; **$p \leq 0.01$.
Benchmark response set at 1 standard deviation from the mean.
BMD = benchmark dose; BMD_L = benchmark dose lower confidence limit; study day 0 = the first day of dosing; study day 4 = the day of necropsy; ND = not determined; N/A = no data collected.
[a]Data are displayed as mean ± standard error of the mean; body weight data are presented in grams.
[b]Statistical analysis performed by the Jonckheere (trend) and Williams or Dunnett (pairwise) tests.

At necropsy, a significant increase in absolute and relative liver weights occurred in dose groups ≥306 mg/kg and ≥153 mg/kg, respectively; both endpoints had positive trends (Table 2). The BMD (BMD_L) for increased absolute liver weight was 400.0 (246.0) mg/kg and for relative liver weight was 55.8 (33.7) mg/kg. Significant trend and pairwise comparisons were not observed in absolute or relative brain weights (Appendix B).

Clinical Chemistry

Total cholesterol and high-density lipoprotein (HDL) cholesterol concentrations were significantly increased in all dosed groups with a positive trend. A BMD_L was not reportable for increased total cholesterol because the BMD was below the lower limit of extrapolation (<25.7 mg/kg) (Table 3). A BMD (BMD_L) was not determined for increased HDL cholesterol because no viable model was available. Albumin concentration had a negative trend with a significant pairwise comparison at 611 mg/kg and a BMD (BMD_L) of 167.6 (63.2) mg/kg. The albumin/globulin (A/G) ratio had a negative trend and significant decreases in the 153 mg/kg and higher dose groups with a BMD (BMD_L) of 51.3 (27.0) mg/kg. There was a minimal but significant decrease in creatinine level in the 306 and 611 mg/kg groups with a negative trend and a BMD (BMD_L) of 223.9 (158.9) mg/kg.

Testing lower doses in future studies will therefore be necessary to calculate a BMD associated with increased cholesterol in the context of isopropylated phenol phosphate (IPP) exposure. There were no other clinical chemistry findings that exhibited significant trend and pairwise comparisons (Appendix B).

Hormones and Enzymes

Serum cholinesterase activity was significantly decreased in all dosed groups by 42%–64%, beginning with the 77 mg/kg group; a BMD_L was not reportable because the BMD was <25.7 mg/kg (Table 4). Testing lower doses in future studies will therefore be necessary to calculate a BMD associated with decreased cholinesterase in the context of IPP exposure. No significant trend and pairwise comparisons were observed in total thyroxine concentration (Appendix B).

Table 2. Organ Weights Summary[a]

Endpoint	0 mg/kg[b,c] n = 5	77 mg/kg n = 5	153 mg/kg n = 5	306 mg/kg n = 5	611 mg/kg n = 4–5[d]	BMD_{1Std} (mg/kg)	$BMDL_{1Std}$ (mg/kg)
Terminal Body Weight (g)	247.2 ± 3.8**	251.2 ± 3.5	245.8 ± 5.3	239.4 ± 4.2	230.6 ± 4.5*	278.2	187.3
Liver Weight Absolute (g)	10.35 ± 0.23**	11.23 ± 0.27	11.29 ± 0.37	11.95 ± 0.42**	11.75 ± 0.46**	400.0	246.0
Liver Weight Relative[e] (mg/g)	41.90 ± 0.77**	44.70 ± 0.72	45.91 ± 0.95*	49.87 ± 1.17**	51.30 ± 1.59**	55.8	33.7

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 $p \leq 0.05$; **$p \leq 0.01$.
Benchmark response set at 1 standard deviation from the mean.
BMD = benchmark dose; BMD_L = benchmark dose lower confidence limit.
[a]Descriptions of organ weight endpoints and changes are provided in Appendix E.
[b]Data are displayed as mean ± standard error of the mean.
[c]Statistical analysis performed by the Jonckheere (trend) and Williams or Dunnett (pairwise) tests.
[d]Change in n reflects organ weights not recorded at necropsy. Liver weight was not measured for one animal in the 611 mg/kg group.
[e]Relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight.

Table 3. Clinical Chemistry Summary

Endpoint	0 mg/kg[a,b] n = 5	77 mg/kg n = 5	153 mg/kg n = 5	306 mg/kg n = 5	611 mg/kg n = 5	BMD_{1Std} (mg/kg)	BMD_{L1Std} (mg/kg)
Creatinine (mg/dL)	0.27 ± 0.01**	0.28 ± 0.00	0.28 ± 0.01	0.25 ± 0.01*	0.25 ± 0.00*	223.9	158.9
A/G Ratio	1.35 ± 0.01**	1.28 ± 0.03	1.23 ± 0.02*	1.18 ± 0.03**	1.17 ± 0.03**	51.3	27.0
Albumin (g/dL)	3.42 ± 0.06**	3.42 ± 0.06	3.30 ± 0.06	3.24 ± 0.05	3.18 ± 0.07*	167.6	63.2
Cholesterol (mg/dL)	113.2 ± 5.1**	140.6 ± 7.6*	131.6 ± 5.2*	134.0 ± 5.1*	149.0 ± 3.9**	<25.7[c]	NR
HDL Cholesterol (mg/dL)	52.0 ± 2.2**	66.8 ± 3.4*	59.0 ± 2.2*	66.0 ± 1.3**	71.2 ± 2.5**	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 $p \leq 0.05$; **$p \leq 0.01$.
Benchmark response set at 1 standard deviation from the mean.
BMD = benchmark dose; BMD_L = benchmark dose lower confidence limit; A/G Ratio = ratio of albumin to globulin; NR = BMD_L is not reportable because the BMD is below the lower limit of extrapolation (<1/3 the lowest nonzero dose tested); HDL = high-density lipoprotein; NVM = nonviable model.
[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]<25.7 = a best-fit model was identified and a BMD was estimated that was <1/3 the lowest nonzero dose tested.

Table 4. Hormones and Enzymes Summary

Endpoint	0 mg/kg[a,b] n = 5	77 mg/kg n = 5	153 mg/kg n = 5	306 mg/kg n = 5	611 mg/kg n = 5	BMD_{1Std} (mg/kg)	BMD_{L1Std} (mg/kg)
Cholinesterase (IU/L)	285.4 ± 16.1**	166.4 ± 8.0**	146.4 ± 13.8**	116.4 ± 6.2**	104.0 ± 3.9**	<25.7[c]	NR

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 $p \leq 0.01$.
Benchmark response set at 1 standard deviation from the mean.
BMD = benchmark dose; BMD_L = benchmark dose lower confidence limit; NR = BMD_L is not reportable because the BMD is below the lower limit of extrapolation (<1/3 the lowest nonzero dose tested).
[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]<25.7 = a best-fit model was identified and a BMD was estimated that was <1/3 the lowest nonzero dose tested

Apical Endpoint Benchmark Dose Summary

A summary of the calculated BMDs for each toxicological endpoint is provided in Table 5. The lowest-observed-effect level (LOEL) and no-observed-effect level (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 <25.7 mg/kg.

Table 5. BMD, BMD_L, LOEL, and NOEL Summary for Apical Endpoints, Sorted by BMD or LOEL from Low to High

Endpoint	BMD_{1Std} (mg/kg)	BMD_{L1Std} (mg/kg)	LOEL (mg/kg)	NOEL (mg/kg)	Direction of Change
Cholesterol	<25.7[a]	NR	77	ND	UP
Cholinesterase	<25.7	NR	77	ND	DOWN
A/G Ratio	51.3	27.0	153	77	DOWN
Liver Weight Relative	55.8	33.7	153	77	UP
Albumin	167.6	63.2	611	306	DOWN
Creatinine	223.9	158.9	306	153	DOWN
Terminal Body Weight	278.2	187.3	611	306	DOWN
Liver Weight Absolute	400.0	246.0	306	153	UP
HDL Cholesterol	NVM	NVM	**77**	ND	UP

Benchmark response set at 1 standard deviation from the mean.
BMD = benchmark dose; BMD_L = benchmark dose lower confidence limit; LOEL = lowest-observed-effect level; NOEL = no-observed-effect level; NR = BMD_L is not reportable because the BMD is below the lower limit of extrapolation (<1/3 the lowest nonzero dose tested); ND = not determined; A/G = ratio of albumin to globulin; HDL = high-density lipoprotein; NVM = nonviable model.
Values in bold text indicate the LOEL of endpoints for which a BMD could not be calculated.
[a]<25.7 = a best-fit model was identified, and a BMD was estimated that was <1/3 the lowest nonzero dose tested.

Gene Set Benchmark Dose Analysis

Chemical-induced alterations in liver gene transcript expression were examined to determine those gene sets most sensitive to IPP 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. 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 with the lowest BMD median values are shown in Table 6. The gene sets in Table 6 should be interpreted with caution from the standpoint of the underlying biology 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 that could serve as a conservative surrogate of estimated biological potency and, by extension, toxicological potency when more definitive toxicological data are unavailable.

The top 10 most sensitive gene sets had estimated BMD median values <25.7 mg/kg. These sensitive gene sets were glycogen biosynthetic process (GO:0005978), glucan biosynthetic process (GO:0009250), steroid hormone mediated signaling pathway (GO:0043401), cardiac

muscle cell differentiation (GO:0055007), regulation of skeletal muscle tissue development (GO:0048641), striated muscle cell differentiation (GO:0051146), regulation of carbohydrate catabolic process (GO:0043470), positive regulation of glucose metabolic process (GO:0010907), response to corticosterone (GO:0051412), and cellular senescence (GO:0090398). The full list of affected gene sets can be found in Appendix F.

Table 6. Top 10 Gene Ontology Biological Process Gene Sets Ranked by Potency of Perturbation, Sorted by Benchmark Dose Median[a]

Category Name	Input Genes/Platform Genes in Gene Set	% Gene Set Coverage	Active Genes	BMD_{1std} Median of Gene Set Transcripts (mg/kg)	Median BMD_{L1Std}–BMD_{U1Std} (mg/kg)	Genes with Changed Direction Up	Genes with Changed Direction Down
GO:0005978 glycogen biosynthetic process	3/17	18%	*Gck*; *Nr1d1*; *Per2*	<25.7[b]	NR	2	1
GO:0009250 glucan biosynthetic process	3/17	18%	*Gck*; *Nr1d1*; *Per2*	<25.7	NR	2	1
GO:0043401 steroid hormone mediated signaling pathway	5/95	5%	*Fkbp4*; *Esrrg*; *Ppard*; *Nr1d1*; *Nr1d2*	<25.7	NR	3	2
GO:0055007 cardiac muscle cell differentiation	3/36	8%	*Sox6*; *Tbx3*; *Syne1*	<25.7	NR	2	1
GO:0048641 regulation of skeletal muscle tissue development	4/50	8%	*Tcf7l2*; *Nr1d2*; *Arntl*; *Tsc22d3*	<25.7	NR	2	2
GO:0051146 striated muscle cell differentiation	5/67	7%	*Sox6*; *Tbx3*; *Chuk*; *Syne1*; *Avpr1a*	<25.7	NR	3	2
GO:0043470 regulation of carbohydrate catabolic process	3/47	6%	*Gck*; *Avpr1a*; *Ddit4*	<25.7	NR	1	2
GO:0010907 positive regulation of glucose metabolic process	3/41	7%	*Gck*; *Tcf7l2*; *Avpr1a*	<25.7	NR	1	2
GO:0051412 response to corticosterone	3/41	7%	*Junb*; *Cdkn1a*; *Avpr1a*	<25.7	NR	0	3

Category Name	Input Genes/Platform Genes in Gene Set	% Gene Set Coverage	Active Genes	BMD_{1std} Median of Gene Set Transcripts (mg/kg)	Median BMD_{L1Std}–BMD_{U1Std} (mg/kg)	Genes with Changed Direction Up	Genes with Changed Direction Down
GO:0090398 cellular senescence	4/30	13%	*Cdkn1a*; *Tbx3*; *Arntl*; *Lmna*	<25.7	NR	2	2

Benchmark response set at 1 standard deviation from the mean.
BMD = benchmark dose; BMD_L = benchmark dose lower confidence limit; BMD_U = benchmark dose upper confidence limit; GO = Gene Ontology; NR = the BMD_L–BMD_U range is not reportable because the BMD median is below the lower limit of extrapolation (<1/3 the lowest nonzero dose tested).
[a]Definitions of GO terms were adapted from the Gene Ontology Resource.[22] Official gene symbols from the Rat Genome Database[23] are shown in the "Active Genes" column.
[b]<25.7 = a best-fit model was identified and a BMD was estimated that was <1/3 the lowest nonzero dose tested.
GO process description version: https://doi.org/10.22427/NTP-DATA-002-00600-0002-000-0.[24]
GO:0005978 glycogen biosynthetic process: The chemical reactions and pathways resulting in the formation of glycogen, a polydisperse, highly branched glucan composed of chains of D-glucose residues.
GO:0009250 glucan biosynthetic process: The chemical reactions and pathways resulting in the formation of glucans, polysaccharides consisting only of glucose residues.
GO:0043401 steroid hormone mediated signaling pathway: A series of molecular signals mediated by a steroid hormone binding to a receptor.
GO:0055007 cardiac muscle cell differentiation: The process by which a cardiac muscle precursor cell acquires specialized features of a cardiac muscle cell. Cardiac muscle cells are striated muscle cells that are responsible for heart contraction.
GO:0048641 regulation of skeletal muscle tissue development: Any process that modulates the frequency, rate, or extent of skeletal muscle tissue development.
GO:0051146 striated muscle cell differentiation: The process in which a relatively unspecialized cell acquires specialized features of a striated muscle cell; striated muscle fibers are divided by transverse bands into striations, and cardiac and voluntary muscle are types of striated muscle.
GO:0043470 regulation of carbohydrate catabolic process: Any process that modulates the frequency, rate, or extent of the chemical reactions and pathways, resulting in the breakdown of carbohydrates.
GO:0010907 positive regulation of glucose metabolic process: Any process that increases the rate, frequency, or extent of glucose metabolism. Glucose metabolic processes are the chemical reactions and pathways involving glucose, the aldohexose gluco-hexose.
GO:0051412 response to corticosterone: 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 a corticosterone stimulus. Corticosterone is a 21-carbon steroid hormone of the corticosteroid type, produced in the cortex of the adrenal glands. In many species, corticosterone is the principal glucocorticoid, involved in regulation of fuel metabolism, immune reactions, and stress responses.
GO:0090398 cellular senescence: A cell aging process stimulated in response to cellular stress, whereby normal cells lose the ability to divide through irreversible cell cycle arrest.

Gene Benchmark Dose Analysis

The top 10 genes (fold change >|2|, significant Williams trend test, global goodness of fit p value >0.1, and BMD_U/BMD_L < 40), ranked by estimated BMD are shown in Table 7. As with the GO analysis, the biological or toxicological significance of the changes in gene expression shown in Table 7 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.

All 10 of the most sensitive genes had an estimated BMD median value <25.7 mg/kg. Six genes exhibited an increase in expression: *Tbx3* (T-box transcription factor 3), *Nr1d1* (nuclear receptor subfamily 1, group D, member 1), *Nr1d2* (nuclear receptor subfamily 1, group D, member 2), *Sik2* (salt-inducible kinase 2), *Oaf* (out at first homolog), and *Dbp* (D-box binding PAR bZIP transcription factor). Four genes exhibited a decrease in expression: *Gck* (glucokinase), *Syne1*

(spectrin repeat containing nuclear envelope protein 1), *Cdkn1a* (cyclin-dependent kinase inhibitor 1A), and *Cldn1* (claudin 1).

Table 7. Top 10 Genes Ranked by Potency of Perturbation, Sorted by Benchmark Dose Median[a]

Gene Symbol	Entrez Gene IDs	Probe IDs	BMD_{1Std} (BMD_{L1std}–BMD_{U1std}) in mg/kg	Maximum Fold Change	Direction of Expression Change
Tbx3	353305	1393160_at	<25.7[b] (NR)	2.2	UP
Gck	24385	1387312_a_at	<25.7 (NR)	6.1	DOWN
Nr1d1	252917	1370816_at	<25.7 (NR)	10.0	UP
Nr1d2	259241	1370541_at,1390430_at	<25.7 (NR)	4.1	UP
Sik2	315649	1376649_at	<25.7 (NR)	2.1	UP
Syne1	499010	1370264_at	<25.7 (NR)	2.3	DOWN
Oaf	315594	1388425_at	<25.7 (NR)	2.2	UP
Dbp	24309	1387874_at	<25.7 (NR)	18.4	UP
Cdkn1a	114851	1388674_at	<25.7 (NR)	6.5	DOWN
Cldn1	65129	1383946_at	<25.7 (NR)	3.0	DOWN

Benchmark response set at 1 standard deviation from the mean.
BMD = benchmark dose; BMD_L = benchmark dose lower confidence limit; BMD_U = benchmark dose upper confidence limit; NR = the BMD_L–BMD_U range is not reportable because the BMD median is below the lower limit of extrapolation (<1/3 the lowest nonzero dose tested).
[a]Descriptions of orthologous human genes are shown due to the increased detail available in public resources such as UniprotKB[25] and Entrez Gene.[26] Human UniprotKB was used as the primary resource due to the greater breadth of annotation and depth of functional detail provided. Rat UniprotKB was used as the secondary resource if the primary source did not provide a detailed description of function. Human Entrez Gene Summary was used as the third resource. Rat Entrez Gene Summary was used as the fourth resource.
[b]<25.7 = a best-fit model was identified and a BMD was estimated that was <1/3 the lowest nonzero dose tested.
Gene definition version: https://doi.org/10.22427/NTP-DATA-002-00600-0002-000-0.[24]
***Tbx3*:** Human Uniprot function (Human *TBX3*): Transcriptional repressor involved in developmental processes. Probably plays a role in limb pattern formation. Acts as a negative regulator of PML function in cellular senescence. {ECO0000269|PubMed10468588, ECO0000269|PubMed22002537}.
***Gck*:** Human Uniprot function (Human *GCK*): Catalyzes the phosphorylation of hexose, such as D-glucose, D-fructose and D-mannose, to hexose 6-phosphate (D-glucose 6-phosphate, D-fructose 6-phosphate, and D-mannose 6-phosphate, respectively) (PubMed7742312, PubMed11916951, PubMed15277402, PubMed17082186, PubMed18322640, PubMed19146401, PubMed25015100, PubMed8325892). Compared to other hexokinases, has a weak affinity for D-glucose, and is effective only when glucose is abundant (by similarity). Mainly expressed in pancreatic beta cells and the liver and constitutes a rate-limiting step in glucose metabolism in these tissues (PubMed18322640, PubMed25015100, PubMed8325892, PubMed11916951, PubMed15277402). Since insulin secretion parallels glucose metabolism and the low glucose affinity of GCK ensures that it can change its enzymatic activity within the physiological range of glucose concentrations, GCK acts as a glucose sensor in the pancreatic beta cell (by similarity). In pancreas, plays an important role in modulating insulin secretion (by similarity). In liver, helps facilitate the uptake and conversion of glucose by acting as an insulin-sensitive determinant of hepatic glucose usage (by similarity). Required to provide D-glucose 6-phosphate for the synthesis of glycogen (PubMed8878425). Mediates the initial step of glycolysis by catalyzing phosphorylation of D-glucose to D-glucose 6-phosphate (PubMed7742312). {ECO0000250|UniProtKBP17712, ECO0000250|UniProtKBP52792, ECO0000269|PubMed11916951, ECO0000269|PubMed15277402, ECO0000269|PubMed17082186, ECO0000269|PubMed18322640, ECO0000269|PubMed19146401, ECO0000269|PubMed25015100, ECO0000269|PubMed7742312, ECO0000269|PubMed8325892, ECO0000269|PubMed8878425}.
***Nr1d1*:** Human Uniprot function (Human *NR1D1*): Transcriptional repressor that coordinates circadian rhythm and metabolic pathways in a heme-dependent manner. Integral component of the complex transcription machinery that governs circadian rhythmicity and forms a critical negative limb of the circadian clock by directly repressing the expression of core clock components, ARTNL/BMAL1, CLOCK, and CRY1. Also regulates genes involved in metabolic functions, including lipid and bile acid metabolism, adipogenesis, gluconeogenesis, and the macrophage inflammatory response. Acts as a receptor for heme,

which stimulates its interaction with the NCOR1/HDAC3 corepressor complex, enhancing transcriptional repression. Recognizes two classes of DNA response elements within the promoter of its target genes and can bind to DNA as either monomers or homodimers, depending on the nature of the response element. Binds as a monomer to a response element composed of the consensus half-site motif 5'-[A/G]GGTCA-3' preceded by an A/T-rich 5' sequence (RevRE), or as a homodimer to a direct repeat of the core motif spaced by two nucleotides (RevDR-2). Acts as a potent competitive repressor of ROR alpha (RORA) function and regulates the levels of its ligand heme by repressing the expression of PPARGC1A, a potent inducer of heme synthesis. Regulates lipid metabolism by repressing the expression of APOC3 and by influencing the activity of sterol response element binding proteins (SREBPs); represses INSIG2, which interferes with the proteolytic activation of SREBPs, which in turn govern the rhythmic expression of enzymes with key functions in sterol and fatty acid synthesis. Regulates gluconeogenesis via repression of G6PC and PEPCK and adipocyte differentiation via repression of PPARG. Regulates glucagon release in pancreatic alpha-cells via the AMPK-NAMPT-SIRT1 pathway and the proliferation, glucose-induced insulin secretion, and expression of key lipogenic genes in pancreatic-beta cells. Positively regulates bile acid synthesis by increasing hepatic expression of CYP7A1 via repression of NR0B2 and NFIL3, which are negative regulators of CYP7A1. Modulates skeletal muscle oxidative capacity by regulating mitochondrial biogenesis and autophagy; controls mitochondrial biogenesis and respiration by interfering with the STK11-PRKAA1/2-SIRT1-PPARGC1A signaling pathway. Represses the expression of SERPINE1/PAI1, an important modulator of cardiovascular disease and the expression of inflammatory cytokines and chemokines in macrophages. Represses gene expression at a distance in macrophages by inhibiting the transcription of enhancer-derived RNAs (eRNAs). Plays a role in the circadian regulation of body temperature and negatively regulates thermogenic transcriptional programs in brown adipose tissue (BAT); imposes a circadian oscillation in BAT activity, increasing body temperature when awake and depressing thermogenesis during sleep. In concert with NR2E3, regulates transcriptional networks critical for photoreceptor development and function. In addition to its activity as a repressor, can also act as a transcriptional activator. In the ovarian granulosa cells, acts as a transcriptional activator of STAR, which plays a role in steroid biosynthesis. In collaboration with SP1, activates GJA1 transcription in a heme-independent manner. Represses the transcription of CYP2B10, CYP4A10 and CYP4A14 (by similarity). Represses the transcription of CES2 (by similarity). Represses and regulates the circadian expression of TSHB in a NCOR1-dependent manner (by similarity). Negatively regulates the protein stability of NR3C1 and influences the time-dependent subcellular distribution of NR3C1, thereby affecting its transcriptional regulatory activity (by similarity). Plays a critical role in the circadian control of neutrophilic inflammation in the lung; under resting, nonstress conditions, acts as a rhythmic repressor to limit inflammatory activity, whereas in the presence of inflammatory triggers, undergoes ubiquitin-mediated degradation thereby relieving inhibition of the inflammatory response (by similarity). Plays a key role in the circadian regulation of microglial activation and neuroinflammation; suppresses microglial activation through the NF-kappaB pathway in the central nervous system (by similarity). Plays a role in the regulation of the diurnal rhythms of lipid and protein metabolism in the skeletal muscle via transcriptional repression of genes controlling lipid and amino acid metabolism in the muscle (by similarity). {ECO0000250|UniProtKBQ3UV55, ECO0000269|PubMed12021280, ECO0000269|PubMed15761026, ECO0000269|PubMed16968709, ECO0000269|PubMed18006707, ECO0000269|PubMed19710360, ECO0000269|PubMed1971514, ECO0000269|PubMed21479263, ECO0000269|PubMed22184247, ECO0000269|PubMed23398316, ECO0000269|PubMed2539258}.

***Nr1d2*:** Human Uniprot function (Human *NR1D2):* Transcriptional repressor that coordinates circadian rhythm and metabolic pathways in a heme-dependent manner. Integral component of the complex transcription machinery that governs circadian rhythmicity and forms a critical negative limb of the circadian clock by directly repressing the expression of core clock components, ARNTL/BMAL1 and CLOCK. Also regulates genes involved in metabolic functions, including lipid metabolism and the inflammatory response. Acts as a receptor for heme, which stimulates its interaction with the NCOR1/HDAC3 corepressor complex, enhancing transcriptional repression. Recognizes two classes of DNA response elements within the promoter of its target genes and can bind to DNA as either monomers or homodimers, depending on the nature of the response element. Binds as a monomer to a response element composed of the consensus half-site motif 5'-[A/G]GGTCA-3' preceded by an A/T-rich 5' sequence (RevRE), or as a homodimer to a direct repeat of the core motif spaced by two nucleotides (RevDR-2). Acts as a potent competitive repressor of ROR alpha (RORA) function and also negatively regulates the expression of *NR1D1*. Regulates lipid and energy homeostasis in the skeletal muscle via repression of genes involved in lipid metabolism and myogenesis including *CD36*, *FABP3*, *FABP4*, *UCP3*, *SCD1*, and *MSTN*. Regulates hepatic lipid metabolism via the repression of *APOC3*. Represses gene expression at a distance in macrophages by inhibiting the transcription of enhancer-derived RNAs (eRNAs). In addition to its activity as a repressor, can also act as a transcriptional activator. Acts as a transcriptional activator of the sterol regulatory element-binding protein 1 (SREBF1) and the inflammatory mediator interleukin-6 (IL6) in the skeletal muscle (by similarity). Plays a role in the regulation of circadian sleep/wake cycle; essential for maintaining wakefulness during the dark phase or active period (by similarity). Key regulator of skeletal muscle mitochondrial function; negatively regulates the skeletal muscle expression of core clock genes and genes involved in mitochondrial biogenesis, fatty acid beta-oxidation and lipid metabolism (by similarity). May play a role in the circadian control of neutrophilic inflammation in the lung (by similarity). {ECO0000250|UniProtKBQ60674, ECO0000269|PubMed17892483, ECO0000269|PubMed17996965}.

***Sik2*:** Human Uniprot function (Human *SIK2*): Phosphorylates 'Ser-794' of IRS1 in insulin-stimulated adipocytes, potentially modulating the efficiency of insulin signal transduction. Inhibits CREB activity by phosphorylating and repressing TORCs, the CREB-specific coactivators. SIK2_HUMAN,Q9H0K1

***Syne1*:** Human Uniprot function (Human *SYNE1*): Multi-isomeric modular protein, which forms a linking network between organelles and the actin cytoskeleton to maintain the subcellular spatial organization. As a component of the LINC (LInker of Nucleoskeleton and Cytoskeleton) complex, involved in the connection between the nuclear lamina and the cytoskeleton. The

nucleocytoplasmic interactions established by the LINC complex play an important role in the transmission of mechanical forces across the nuclear envelope and in nuclear movement and positioning. May be involved in nucleus-centrosome attachment and nuclear migration in neural progenitors implicating LINC complex association with SUN1/2 and probably association with cytoplasmic dynein-dynactin motor complexes; SYNE1 and SYNE2 may act redundantly. Required for centrosome migration to the apical cell surface during early ciliogenesis. May be involved in nuclear remodeling during sperm head formation in spermatogenesis; a probable SUN3SYNE1/KASH1 LINC complex may tether spermatid nuclei to posterior cytoskeletal structures such as the manchette. {ECO0000250|UniProtKBQ6ZWR6, ECO0000269|PubMed11792814, ECO0000269|PubMed18396275}.

***Oaf*:** No description available.

***Dbp*:** Human Uniprot function (Human *DBP*): This transcriptional activator recognizes and binds to the sequence 5'-RTTAYGTAAY-3' found in the promoter of genes such as albumin, *CYP2A4* and *CYP2A5*. It is not essential for circadian rhythm generation but modulates important clock output genes. May be a direct target for regulation by the circadian pacemaker component clock. May affect circadian period and sleep regulation.

***Cdkn1a*:** Human Uniprot function (Human *CDKN1A*): May be involved in p53/TP53-mediated inhibition of cellular proliferation in response to DNA damage. Binds to and inhibits cyclin-dependent kinase activity, preventing phosphorylation of critical cyclin-dependent kinase substrates and blocking cell cycle progression. Functions in the nuclear localization and assembly of cyclin D-CDK4 complex and promotes its kinase activity toward RB1. At higher stoichiometric ratios, inhibits the kinase activity of the cyclin D-CDK4 complex. Inhibits DNA synthesis by DNA polymerase delta by competing with POLD3 for PCNA binding (PubMed11595739). Plays an important role in controlling cell cycle progression and DNA damage-induced G2 arrest (PubMed9106657). {ECO0000269|PubMed11595739, ECO0000269|PubMed8242751, ECO0000269|PubMed9106657}.

***Cldn1*:** Human Uniprot function (Human *CLDN1*): Claudins function as major constituents of the tight junction complexes that regulate the permeability of epithelia. While some claudin family members play essential roles in the formation of impermeable barriers, others mediate the permeability to ions and small molecules. Often, several claudin family members are coexpressed and interact with each other, and this determines the overall permeability. CLDN1 is required to prevent the paracellular diffusion of small molecules through tight junctions in the epidermis and is required for the normal barrier function of the skin. Required for normal water homeostasis and to prevent excessive water loss through the skin, probably via an indirect effect on the expression levels of other proteins, since CLDN1 itself seems to be dispensable for water barrier formation in keratinocyte tight junctions (PubMed23407391). {ECO0000269|PubMed23407391} FUNCTION (Microbial infection) Acts as a co-receptor for hepatitis C virus (HCV) in hepatocytes (PubMed17325668, PubMed20375010, PubMed24038151). Associates with CD81, and the CLDN1-CD81 receptor complex is essential for HCV entry into host cell (PubMed20375010). Acts as a receptor for dengue virus (PubMed24074594). {ECO0000269|PubMed17325668, ECO0000269|PubMed20375010, ECO0000269|PubMed24038151, ECO0000269|PubMed24074594}.

Summary

Isopropylated phenol phosphate (IPP) is an organophosphorus flame retardant with widespread human exposure. The literature contains few toxicological data for estimating the potential adverse health effects of IPP. This study used a transcriptomic approach and standard toxicological endpoints to estimate the in vivo biological potency of IPP.

Serum cholinesterase activity was significantly and markedly decreased for all dosed groups. These findings are consistent with several reports that show the classic cholinesterase inhibition in organophosphates.[27] As one of the endpoints with the lowest-observed-effect level for the study, cholinesterase inhibition appeared to be one of the most sensitive apical measures; the estimated benchmark dose (BMD) was below the lower limit of extrapolation (<25.7 mg/kg). Two additional sensitive apical measures were serum total cholesterol and high-density lipoprotein (HDL) cholesterol concentrations, which were significantly increased in all dosed groups. Similar to cholinesterase, the estimated BMD for increased total cholesterol was <25.7 mg/kg; a BMD (BMD_L) was not determined for increased HDL cholesterol because no viable model was available. Further studies are warranted to assess cholinesterase and cholesterol effects at lower doses to obtain accurate points of departure. The most sensitive apical endpoints for which a BMD could be determined were a decrease in serum albumin/globulin ratio and an increase in relative liver weight with BMDs and benchmark dose lower confidence limits (BMD_Ls) of 51.3 (27.0) and 55.8 (33.7) mg/kg, respectively. The next most sensitive apical endpoints observed were a decrease in serum albumin concentration and a decrease in creatinine level with BMDs (BMD_Ls) of 167.6 (63.2) and 223.9 (158.9) mg/kg, respectively.

Gene set-level transcriptional changes in the liver following IPP exposure were estimated to occur at a BMD <25.7 mg/kg for the top 10 most sensitive gene sets. The top 10 most sensitive individual genes also exhibited changes in expression at dose levels below which a reliable estimate of potency could be achieved (<25.7 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 was a decrease in serum albumin/globulin ratio, which had a BMD (BMD_L) of 51.3 (27.0) mg/kg. Gene sets and individual gene transcriptional changes provided potency estimates <25.7 mg/kg. Follow-up studies that investigate transcriptional and apical endpoint changes at lower doses will be a useful future direction to determine the biological potency of IPP more accurately.

References

1. Yang J, Zhao Y, Li M, Du M, Li X, Li Y. A review of a class of emerging contaminants: The classification, distribution, intensity of consumption, synthesis routes, environmental effects and expectation of pollution abatement to organophosphate flame retardants (OPFRs). Int J Mol Sci. 2019; 20(12):2874. https://doi.org/10.3390/ijms20122874

2. U.S. Environmental Protection Agency (USEPA). Fact sheet: Assessing risks from flame retardants. Washington, DC: U.S. Environmental Protection Agency; 2016. https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/fact-sheet-assessing-risks-flame-retardants [Accessed: May 28, 2018]

3. Meeker JD, Cooper EM, Stapleton HM, Hauser R. Urinary metabolites of organophosphate flame retardants: Temporal variability and correlations with house dust concentrations. Environ Health Perspect. 2013; 121(5):580-585. http://dx.doi.org/10.1289/ehp.1205907

4. Porte C, Barcelo D, Albaigés J. Quantitation of total versus selected polychlorinated biphenyl congeners in marine biota samples. J Chromatogr. 1988; 442:386-393. http://dx.doi.org/10.1016/S0021-9673(00)94488-1

5. Sundkvist AM, Olofsson U, Haglund P. Organophosphorus flame retardants and plasticizers in marine and fresh water biota and in human milk. J Environ Monit. 2010; 12(4):943-951. http://dx.doi.org/10.1039/b921910b

6. van der Veen I, de Boer J. Phosphorus flame retardants: Properties, production, environmental occurrence, toxicity and analysis. Chemosphere. 2012; 88(10):1119-1153. http://dx.doi.org/10.1016/j.chemosphere.2012.03.067

7. Thomas RS, Wesselkamper SC, Wang NC, Zhao QJ, Petersen DD, Lambert JC, Cote I, Yang L, Healy E, Black MB et al. Temporal concordance between apical and transcriptional points of departure for chemical risk assessment. Toxicol Sci. 2013; 134(1):180-194. http://dx.doi.org/10.1093/toxsci/kft094

8. Dean JL, Zhao QJ, Lambert JC, Hawkins BS, Thomas RS, Wesselkamper SC. Editor's Highlight: Application of gene set enrichment analysis for identification of chemically induced, biologically relevant transcriptomic networks and potential utilization in human health risk Assessment. Toxicol Sci. 2017; 157(1):85-99. http://dx.doi.org/10.1093/toxsci/kfx021

9. Wilson CL, Miller CJ. Simpleaffy: A BioConductor package for Affymetrix quality control and data analysis. Bioinformatics. 2005; 21(18):3683-3685. http://dx.doi.org/10.1093/bioinformatics/bti605

10. Williams DA. A test for differences between treatment means when several dose levels are compared with a zero dose control. Biometrics. 1971; 27(1):103-117. http://dx.doi.org/10.2307/2528930

11. Williams DA. The comparison of several dose levels with a zero dose control. Biometrics. 1972; 28(2):519-531. http://dx.doi.org/10.2307/2556164

12. Dunnett CW. A multiple comparison procedure for comparing several treatments with a control. J Am Stat Assoc. 1955; 50(272):1096-1121. http://dx.doi.org/10.1080/01621459.1955.10501294

13. Shirley E. A non-parametric equivalent of Williams' test for contrasting increasing dose levels of a treatment. Biometrics. 1977; 33(2):386-389. http://dx.doi.org/10.2307/2529789

14. Dunn OJ. Multiple comparisons using rank sums. Technometrics. 1964; 6(3):241-252. http://dx.doi.org/10.1080/00401706.1964.10490181

15. Jonckheere AR. A distribution-free k-sample test against ordered alternatives. Biometrika. 1954; 41(1/2):133-145. http://dx.doi.org/10.2307/2333011

16. Dixon WJ, Massey FJ. Introduction to statistical analysis. New York, NY: McGraw-Hill; 1951.

17. U.S. Environmental Protection Agency (USEPA). Benchmark dose technical guidance. Washington, DC: U.S. Environmental Protection Agency, Risk Assessment Forum; 2012. https://www.epa.gov/sites/production/files/2015-01/documents/benchmark_dose_guidance.pdf

18. Wignall JA, Shapiro AJ, Wright FA, Woodruff TJ, Chiu WA, Guyton KZ, Rusyn I. Standardizing benchmark dose calculations to improve science-based decisions in human health assessments. Environ Health Perspect. 2014; 122(5):499-505. http://dx.doi.org/10.1289/ehp.1307539

19. National Toxicology Program (NTP). NTP research report on National Toxicology Program approach to genomic dose-response modeling. Research Triangle Park, NC: U.S. Department of Health and Human Services, National Institute of Environmental Health Sciences, National Toxicology Program; 2018. Research Report 5. https://doi.org/10.22427/NTP-RR-5

20. Phillips JR, Svoboda DL, Tandon A, Patel S, Sedykh A, Mav D, Kuo B, Yauk CL, Yang L, Thomas RS et al. BMDExpress 2: Enhanced transcriptomic dose-response analysis workflow. Bioinformatics. 2019; 35(10):1780-1782. http://dx.doi.org/10.1093/bioinformatics/bty878

21. National Toxicology Program (NTP). NIEHS 5: Chemical Effects in Biological Systems (CEBS) data repository. Research Triangle Park, NC: U.S. Department of Health and Human Services, National Institute of Environmental Health Sciences, National Toxicology Program; 2022. https://doi.org/10.22427/NIEHS-DATA-NIEHS-05

22. Geneontology. The gene ontology resource. The Gene Ontology Consortium; 2020. http://geneontology.org/

23. Medical College of Wisconsin. Rat Genome Database. Milwaukee, WI: Milwaukee Regional Medical Center; 2020. https://rgd.mcw.edu/

24. National Toxicology Program (NTP). Aromatic Phosphate Annotation files for 5 day studies: Chemical Effects in Biological Systems (CEBS) data repository. Research Triangle Park, NC: U.S. Department of Health and Human Services, National Institute of Environmental Health Sciences, National Toxicology Program; 2022. https://doi.org/10.22427/NTP-DATA-002-00600-0002-000-0

25. UniProt. UniProtKB results. UniProt Consortium; 2020. https://www.uniprot.org/uniprot/

26. Entrez Gene. Gene. Bethesda, MD: National Library of Medicine, National Center for Biotechnology Information; 2020. https://www.ncbi.nlm.nih.gov/gene/

27. Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological profile for phosphate ester flame retardants. Atlanta, GA: US Department of Health and Human Services, Public Health Service; 2012. https://www.atsdr.cdc.gov/toxprofiles/tp202.pdf

28. Thomas RS, Allen BC, Nong A, Yang L, Bermudez E, Clewell HJ, III, Andersen ME. A method to integrate benchmark dose estimates with genomic data to assess the functional effects of chemical exposure. Toxicol Sci. 2007; 98(1):240-248. http://dx.doi.org/10.1093/toxsci/kfm092

Appendix A. Animal Identifiers

Tables

Table A-1. Animal Numbers and Microarray Data File Names

Animal Number	Group	Dose (mmol/kg/day)	Dose (mg/kg/day)	Survived to Study Termination	Array ID
77	Corn Oil	0	0	Yes	057-052114-MW_(Rat230_2).CEL
100	Corn Oil	0	0	Yes	084-052714-JAP_(Rat230_2).CEL
104	Corn Oil	0	0	Yes	013-051914-MW_(Rat230_2).CEL
122	Corn Oil	0	0	Yes	017-051914-MW_(Rat230_2).CEL
133	Corn Oil	0	0	Yes	049-052114-MW_(Rat230_2).CEL
97	IPP	0.169	77	Yes	NA
110	IPP	0.169	77	Yes	20R-082514-MW_(Rat230_2).CEL
117	IPP	0.169	77	Yes	55R-082514-MW_(Rat230_2).CEL
124	IPP	0.169	77	Yes	090-052814-MW_(Rat230_2).CEL
125	IPP	0.169	77	Yes	NA
73	IPP	0.338	153	Yes	021-051914-MW_(Rat230_2).CEL
74	IPP	0.338	153	Yes	NA
90	IPP	0.338	153	Yes	NA
105	IPP	0.338	153	Yes	056-052114-MW_(Rat230_2).CEL
106	IPP	0.338	153	Yes	091-052814-MW_(Rat230_2).CEL
71	IPP	0.675	306	Yes	NA
82	IPP	0.675	306	Yes	022-051914-MW_(Rat230_2).CEL
116	IPP	0.675	306	Yes	58R-082514-MW_(Rat230_2).CEL
121	IPP	0.675	306	Yes	092-052814-MW_(Rat230_2).CEL
144	IPP	0.675	306	Yes	NA
86	IPP	1.35	611	Yes	NA
108	IPP	1.35	611	Yes	NA
114	IPP	1.35	611	Yes	023-051914-MW_(Rat230_2).CEL
130	IPP	1.35	611	Yes	059-052114-MW_(Rat230_2).CEL
145	IPP	1.35	611	Yes	093-052814-MW_(Rat230_2).CEL
72	IPP	2.7	1,222	No	NA
81	IPP	2.7	1,222	No	NA
84	IPP	2.7	1,222	No	060-052114-MW_(Rat230_2).CEL
85	IPP	2.7	1,222	No	024-051914-MW_(Rat230_2).CEL
93	IPP	2.7	1,222	No	NA
96	IPP	2.7	1,222	No	094-052814-MW_(Rat230_2).CEL
101	IPP	2.7	1,222	No	NA

NA = no transcriptomics data collected for selected animal.

Appendix B. Toxicology Data Tables

Tables

Table B-1. I04: Body Weight Summary[a,b]

Study Day	0 mg/kg n = 5	77 mg/kg n = 5	153 mg/kg n = 5	306 mg/kg n = 5	611 mg/kg n = 5	1,222 mg/kg n = 7
0	225.8 ± 3.7	227.7 ± 2.1	226.6 ± 3.6	224.0 ± 2.8	225.4 ± 1.9	226.4 ± 2.2
4	247.2 ± 3.8**	251.2 ± 3.5	245.8 ± 5.3	239.4 ± 4.2	230.6 ± 4.5*	N/A

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 $p \leq 0.05$; **$p \leq 0.01$.
Study day 0 = the first day of dosing; study day 4 = the day of necropsy; N/A = no data collected.
[a]Data are displayed as mean ± standard error of the mean; body weight data are presented in grams.
[b]Statistical analysis performed by the Jonckheere (trend) and Williams or Dunnett (pairwise) tests.

Table B-2. PA06: Organ Weights Summary[a,b,c]

Endpoint	0 mg/kg n = 5	77 mg/kg n = 5	153 mg/kg n = 5	306 mg/kg n = 5	611 mg/kg n = 4–5[d]
Terminal Body Weight (g)	247.2 ± 3.8**	251.2 ± 3.5	245.8 ± 5.3	239.4 ± 4.2	230.6 ± 4.5*
Brain Weight Absolute (g)	1.68 ± 0.04	1.74 ± 0.02	1.71 ± 0.05	1.72 ± 0.02	1.60 ± 0.13
Brain Weight Relative[e] (mg/g)	6.82 ± 0.22*	6.91 ± 0.07	6.96 ± 0.13	7.19 ± 0.12	6.95 ± 0.61
Liver Weight Absolute (g)	10.35 ± 0.23**	11.23 ± 0.27	11.29 ± 0.37	11.95 ± 0.42**	11.75 ± 0.46**
Liver Weight Relative (mg/g)	41.90 ± 0.77**	44.70 ± 0.72	45.91 ± 0.95*	49.87 ± 1.17**	51.30 ± 1.59**

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 $p \leq 0.05$; **$p \leq 0.01$.
[a]Descriptions of organ weight endpoints and changes are provided in Appendix E.
[b]Data are displayed as mean ± standard error of the mean.
[c]Statistical analysis performed by the Jonckheere (trend) and Williams or Dunnett (pairwise) tests.
[d]Change in n reflects organ weights not recorded at necropsy. Liver weight was not measured for one animal in the 611 mg/kg group.
[e]Relative organ weights (organ weight-to-body weight ratios) are given as mg organ weight/g body weight.

Table B-3. PA41: Clinical Chemistry Summary[a,b]

Endpoint	0 mg/kg n = 5	77 mg/kg n = 5	153 mg/kg n = 5	306 mg/kg n = 5	611 mg/kg n = 5
Urea Nitrogen (mg/dL)	9.6 ± 0.2	10.8 ± 1.2	11.8 ± 2.1	12.6 ± 0.7*	11.2 ± 0.7
Creatinine (mg/dL)	0.27 ± 0.01**	0.28 ± 0.00	0.28 ± 0.01	0.25 ± 0.01*	0.25 ± 0.00*
Total Protein (g/dL)	5.96 ± 0.12	6.10 ± 0.08	5.98 ± 0.11	6.00 ± 0.07	5.90 ± 0.11
Globulin (g/dL)	2.54 ± 0.07*	2.68 ± 0.05	2.68 ± 0.06	2.76 ± 0.05	2.72 ± 0.07
A/G Ratio	1.35 ± 0.01**	1.28 ± 0.03	1.23 ± 0.02*	1.18 ± 0.03**	1.17 ± 0.03**
Albumin (g/dL)	3.42 ± 0.06**	3.42 ± 0.06	3.30 ± 0.06	3.24 ± 0.05	3.18 ± 0.07*
Cholesterol (mg/dL)	113.2 ± 5.1**	140.6 ± 7.6*	131.6 ± 5.2*	134.0 ± 5.1*	149.0 ± 3.9**
Triglyceride (mg/L)	52.2 ± 1.1	51.8 ± 11.8	61.4 ± 4.3	57.4 ± 6.0	59.2 ± 2.9
LDL Cholesterol (mg/dL)	23.2 ± 1.3	22.6 ± 1.1	25.4 ± 1.0	21.0 ± 1.4	22.6 ± 0.6
HDL Cholesterol (mg/dL)	52.0 ± 2.2**	66.8 ± 3.4*	59.0 ± 2.2*	66.0 ± 1.3**	71.2 ± 2.5**
Alanine Aminotransferase (IU/L)	59.60 ± 3.99	52.60 ± 3.76	57.60 ± 4.70	60.80 ± 3.50	60.60 ± 4.21
Aspartate Aminotransferase (U/L)	109.80 ± 5.58	79.80 ± 10.75	76.0 ± 15.80	81.00 ± 1.73	89.00 ± 8.01
Sorbitol Dehydrogenase (IU/L)	15.0 ± 1.5	10.0 ± 0.6*	13.4 ± 2.0	11.3 ± 0.8	10.0 ± 1.2
Bile Salts/Acids (μmol/L)	34.9 ± 4.4*	33.5 ± 11.1	23.2 ± 2.2	26.4 ± 5.9	18.9 ± 4.1

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 $p \leq 0.05$; **$p \leq 0.01$.
A/G Ratio = ratio of albumin to globulin; LDL = low-density lipoprotein; HDL = high-density lipoprotein.
[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.

Table B-4. R07: Hormones and Enzymes Summary[a,b]

Endpoint	0 mg/kg n = 5	77 mg/kg n = 5	153 mg/kg n = 5	306 mg/kg n = 5	611 mg/kg n = 5
Total Thyroxine (µg/dL)	3.98 ± 0.40	4.92 ± 0.35	5.35 ± 0.12	5.24 ± 0.33	5.02 ± 0.24
Cholinesterase (IU/L)	285.4 ± 16.1**	166.4 ± 8.0**	146.4 ± 13.8**	116.4 ± 6.2**	104.0 ± 3.9**

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 $p \leq 0.01$.
[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.

Appendix C. Transcriptomic Quality Control and Additional Data Analysis

Table of Contents

Figures

C.1. Gene Expression Quality Control

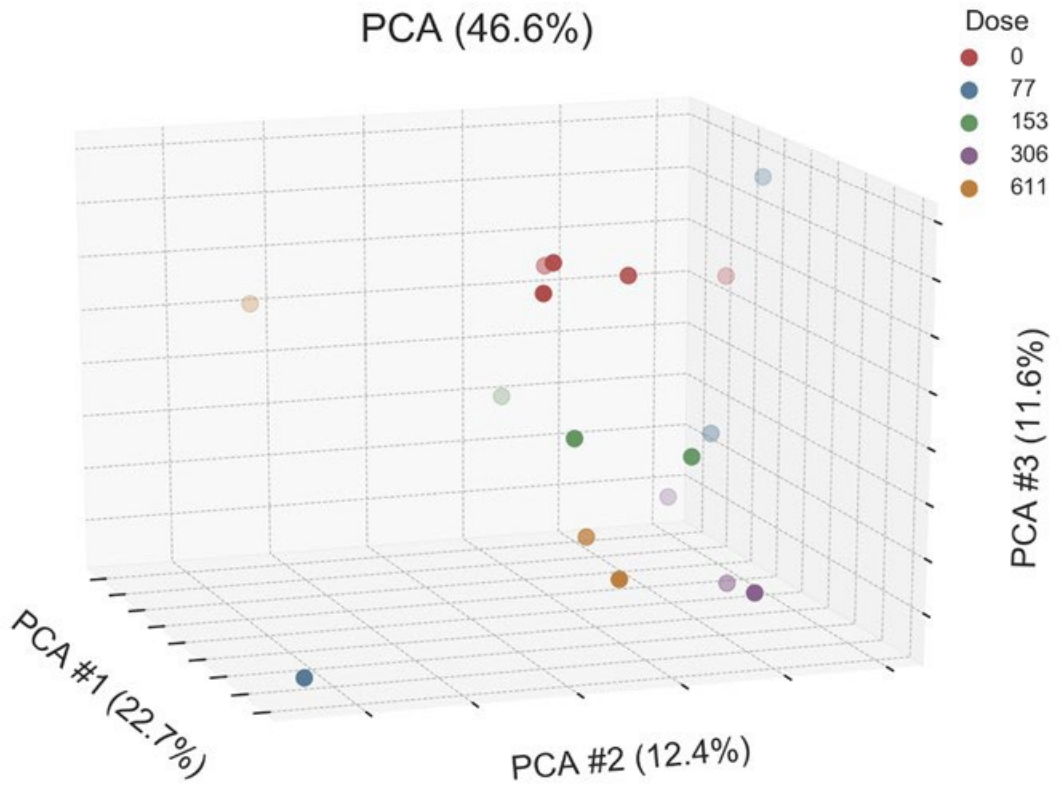

Figure C-1. A Principal Component Analysis of the Robust Multi-array Average-normalized Data

The principal component analysis (PCA) plot enables three-dimensional visualization of global transcriptional changes and the divergence of transcript expression from 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. Lighter color (fogging) indicates data point is farther back on the z-plane [principal component (PC) #1].

C.2. Additional Data Analysis

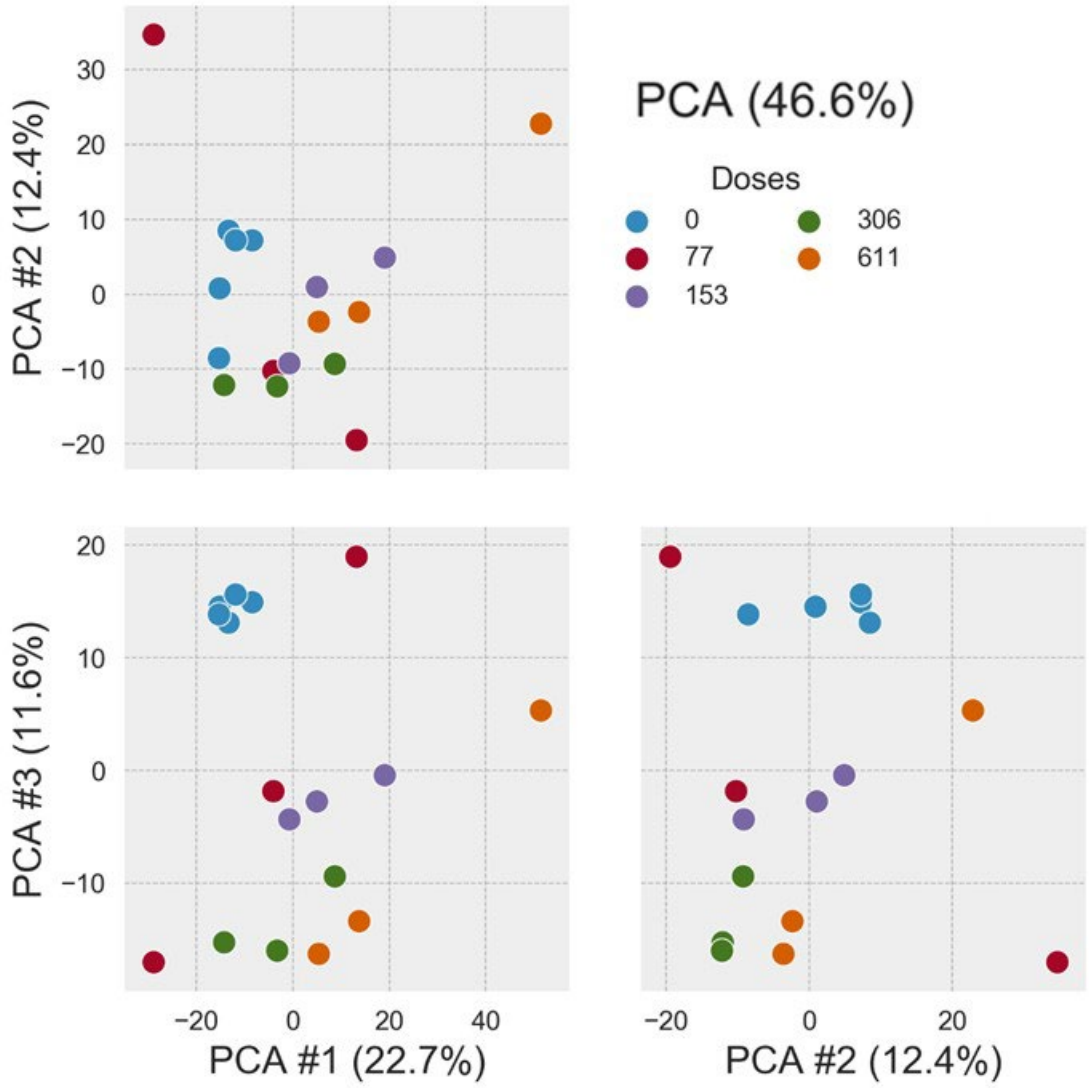

Figure C-2. An Alternative View of the Principal Component Analysis of the Robust Multi-array Average-normalized Data

This alternative view of the 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.

Appendix D. Benchmark Dose Model Recommendation and Selection Methodologies

Tables

Figures

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

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

BMD = benchmark dose; N/A = not applicable; BMD_L = benchmark dose lower confidence limit; AIC = Akaike information criterion; BMDS = Benchmark Dose Software.

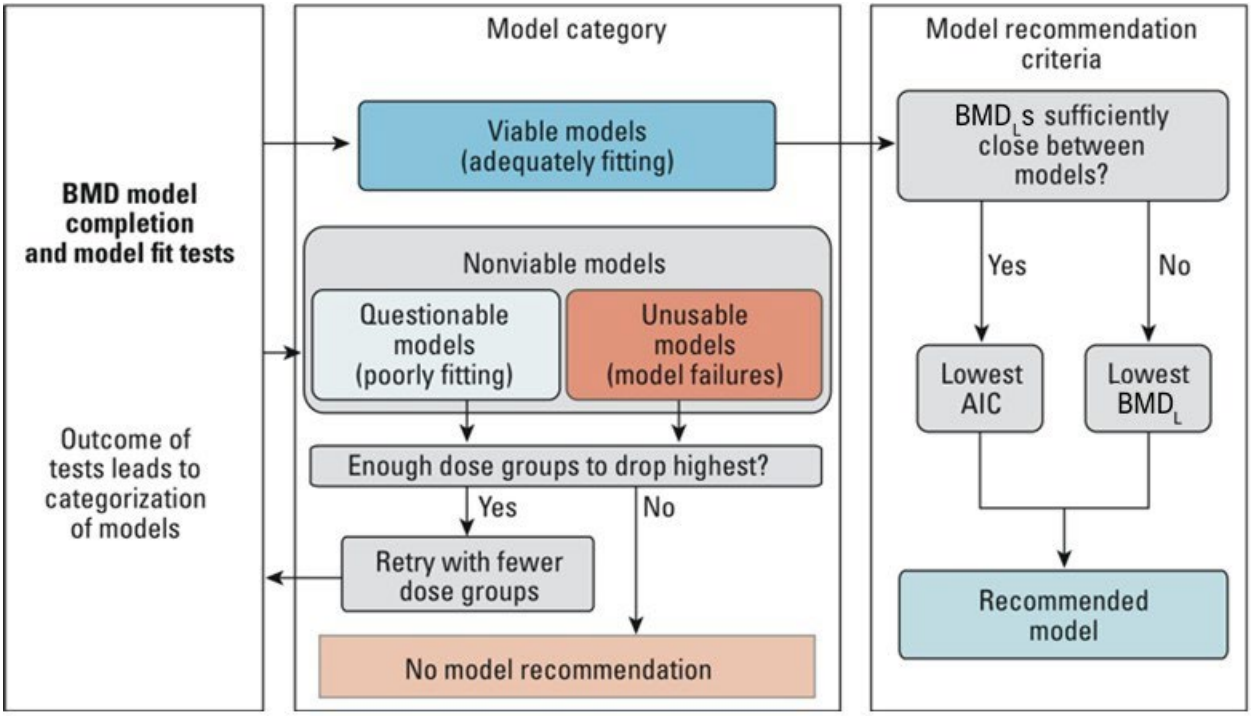

Figure D-1. Benchmark Dose Model Recommendation/Selection Methodology for Automated Benchmark Dose Execution of Apical Endpoints

Source: Figure adapted from Wignall et al. (2014)[18]
BMD = benchmark dose; BMD_L = benchmark dose lower confidence limit; AIC = Akaike information criterion.

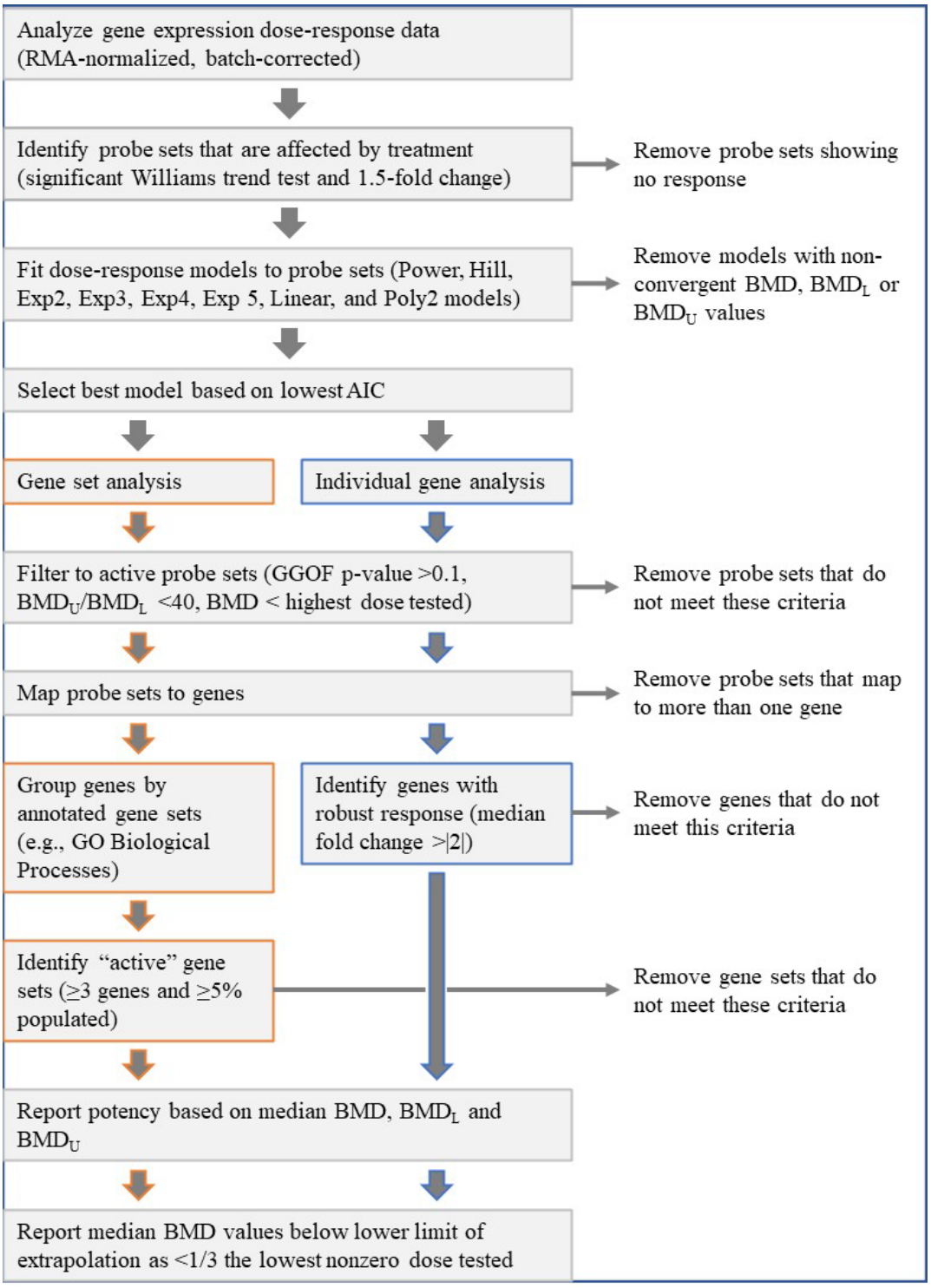

Figure D-2. Benchmark Dose Model Recommendation/Selection Methodology for Benchmark Dose Execution of Gene Sets with Expression Changes Enacted by Chemical Exposure

Adapted from Thomas et al. (2007)[28]
RMA = Robust Multi-array Average; BMD = benchmark dose; BMD_L = benchmark dose lower confidence limit; BMD_U = benchmark dose upper confidence limit; AIC = Akaike information criterion; GGOF = global goodness of fit; GO = Gene Ontology.

Appendix E. Organ Weight Descriptions

Table of Contents

E.1. Organ Weight Descriptions

Brain: As the principal organ responsible for cognition and control of organ systems and bodily functions, the brain is largely shielded from toxic insults sufficiently severe to affect its weight. Because of this resistance to change, brain weight is often used as a denominator in determinations of other organ weight ratio changes. Other than in cases of grossly observable effects in the brain at necropsy, significant differences in brain weight in subacute toxicity studies are unlikely an effect of chemical exposure. More likely, changes in brain weight are the result of randomization (i.e., sorting of animals into groups for which the mean and standard deviation are significantly different at the outset of study, making it appear that there is an exposure-related difference when it is rather a byproduct of natural variation and chance).

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.

Appendix F. Supplemental Data

The following supplemental files are available at https://doi.org/10.22427/NIEHS-DATA-NIEHS-05.[21]

F.1. Apical Benchmark Dose Analysis

BMD Apical Endpoints Model Fits
BMD_Apical_Endpoints_Model_Fits.docx

BMD Model Recommendation Selection Rules
BMD_Model_Recommendation_Selection_Rules.docx

Read Me
Read_Me.docx

Model Parameters
Model_Parameters.xlsx

BMDs Code Package
BMDs_code_package.zip

F.2. Genomic Benchmark Dose Analysis

BMDExpress Project File (bm2 format)
BMDExpress_Project_File_(bm2_format).bm2

Gene Description
Gene_Description.csv

Top 10 GO Biological Process Gene Sets
Top_10_GO_Biological_Process_Gene_Sets.docx

Top 10 Genes Ranked by Potency of Perturbation
Top_10_Genes_Ranked_by_Potency_of_Perturbation.docx

BMDExpress Project File (JSON format)
BMDExpress_Project_File_(JSON_format).json

GO Biological Process Description
GO_Biological_Process_Description.tsv

BMDExpress Expression Data
BMDExpress_Expression_Data.txt

BMDExpress GO Biological Process Deduplicated BMD Results
BMDExpress_GO_Biological_Process_Deduplicated_BMD_Results.txt

BMDExpress Individual Probe Set BMD Results
BMDExpress_Individual_Probe_Set_BMD_Results.txt

BMDExpress Individual Gene BMD Results
BMDExpress_Individual_Gene_BMD_Results.txt

BMDExpress Prefilter Results
BMDExpress_Prefilter_Results.txt

Animal and Microarray Metadata
Animal_and_Microarray_Metadata.zip

Array Platform Gene and GO Term Annotation File
Array_platform_gene_and_GO_term_annotation_file.zip

BMDExpress Software
BMDExpress_Software.zip

Batch Correction Documentation
Batch_Correction_Documentation.zip

Individual Gene BMD Analysis Results File
Individual_Gene_BMD_Analysis_Results_File.zip

Principal Components Analysis Files
Principal_Components_Analysis_Files.zip

Raw Data CEL Files
Raw_data_CEL_files.zip

F.3. Study Tables

I04 - Mean Body Weight Summary
C10866B_I04_Mean_Body_Weight_Summary.pdf

I05 - Clinical Observations Summary
C10866B_I05_Clinical_Observations_Summary.pdf

PA06 - Organ Weights Summary
C10866B_PA06_Organ_Weights_Summary.pdf

PA41 - Clinical Chemistry Summary
C10866B_PA41_Clinical_Chemistry_Summary.pdf

R07 - Hormone Summary
C10866B_R07_Hormone_Summary.pdf

F.4. Individual Animal Data

Individual Animal Body Weight Data
C10866B_Individual_Animal_Body_Weight_Data.xlsx

Individual Animal Clinical Chemistry Data
C10866B_Individual_Animal_Clinical_Chemistry_Data.xlsx

Individual Animal Clinical Observations Data
C10866B_Individual_Animal_Clinical_Observations_Data.xlsx

Individual Animal Hormone Data
C10866B_Individual_Animal_Hormone_Data.xlsx

Individual Animal Organ Weight Data
C10866B_Individual_Animal_Organ_Weight_Data.xlsx

National Institute of
Environmental Health Sciences
Division of Translational Toxicology
Office of Policy, Review, and Outreach
P.O. Box 12233
Durham, NC 27709

www.niehs.nih.gov/reports

ISSN 2768-5632