National Institute of Environmental Health Sciences

NIEHS Report on In Vivo Repeat Dose Biological Potency Study of *tert*-Butylphenyl Diphenyl Phosphate (CASRN 56803-37-3) in Male Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats(Gavage Studies)

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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/DNTP 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).

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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 tert-Butylphenyl Diphenyl Phosphate (CASRN 56803-37-3) in Male Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats (Gavage Studies)* was not subjected to further external peer review.

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Abstract

Background: *tert*-Butylphenyl diphenyl phosphate (BPDP) is an organophosphate flame retardant currently on the market that is used as a replacement for phased-out polybrominated diphenyl ethers. Toxicological information on this class of chemicals is sparse. A short-term, in vivo transcriptomic study was used to assess the biological potency of BPDP.

Methods: Scientists at the Division of Translational Toxicology, National Institute of Environmental Health Sciences conducted this short-term in vivo biological potency study on BPDP in young adult male Sprague Dawley (Hsd:Sprague Dawley® SD®) rats. BPDP was formulated in corn oil and administered once daily for 4 consecutive days by gavage. BPDP was tested at six doses (0, 65, 129, 258, 516, and 1,033 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 increased relative and absolute liver weights and decreased serum bile salt/acid concentration. The BMDs and benchmark dose lower confidence limits (BMD_Ls) were 288.7 (223.5), 313.0 (121.5), and 763.1 (434.1) mg/kg, respectively. Although serum cholinesterase activity was significantly decreased in all dosed groups (19%–56% decrease), beginning with 65 mg/kg (the lowest-observed-effect level), a BMD (BMD_L) was not determined because no viable model was available.

Two Gene Ontology biological processes had BMD median values below the lower limit of extrapolation (<21.7 mg/kg), which relate to entrainment of circadian clock by photoperiod and cellular response to thyroid hormone stimulus. The most sensitive gene sets for which a reliable estimate of the BMD could be made were neutral amino acid transport and negative regulation of collagen biosynthetic process with median BMDs of 23.8 and 29.4 mg/kg and median BMD_Ls of 15.2 and 18.8 mg/kg, respectively. The top 10 most sensitive individual genes exhibited changes in expression at dose levels below which a reliable estimate of potency could be achieved (<21.7 mg/kg). Of these genes, eight were upregulated: *Hsd17b2*, *Nr1d2*, *Jade1*, *Sdr42e1, Tef, Per3*, *Bcar3 and Akr7a3*. Two genes, *Lgalsl* and *Nfil3*, were downregulated.

Summary: Taken together, the most sensitive gene set BMD (BMD_L) median and apical endpoint BMD (BMD_L) values that could be reliably determined occurred at 23.8 (15.2) and 288.7 (223.5) mg/kg, respectively. The BMDs (and BMD_Ls) could not be determined for the top 10 most sensitive individual genes and were estimated to be <21.7 mg/kg. Serum cholinesterase activity was significantly and markedly decreased for all dosed groups and appeared to be one of the most sensitive apical measures, although a 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 and genes.

Background

tert-Butylphenyl diphenyl phosphate (BPDP) 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 BPDP 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 rats. The rats in each dose group then were administered *tert*-butylphenyl diphenyl phosphate (BPDP) by gavage in corn oil at a dose level of 0, 65, 129, 258, 516, or 1,033 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 BPDP. 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 BPDP was estimated to be approximately 1,033 mg/kg/day; thus, this dose was selected as the highest dose in the present study.

Chemistry

BPDP (CASRN 56803-37-3; $C_{22}H_{23}O_4P$; molar mass 382.39 g/mol) was obtained in two lots from Ubichem PLC (Eastleigh, Hampshire, UK; lots 2010-270439 and 2011020199), which were filtered and blended to form a single lot (M062011NS, batch 02). The identity was confirmed and the purity was determined using gas chromatography coupled with mass spectrometry. The BPDP test article was a mixture of triphenyl phosphate (TPHP) and two isomers of BPDP, two isomers of di(*tert*-butylphenyl) phenyl phosphate, and tris(*tert*-butylphenyl) phosphate. BPDP lot purity, determined by summing the relative peak areas of the butylphenyl isomers identified in the total ion chromatogram, was 64.5%. The estimated TPHP in the lot was approximately 35%.

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 gas chromatography with flame ionization detection, and shipped to Alion (Research Triangle Park, NC). All formulations were within ±10% of target concentrations and no BPDP 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 22 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 BMD_L 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 BPDP 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-03.[21]

Results

Animal Condition, Body Weights, and Organ Weights

Four rats died early in the study; one vehicle control rat was found dead on study day 3, two rats in the 65 mg/kg group died due to gavage error on study day 0, and one rat in the 1,033 mg/kg group was found dead on study day 1. No clinical observations were noted. No significant change in terminal body weight was observed with exposure to *tert*-butylphenyl diphenyl phosphate (BPDP) (Table 1).

Table 1. Body Weight Summary

Study Day	0 mg/kg[a,b] n = 4–5[c]	65 mg/kg n = 3[c]	129 mg/kg n = 5	258 mg/kg n = 5	516 mg/kg n = 5	1,033 mg/kg n = 4–5[c]	BMD_{1Std} (mg/kg)	BMD_{L1Std} (mg/kg)
0	254.6 ± 1.8	260.0 ± 5.1	262.7 ± 1.4	257.4 ± 1.8	256.9 ± 2.6	258.0 ± 2.4	ND	ND
4	272.2 ± 0.4	257.6 ± 5.7	284.5 ± 2.9	275.0 ± 5.4	273.7 ± 3.1	262.2 ± 6.0	ND	ND

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.
[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.
[c]Changes in n are the result of early deaths related to gavage error and not related to chemical exposure.

At necropsy, a significant increase in absolute and relative liver weights occurred in dose groups ≥129 mg/kg; both endpoints had positive trends (Table 2). The benchmark dose (benchmark dose lower confidence limit)—BMD (BMD_L)—for increased absolute liver weight was 313.0 (121.5) mg/kg and for relative liver weight was 288.7 (223.5) mg/kg. Significant trend and pairwise comparisons were not observed in absolute or relative brain weights (Appendix B).

Clinical Chemistry

Serum bile salt/acid concentration was significantly decreased at 1,033 mg/kg and had a negative trend with a BMD (BMD_L) of 763.1 (434.1) mg/kg (Table 3). There were no other clinical chemistry findings that exhibited significant trend and pairwise comparisons (Appendix B).

Table 2. Organ Weights Summary[a]

Endpoint	0 mg/kg[b,c] n = 4	65 mg/kg n = 3	129 mg/kg n = 5	258 mg/kg n = 5	516 mg/kg n = 5	1,033 mg/kg n = 4	BMD_{1Std} (mg/kg)	BMD_{L1Std} (mg/kg)
Terminal Body Weight (g)	272.2 ± 0.4	257.6 ± 5.7	284.5 ± 2.9	275.0 ± 5.4	273.7 ± 3.1	262.2 ± 6.0	ND	ND
Liver Weight Absolute (g)	10.40 ± 0.31**	9.70 ± 0.10	12.63 ± 0.44**	12.96 ± 0.25**	15.00 ± 0.49**	14.62 ± 0.47**	313.0	121.5
Liver Weight Relative[d] (mg/g)	38.21 ± 1.15**	37.70 ± 1.18	44.37 ± 1.47**	47.13 ± 0.52**	54.82 ± 1.66**	55.79 ± 1.51**	288.7	223.5

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; ND = not determined.
[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]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 = 4	65 mg/kg n = 3	129 mg/kg n = 5	258 mg/kg n = 5	516 mg/kg n = 5	1,033 mg/kg n = 4	BMD_{1Std} (mg/kg)	BMD_{L1Std} (mg/kg)
Bile Salts/Acids (µmol/L)	52.2 ± 5.2*	35.1 ± 2.3	41.9 ± 6.4	30.9 ± 9.0	41.5 ± 6.5	23.4 ± 5.8*	763.1	434.1

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 $p \leq 0.05$.
Benchmark response set at 1 standard deviation from the mean.
BMD = benchmark dose; BMD_L = benchmark dose lower confidence limit.
[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.

Hormones and Enzymes

Serum cholinesterase activity was significantly decreased in all dosed groups by 19%–56%, beginning with the 65 mg/kg group; a BMD (BMD_L) was not determined because no viable model was available (Table 4). No significant trend and pairwise comparisons were observed in total thyroxine concentration (Appendix B).

Table 4. Hormones and Enzymes Summary

Endpoint	0 mg/kg[a,b] n = 4	65 mg/kg n = 3	129 mg/kg n = 5	258 mg/kg n = 5	516 mg/kg n = 5	1,033 mg/kg n = 4	BMD_{1Std} (mg/kg)	BMD_{L1Std} (mg/kg)
Cholinesterase (IU/L)	291.3 ± 9.4**	209.0 ± 13.9*	236.0 ± 6.7*	183.4 ± 8.8**	152.4 ± 20.0**	129.0 ± 14.8**	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; 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.

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 below the lower limit of extrapolation (<21.7 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
Liver Weight Relative	288.7	223.5	129	65	UP
Liver Weight Absolute	313.0	121.5	129	65	UP
Bile Salts/Acids	763.1	434.1	1,033	516	DOWN
Cholinesterase	NVM	NVM	**65**	ND	DOWN

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; NVM = nonviable model; ND = not determined.
Values in bold text indicate the LOEL of endpoints for which a BMD could not be calculated.

Gene Set Benchmark Dose Analysis

Chemical-induced alterations in liver gene transcript expression were examined to determine those gene sets most sensitive to BPDP 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.

Two gene sets had estimated BMD median values <21.7 mg/kg, which relate to entrainment of circadian clock by photoperiod and cellular response to thyroid hormone stimulus. The most sensitive GO biological processes for which a BMD value could be reliably calculated were neutral amino acid transport (GO:0015804) and negative regulation of collagen biosynthetic process (GO:0032966) with BMDs (BMD_{LS}) of 23.8 (15.2) and 29.4 (18.8) mg/kg, respectively. 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:0043153 entrainment of circadian clock by photoperiod	3/27	11	*Per3*; *Cry2*; *Bhlhe40*	<21.7[b]	NR	3	0
GO:0097067 cellular response to thyroid hormone stimulus	3/24	13	*Gclc*; *Klf9*; *Gclm*	<21.7	NR	3	0
GO:0015804 neutral amino acid transport	3/35	9	*Slc3a2*; *Nfe2l1*; *Slc6a6*	23.8	15.2–73.8	2	1
GO:0032966 negative regulation of collagen biosynthetic process	3/17	18	*Cyp2j4*; *Errfi1*; *Pparg*	29.4	18.8–53.5	3	0
GO:0009404 toxin metabolic process	4/18	22	*Gsta3*; *Cyp1a1*; *Akr7a3*; *Ddc*	29.7	14.9–66.7	4	0
GO:0120163 negative regulation of cold-induced thermogenesis	3/42	7	*Tle3*; *Id1*; *Aldh1a1*	33.1	22.1–57.0	2	1
GO:0006825 copper ion transport	3/15	20	*Fkbp4*; *Steap4*; *Mmgt1*	33.7	15.1–95.6	1	2

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:0045599 negative regulation of fat cell differentiation	3/51	6	*Zadh2*; *Id4*; *Trib3*	36.9	19.3–87.8	2	1
GO:0006801 superoxide metabolic process	3/29	10	*Cbs*; *Cybb*; *Apoa4*	37.8	23.8–70.0	0	3
GO:0033194 response to hydroperoxide	3/20	15	*Dapk1*; *Chuk*; *Apoa4*	37.8	23.8–70.0	1	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]<21.7 = 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:0043153 entrainment of circadian clock by photoperiod: The synchronization of a circadian rhythm to photoperiod, the intermittent cycle of light (day) and dark (night).
GO:0097067 cellular response to thyroid hormone stimulus: A change in state or activity of a cell (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a thyroid hormone stimulus.
GO:0015804 neutral amino acid transport: The directed movement of neutral amino acids—amino acids with no net charge—into, out of, or within a cell, or between cells, by means of some agent such as a transporter or pore.
GO:0032966 negative regulation of collagen biosynthetic process: Any process that stops, prevents, or reduces the frequency, rate, or extent of the chemical reactions and pathways resulting in the formation of collagen, any of a group of fibrous proteins of very high tensile strength that form the main component of connective tissue in animals.
GO:0009404 toxin metabolic process: The chemical reactions and pathways involving a toxin, a poisonous compound (typically a protein) that is produced by cells or organisms and that can cause disease when introduced into the body or tissues of an organism.
GO:0120163 negative regulation of cold-induced thermogenesis: Any process that stops, prevents, or reduces the rate of cold-induced thermogenesis.
GO:0006825 copper ion transport: The directed movement of copper (Cu) ions into, out of, or within a cell, or between cells, by means of some agent such as a transporter or pore.
GO:0045599 negative regulation of fat cell differentiation: Any process that stops, prevents, or reduces the frequency, rate, or extent of adipocyte differentiation.
GO:0006801 superoxide metabolic process: The chemical reactions and pathways involving superoxide, the superoxide anion O_2^- (superoxide free radical), or any compound containing this species.
GO:0033194 response to hydroperoxide: 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 hydroperoxide stimulus. Hydroperoxides are monosubstitution products of hydrogen peroxide, HOOH.

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 <21.7 mg/kg. Eight genes exhibited an increase in expression: *Hsd17b2* [hydroxysteroid (17-beta) dehydrogenase 2], *Nr1d2* (nuclear receptor subfamily 1, group D, member 2), *Jade1* (jade family PHD finger 1), *Sdr42e1* (short chain dehydrogenase/reductase family 42E, member 1), *Tef* (TEF transcription factor, PAR bZIP family member), *Per3* (period circadian regulator 3), *Bcar3* (BCAR3 adaptor protein, NSP family member), and *Akr7a3* (aldo-keto reductase family 7 member A3). Two genes exhibited a decrease in expression: *Lgalsl* (galectin-like) and *Nfil3* (nuclear factor, interleukin 3 regulated).

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
Hsd17b2	79243	1387156_at	<21.7[b] (NR)	6.0	UP
Nr1d2	259241	1370541_at,1390430_at	<21.7 (NR)	3.8	UP
Jade1	310352	1374636_at	<21.7 (NR)	2.1	UP
Lgalsl	360983	1376867_at	<21.7 (NR)	3.0	DOWN
Sdr42e1	307897	1394960_at	<21.7 (NR)	2.1	UP
Tef	29362	1385374_at	<21.7 (NR)	2.1	UP
Per3	78962	1378745_at	<21.7 (NR)	5.5	UP
Bcar3	310838	1374947_at	<21.7 (NR)	2.2	UP
Nfil3	114519	1368488_at	<21.7 (NR)	3.3	DOWN
Akr7a3	26760	1368121_at	<21.7 (NR)	4.5	UP

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]<21.7 = 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]
***Hsd17b2*:** Human Uniprot function (Human *HSD17B2*): Capable of catalyzing the interconversion of testosterone and androstenedione, as well as estradiol and estrone. Also has 20-alpha-HSD activity. Uses NADH, whereas EDH17B3 uses NADPH. {ECO0000269|PubMed8099587}.
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}.
Jade1: Human Uniprot function (Human *JADE1*): Scaffold subunit of some HBO1 complexes, which have a histone H4 acetyltransferase activity (PubMed16387653, PubMed19187766, PubMed20129055, PubMed24065767). Plays a key role in HBO1 complex by directing KAT7/HBO1 specificity towards histone H4 acetylation (H4K5ac, H4K8ac and H4K12ac), regulating DNA replication initiation, regulating DNA replication initiation (PubMed20129055, PubMed24065767). May also promote acetylation of nucleosomal histone H4 by KAT5 (PubMed15502158). Promotes apoptosis (PubMed16046545). May act as a renal tumor suppressor (PubMed16046545). Negatively regulates canonical Wnt signaling; at least in part, cooperates with NPHP4 in this function (PubMed22654112). {ECO:0000269|PubMed15502158, ECO:0000269|PubMed16046545, ECO:0000269|PubMed16387653, ECO:0000269|PubMed19187766, ECO:0000269|PubMed20129055, ECO:0000269|PubMed22654112, ECO:0000269|PubMed24065767}.
Lgalsl: Human Uniprot function (Human *LGALSL*): Does not bind lactose and may not bind carbohydrates. {ECO0000269|PubMed18320588, ECO0000269|PubMed18433051}.
Sdr42e1: No description available.
Tef: Human Uniprot function (Human *TEF*): Transcription factor that binds to and transactivates the TSHB promoter. Binds to a minimal DNA-binding sequence 5'-[TC][AG][AG]TTA[TC][AG]-3'.
Per3: Human Uniprot function (Human *PER3*): Originally described as a core component of the circadian clock. The circadian clock, an internal time-keeping system, regulates various physiological processes through the generation of approximately 24-hour circadian rhythms in gene expression, which are translated into rhythms in metabolism and behavior. It is derived from the Latin roots “circa” (about) and “diem” (day) and acts as an important regulator of a wide array of physiological functions including metabolism, sleep, body temperature, blood pressure, endocrine, immune, cardiovascular, and renal function. Consists of two major components: the central clock, residing in the suprachiasmatic nucleus (SCN) of the brain, and the peripheral clocks that are present in nearly every tissue and organ system. Both the central and peripheral clocks can be reset by environmental cues, also known as Zeitgebers (German for “time givers”). The predominant Zeitgeber for the central clock is light, which is sensed by the retina and signals directly to the SCN. The central clock entrains the peripheral clocks through neuronal and hormonal signals, body temperature, and feeding-related cues, aligning all clocks with the external light/dark cycle. Circadian rhythms allow an organism to achieve temporal homeostasis with its environment at the molecular level by regulating gene expression to create a peak of protein expression once every 24 hours to control when a particular physiological process is most active with respect to the solar day. Transcription and translation of core clock components (CLOCK, NPAS2, ARNTL/BMAL1, ARNTL2/BMAL2, PER1, PER2, PER3, CRY1, and CRY2) play a critical role in rhythm generation, whereas delays imposed by post-translational modifications (PTMs) are important for determining the period (tau) of the rhythms (tau refers to the period of a rhythm and is the length, in time, of one complete cycle). A diurnal rhythm is synchronized with the day/night cycle, whereas the ultradian and infradian rhythms have a period shorter and longer than 24 hours, respectively. Disruptions in the circadian rhythms contribute to the pathology of cardiovascular diseases, cancer, metabolic syndromes, and aging. A transcription/translation feedback loop (TTFL) forms the core of the molecular circadian clock mechanism. Transcription factors, CLOCK or NPAS2 and ARNTL/BMAL1 or ARNTL2/BMAL2, form the positive limb of the feedback loop, act in the form of a heterodimer, and activate the transcription of core clock genes and clock-controlled genes (involved in key metabolic processes), harboring E-box elements (5'-CACGTG-3') within their promoters. The core clock genes, PER1/2/3 and CRY1/2, which are transcriptional repressors, form the negative limb of the feedback loop and interact with the CLOCK|NPAS2-ARNTL/BMAL1|ARNTL2/BMAL2 heterodimer, inhibiting its activity and thereby negatively regulating their own expression. This heterodimer also activates nuclear receptors NR1D1, NR1D2, RORA, RORB, and RORG, which form a second feedback loop and which activate and repress ARNTL/BMAL1 transcription, respectively. Has a redundant role with the other PER proteins, PER1 and PER2, and is not essential for the circadian rhythms’ maintenance. In contrast, plays an important role in sleep-wake timing and sleep homeostasis, probably through the transcriptional regulation of sleep homeostasis-related genes, without influencing circadian parameters. Can bind heme. {ECO0000269|PubMed17346965, ECO0000269|PubMed19716732, ECO0000269|PubMed24439663, ECO0000269|PubMed24577121, ECO0000269|PubMed26903630}.
Bcar3: Human Uniprot function (Human *BCAR3*): Acts as an adapter protein downstream of several growth factor receptors to promote cell proliferation, migration, and redistribution of actin fibers (PubMed24216110). Specifically involved in INS/insulin signaling pathway by mediating MAPK1/ERK2-MAPK3/ERK1 activation and DNA synthesis (PubMed24216110). Promotes insulin-mediated membrane ruffling (by similarity). In response to vasoconstrictor peptide EDN1, involved in the activation of RAP1 downstream of PTK2B via interaction with phosphorylated BCAR1 (PubMed19086031). Inhibits cell migration and invasion via regulation of TGFB-mediated matrix digestion, actin filament rearrangement, and inhibition of invadopodia activity (by similarity). May inhibit TGFB-SMAD signaling, via facilitating BCAR1 and SMAD2 and/or SMAD3 interaction (by

similarity). Regulates EGF-induced DNA synthesis (PubMed18722344). Required for the maintenance of ocular lens morphology and structural integrity, potentially via regulation of focal adhesion complex signaling (by similarity). Acts upstream of PTPRA to regulate the localization of BCAR1 and PTPRA to focal adhesions, via regulation of SRC-mediated phosphorylation of PTPRA (by similarity). Positively regulates integrin-induced tyrosine phosphorylation of BCAR1 (by similarity). Acts as a guanine nucleotide exchange factor (GEF) for small GTPases RALA, RAP1A, and RRAS (by similarity). However, in a contrasting study, lacks GEF activity toward RAP1 (PubMed22081014).

Nfil3: Human Uniprot function (Human *NFIL3*): Acts as a transcriptional regulator that recognizes and binds to the sequence 5'[GA]TTA[CT]GTAA[CT]-3', a sequence present in many cellular and viral promoters. Represses transcription from promoters with activating transcription factor (ATF) sites. Represses promoter activity in osteoblasts (by similarity). Represses transcriptional activity of PER1 (by similarity). Represses transcriptional activity of PER2 via the B-site on the promoter (by similarity). Activates transcription from the interleukin-3 promoter in T-cells. Competes for the same consensus-binding site with PAR DNA-binding factors (DBP, HLF, and TEF) (by similarity). Component of the circadian clock that acts as a negative regulator for the circadian expression of PER2 oscillation in the cell-autonomous core clock (by similarity). Protects pro-B cells from programmed cell death (by similarity). Represses the transcription of CYP2A5 (by similarity). Positively regulates the expression and activity of CES2 by antagonizing the repressive action of NR1D1 on CES2 (by similarity). {ECO0000250|UniProtKBO08750, ECO0000269|PubMed1620116, ECO0000269|PubMed7565758, ECO0000269|PubMed8836190}.

Akr7a3: Human Uniprot function (Human *AKR7A3*): Can reduce the dialdehyde protein-binding form of aflatoxin B1 (AFB1) to the nonbinding AFB1 dialcohol. May be involved in protection of the liver against the toxic and carcinogenic effects of AFB1, a potent hepatocarcinogen. {ECO:0000269|PubMed18416522}.

Summary

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

Serum cholinesterase activity was significantly and markedly decreased for all dosed groups and appeared to be one of the most sensitive apical measures, although a benchmark dose (benchmark dose lower confidence limit)—BMD (BMD_L)—was not determined because no viable model was available. These findings are consistent with several reports that show the classic cholinesterase inhibition in organophosphates.[27] The most sensitive apical endpoints for which a BMD could be determined were an increase in relative and absolute liver weights with BMDs (BMD_Ls) of 288.7 (223.5) and 313.0 (121.5) mg/kg, respectively. The next most sensitive apical endpoint observed was a decrease in serum bile salt/acid concentration with a BMD (BMD_L) of 763.1 (434.1) mg/kg.

Gene set-level transcriptional changes in the liver following BPDP exposure were estimated to occur at a BMD (BMD_L) as low as 23.8 (15.2) mg/kg, corresponding to neutral amino acid transport (GO:0015804). Two gene sets had BMD estimates below the lower limit of extrapolation (<21.7 mg/kg). The top 10 most sensitive genes exhibited changes in expression at dose levels below which a reliable estimate of potency could be achieved (<21.7 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 transcriptional change in a gene set, GO:0015804, with a BMD (BMD_L) of 23.8 (15.2) mg/kg. Individual gene transcriptional changes provided potency estimates <21.7 mg/kg, while apical endpoints provided potency estimates that were higher than GO:0015804. Follow-up studies that investigate transcriptional changes at lower doses will be a useful future direction to determine the biological potency of BPDP more accurately.

References

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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[a]	Array ID
7	Corn Oil	0	0	No	NA
26	Corn Oil	0	0	Yes	025-052014-MW_(Rat230_2).CEL
34	Corn Oil	0	0	Yes	061-052114-MW_(Rat230_2).CEL
35	Corn Oil	0	0	Yes	033-052014-MW_(Rat230_2).CEL
57	Corn Oil	0	0	Yes	065-052714-JAP_(Rat230_2).CEL
2	BPDP	0.169	65	Yes	031-052014-MW_(Rat230_2).CEL
13	BPDP	0.169	65	No	NA
21	BPDP	0.169	65	Yes	068-052714-JAP_(Rat230_2).CEL
31	BPDP	0.169	65	No	NA
65	BPDP	0.169	65	Yes	101R-082514-MW_(Rat230_2).CEL
8	BPDP	0.338	129	Yes	032-052014-MW_(Rat230_2).CEL
18	BPDP	0.338	129	Yes	069-052714-JAP_(Rat230_2).CEL
19	BPDP	0.338	129	Yes	102-052814-MW_(Rat230_2).CEL
43	BPDP	0.338	129	Yes	NA
64	BPDP	0.338	129	Yes	NA
20	BPDP	0.675	258	Yes	NA
33	BPDP	0.675	258	Yes	034-052114-MW_(Rat230_2).CEL
48	BPDP	0.675	258	Yes	NA
59	BPDP	0.675	258	Yes	070-052714-JAP_(Rat230_2).CEL
62	BPDP	0.675	258	Yes	103-052814-MW_(Rat230_2).CEL
22	BPDP	1.35	516	Yes	035-052014-MW_(Rat230_2).CEL
42	BPDP	1.35	516	Yes	NA
49	BPDP	1.35	516	Yes	071-052714-JAP_(Rat230_2).CEL
51	BPDP	1.35	516	Yes	NA
66	BPDP	1.35	516	Yes	104-052814-MW_(Rat230_2).CEL
28	BPDP	2.7	1,033	Yes	NA
32	BPDP	2.7	1,033	No	NA
36	BPDP	2.7	1,033	Yes	036-052014-MW_(Rat230_2).CEL
39	BPDP	2.7	1,033	Yes	072-052714-JAP_(Rat230_2).CEL
61	BPDP	2.7	1,033	Yes	105-052814-MW_(Rat230_2).CEL

NA = no transcriptomics data collected for selected animal.
[a]One vehicle control rat was found dead on study day 3, and one rat in the 1,033 mg/kg/day group was found dead on study day 1. Two unscheduled deaths occurred due to gavage error during exposure (study day 0: animals #13, #31).

Appendix B. Toxicology Data Tables

Tables

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

Study Day	0 mg/kg n = 4–5[c]	65 mg/kg n = 3–5[c]	129 mg/kg n = 5	258 mg/kg n = 5	516 mg/kg n = 5	1,033 mg/kg n = 4–5[c]
0	254.6 ± 1.8	258.7 ± 3.1	262.7 ± 1.4	257.4 ± 1.8	256.9 ± 2.6	258.0 ± 2.4
4	272.2 ± 0.4	257.6 ± 5.7	284.5 ± 2.9	275.0 ± 5.4	273.7 ± 3.1	262.2 ± 6.0

Study day 0 = the first day of dosing; study day 4 = the day of necropsy.
[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.
[c]Changes in n are the result of early deaths related to gavage error and not related to chemical exposure.

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

Endpoint	0 mg/kg n = 4	65 mg/kg n = 3	129 mg/kg n = 5	258 mg/kg n = 5	516 mg/kg n = 5	1,033 mg/kg n = 4
Terminal Body Weight (g)	272.2 ± 0.4	257.6 ± 5.7	284.5 ± 2.9	275.0 ± 5.4	273.7 ± 3.1	262.2 ± 6.0
Brain Weight Absolute (g)	1.67 ± 0.07	1.80 ± 0.04	1.82 ± 0.01*	1.78 ± 0.02	1.77 ± 0.03	1.77 ± 0.04
Brain Weight Relative[d] (mg/g)	6.14 ± 0.26	6.99 ± 0.29*	6.39 ± 0.08	6.50 ± 0.14	6.49 ± 0.14	6.77 ± 0.16
Liver Weight Absolute (g)	10.40 ± 0.31**	9.70 ± 0.10	12.63 ± 0.44**	12.96 ± 0.25**	15.00 ± 0.49**	14.62 ± 0.47**
Liver Weight Relative (mg/g)	38.21 ± 1.15**	37.70 ± 1.18	44.37 ± 1.47**	47.13 ± 0.52**	54.82 ± 1.66**	55.79 ± 1.51**

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]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 = 4	65 mg/kg n = 3	129 mg/kg n = 5	258 mg/kg n = 5	516 mg/kg n = 5	1,033 mg/kg n = 4
Urea Nitrogen (mg/L)	12.8 ± 0.5	11.0 ± 0.6	11.6 ± 1.4	14.4 ± 1.0	14.4 ± 1.2	13.0 ± 1.3
Creatinine (mg/dL)	0.35 ± 0.03*	0.33 ± 0.00	0.33 ± 0.01	0.31 ± 0.02	0.30 ± 0.02	0.28 ± 0.02
Total Protein (g/dL)	6.45 ± 0.13	6.67 ± 0.15	6.48 ± 0.12	6.42 ± 0.17	6.52 ± 0.11	6.33 ± 0.09
Globulin (g/dL)	2.90 ± 0.04	3.03 ± 0.18	2.90 ± 0.05	2.76 ± 0.13	2.90 ± 0.10	2.80 ± 0.07
A/G Ratio	1.23 ± 0.06	1.21 ± 0.08	1.23 ± 0.01	1.34 ± 0.06	1.26 ± 0.06	1.26 ± 0.04
Albumin (g/dL)	3.55 ± 0.14	3.63 ± 0.03	3.58 ± 0.07	3.66 ± 0.06	3.62 ± 0.07	3.53 ± 0.05
Cholesterol (mg/dL)	115.0 ± 9.1	112.7 ± 5.8	116.6 ± 5.0	108.6 ± 7.2	122.8 ± 7.4	131.5 ± 14.7
Triglyceride (mg/dL)	63.5 ± 11.1	54.3 ± 5.7	54.6 ± 9.6	54.4 ± 4.2	68.4 ± 7.6	68.3 ± 1.7
LDL Cholesterol (mg/dL)	25.8 ± 0.9	28.7 ± 1.7	28.4 ± 4.5	25.6 ± 2.1	28.2 ± 1.7	27.8 ± 2.3
HDL Cholesterol (mg/dL)	50.0 ± 2.7	51.3 ± 3.8	62.2 ± 2.1	58.0 ± 3.8	62.8 ± 3.4	55.3 ± 6.1
Alanine Aminotransferase (IU/L)	59.50 ± 9.92	44.00 ± 3.51	67.20 ± 3.79	64.80 ± 0.92	84.00 ± 16.06	132.50 ± 72.86
Aspartate Aminotransferase (U/L)	169.75 ± 46.17	71.00 ± 4.36	106.40 ± 14.26	93.20 ± 16.07	91.40 ± 15.00	154.50 ± 96.20
Sorbitol Dehydrogenase (IU/L)	14.8 ± 5.4	12.1 ± 0.8	9.6 ± 1.6	11.5 ± 1.5	15.1 ± 2.3	22.1 ± 13.8
Bile Salts/Acids (µmol/L)	52.2 ± 5.2*	35.1 ± 2.3	41.9 ± 6.4	30.9 ± 9.0	41.5 ± 6.5	23.4 ± 5.8*

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 $p \leq 0.05$.
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 = 4	65 mg/kg n = 3	129 mg/kg n = 5	258 mg/kg n = 5	516 mg/kg n = 5	1,033 mg/kg n = 4
Total Thyroxine (µg/dL)	3.97 ± 0.23**	4.16 ± 0.54	4.69 ± 0.22	4.11 ± 0.28	3.31 ± 0.23	2.77 ± 0.25
Cholinesterase (IU/L)	291.2 ± 9.4**	209.0 ± 13.9*	236.0 ± 6.7*	183.4 ± 8.8**	152.4 ± 20.0**	129.0 ± 14.8**

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]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

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Figures

C.1. Gene Expression Quality Control

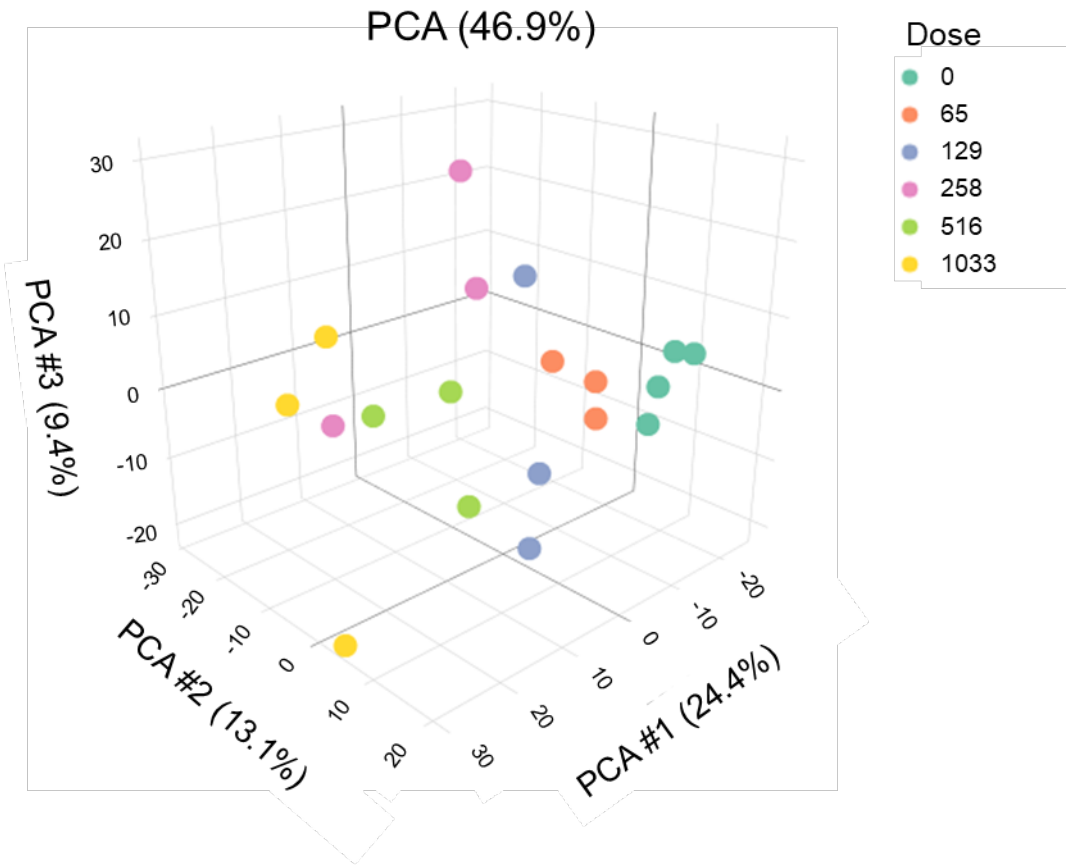

Figure C-1. A Principal Component Analysis of the Robust Multi-array Average-normalized Data

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.

C.2. Additional Data Analysis

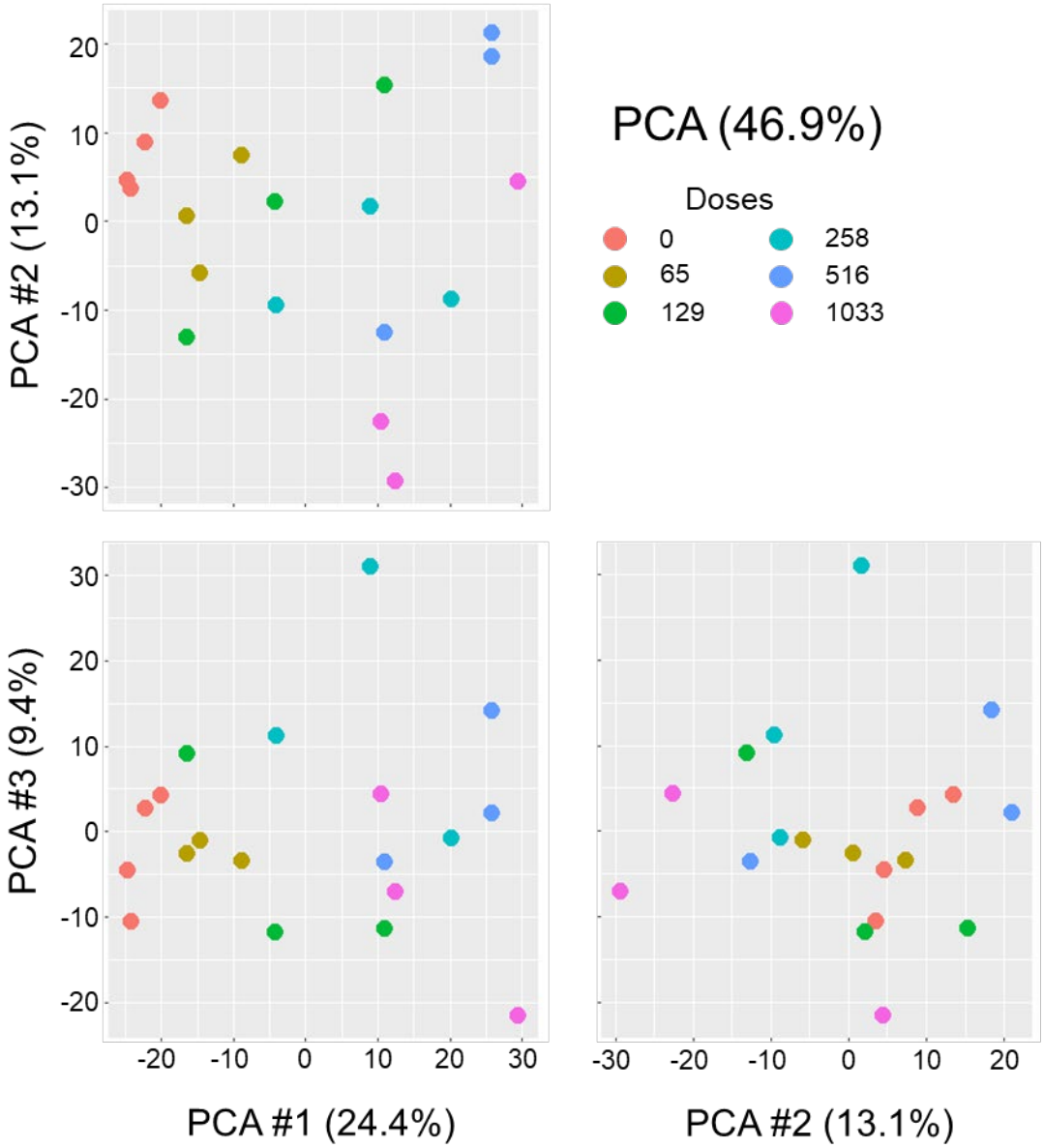

Figure C-2. An Alternative View of the Principal Component Analysis of the Robust Multi-array Average-normalized Data

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.

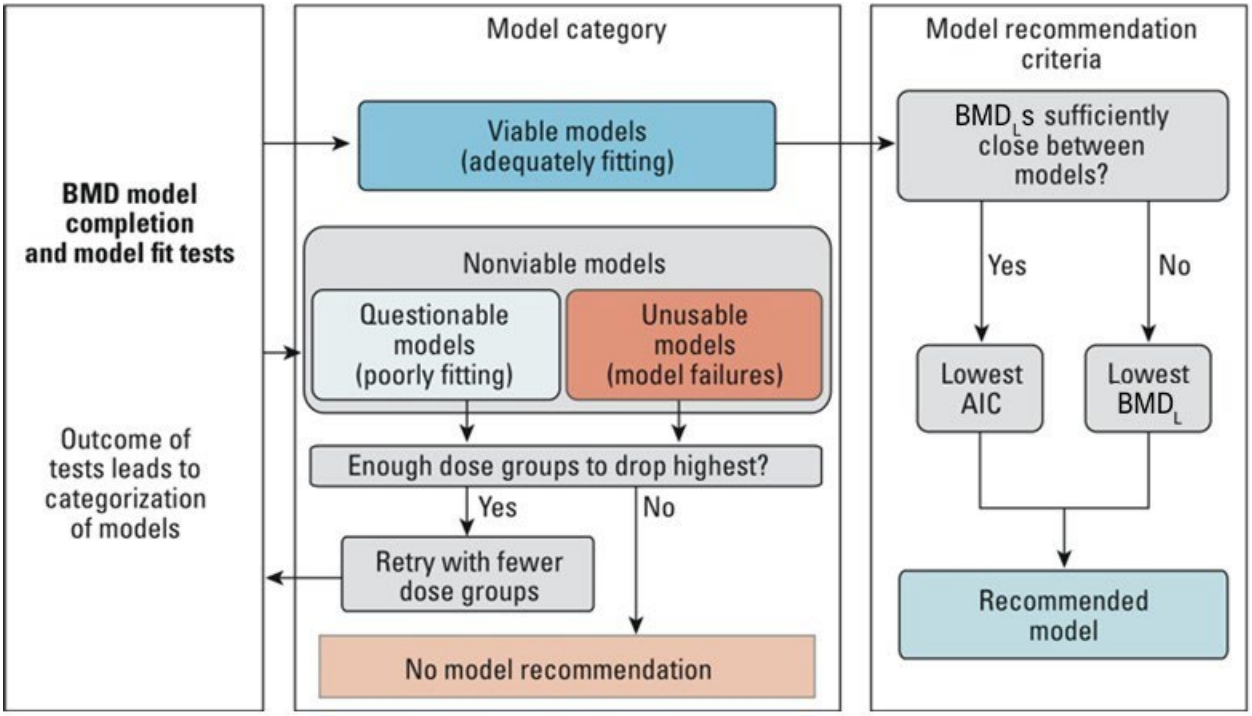

Figure D-1. Benchmark Dose Model Recommendation/Selection Methodology for Automated Benchmark Dose Execution of Apical Endpoints

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

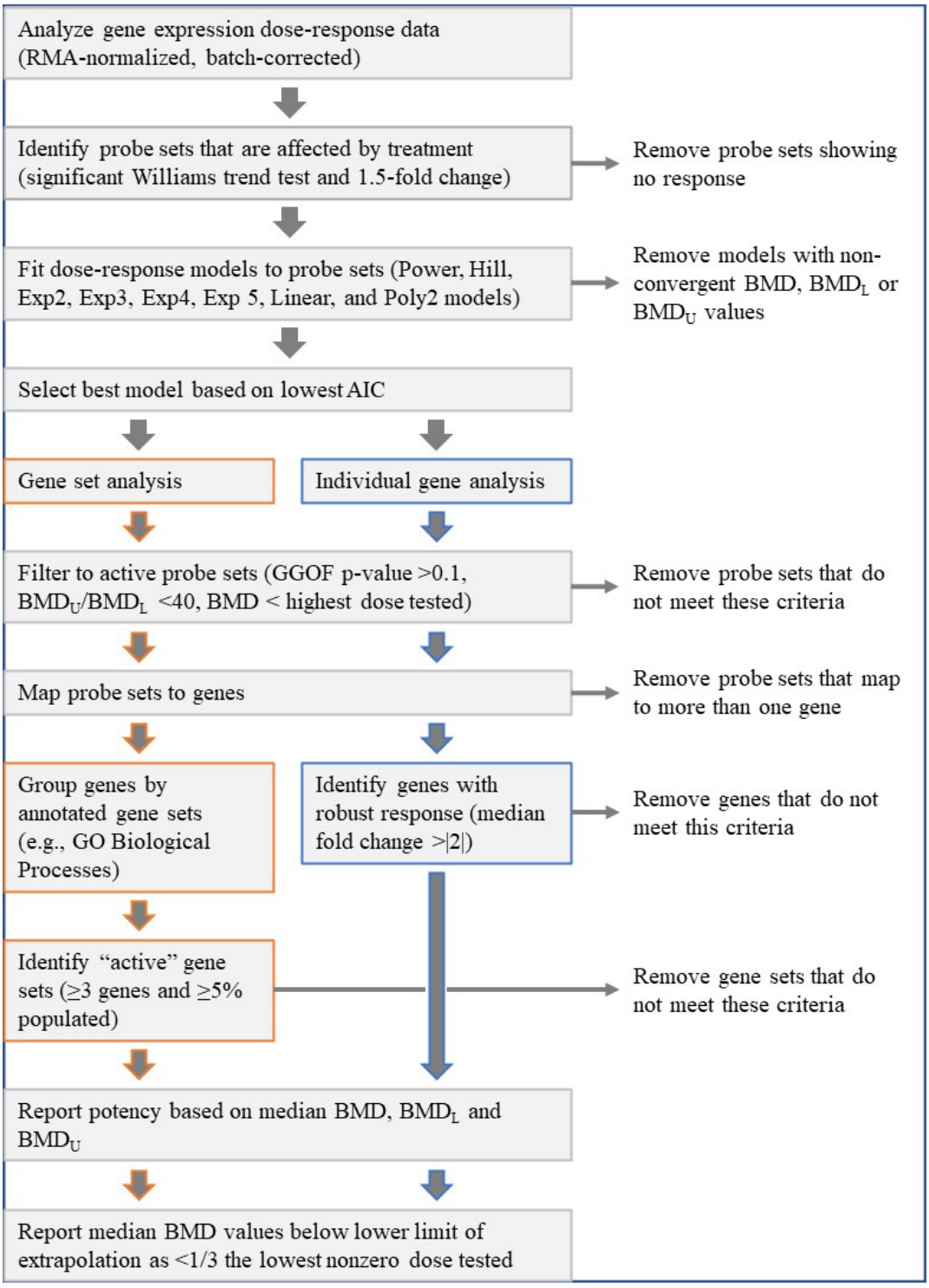

Figure D-2. Benchmark Dose Model Recommendation/Selection Methodology for Benchmark Dose Execution of Gene Sets with Expression Changes Enacted by Chemical Exposure

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-03.[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
C10978_I04_Mean_Body_Weight_Summary.pdf

I05 – Clinical Observations Summary
C10978_I05_Clinical_Observations_Summary.pdf

PA06 – Organ Weights Summary
C10978_PA06_Organ_Weights_Summary.pdf

PA41 – Clinical Chemistry Summary
C10978_PA41_Clinical_Chemistry_Summary.pdf

R07 – Hormone Summary
C10978_R07_Hormone_Summary.pdf

F.4. Individual Animal Data

Individual Animal Body Weight Data
C10978_Individual_Animal_Body_Weight_Data.xlsx

Individual Animal Clinical Chemistry Data
C10978_Individual_Animal_Clinical_Chemistry_Data.xlsx

Individual Animal Clinical Observations Data
C10978_Individual_Animal_Clinical_Observations_Data.xlsx

Individual Animal Hormone Data
C10978_Individual_Animal_Hormone_Data.xlsx

Individual Animal Organ Weight Data
C10978_Individual_Animal_Organ_Weight_Data.xlsx

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