National Institute of Environmental Health Sciences

NIEHS Report on the In Vivo Repeat Dose Biological Potency Study of Isodecyl Diphenyl Phosphate (CASRN 29761-21-5) in Male Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats (Gavage Studies)

NIEHS 04

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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/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).

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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 Isodecyl Diphenyl Phosphate (CASRN 29761-21-5) 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: Isodecyl diphenyl phosphate (IDDP) 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 IDDP.

Methods: Scientists at the Division of Translational Toxicology, National Institute of Environmental Health Sciences conducted this short-term in vivo biological potency study on IDDP in young adult male Sprague Dawley (Hsd:Sprague Dawley® SD®) rats. IDDP was formulated in corn oil and administered once daily for 4 consecutive days by gavage. IDDP was tested at six doses (0, 66, 132, 264, 527, and 1,054 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 liver weight, decreased total thyroxine concentration, decreased serum bile salt/acid concentration, increased absolute liver weight, increased serum total cholesterol concentration, decreased serum albumin/globulin ratio, and increased serum high-density lipoprotein cholesterol concentration. The BMDs and benchmark dose lower confidence limits (BMD_{LS}) were 39.8 (22.4), 67.0 (15.6), 72.9 (49.7), 74.6 (30.2), 89.3 (55.9), 146.2 (85.8), and 161.7 (68.1) mg/kg, respectively. Although serum cholinesterase activity was significantly decreased in all dosed groups (28%–60% decrease), beginning with 66 mg/kg (the lowest-observed-effect level), its BMD value was below the lower limit of extrapolation (<22.0 mg/kg).

No Gene Ontology biological processes had BMD median values <22.0 mg/kg. The most sensitive gene sets for which a reliable estimate of the BMD could be made were monosaccharide biosynthetic process and negative regulation of protein import into nucleus with median BMDs of 28.8 and 32.1 mg/kg and median BMD_{LS} of 14.6 and 13.7 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 (<22.0 mg/kg). Six of these genes were upregulated: *Cidea, Ugt2b17*, *Cyp3a23/3a1*, *Cyrl1*, *Abcc3*, and *Akr7a3.* Four genes, *Sds*, *G6pc*, *C6*, and *Slc6a6*, 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 28.8 (14.6) and 39.8 (22.4) mg/kg, respectively. The BMDs (BMD_{LS}) could not be determined for any of the top 10 most sensitive individual genes and were estimated to be <22.0 mg/kg. Serum cholinesterase inhibition was also estimated to be <22.0 mg/kg. Future studies investigating lower doses would be helpful to obtain more accurate estimates of BMD values for the most sensitive individual genes and for cholinesterase inhibition.

Background

Isodecyl diphenyl phosphate (IDDP) 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 IDDP 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 isodecyl diphenyl phosphate (IDDP) by gavage in corn oil at a dose level of 0, 66, 132, 264, 527, or 1,054 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 IDDP. 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 IDDP was estimated to be approximately 1,054 mg/kg/day; thus, this dose was selected as the highest dose in the present study.

Chemistry

IDDP (CASRN 29761-21-5; $C_{22}H_{31}O_4P$; molar mass 390.45 g/mol) was obtained from the Ferro Corporation (Mayfield Heights, OH; lot F12611AN). The identity was confirmed using gas chromatography/mass spectrometry (GC/MS). GC/MS analysis found triphenyl phosphate (3.9%) and 32 peaks identified as isomers of IDDP. The sum of the relative peak areas of the IDDP isomers yielded an IDDP purity of approximately 96%.

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 IDDP 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 28 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 IDDP 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-04.[21]

Results

Animal Condition, Body Weights, and Organ Weights

No premature mortality occurred, and no clinical observations were noted. No significant change in terminal body weight was observed with exposure to isodecyl diphenyl phosphate (IDDP) (Table 1).

Table 1. Body Weight Summary

Study Day	0 mg/kg[a,b] n = 5	66 mg/kg n = 5	132 mg/kg n = 5	264 mg/kg n = 5	527 mg/kg n = 5	1,054 mg/kg n = 5	BMD_{1Std} (mg/kg)	$BMDL_{1Std}$ (mg/kg)
0	258.9 ± 3.6	256.1 ± 6.6	258.5 ± 2.3	257.2 ± 5.7	259.5 ± 4.3	262.0 ± 2.2	ND	ND
4	276.3 ± 4.6	273.6 ± 6.3	274.6 ± 2.5	271.2 ± 7.9	277.3 ± 7.1	269.1 ± 6.3	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.

At necropsy, a significant increase in absolute and relative liver weights occurred in dose groups ≥132 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 74.6 (30.2) mg/kg and for relative liver weight was 39.8 (22.4) 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 in the two highest dose groups (527 and 1,054 mg/kg) and had a negative trend with a BMD (BMD_L) of 72.9 (49.7) mg/kg (Table 3). Serum total cholesterol and high-density lipoprotein (HDL) cholesterol concentrations were significantly increased in dose groups ≥264 mg/kg; both endpoints had positive trends. The BMDs (BMD_Lx) for increased total cholesterol and increased HDL cholesterol were 89.3 (55.9) and 161.7 (68.1) mg/kg, respectively. The albumin/globulin ratio had a negative trend with significant pairwise comparisons in the two highest dose groups and a BMD (BMD_L) of 146.2 (85.8) mg/kg. 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 = 5	66 mg/kg n = 5	132 mg/kg n = 5	264 mg/kg n = 5	527 mg/kg n = 5	1,054 mg/kg n = 5	BMD_{1Std} (mg/kg)	BMD_{L1Std} (mg/kg)
Terminal Body Weight (g)	276.3 ± 4.6	273.6 ± 6.3	274.6 ± 2.5	271.2 ± 7.9	277.3 ± 7.1	269.1 ± 6.3	ND	ND
Liver Weight Absolute (g)	11.46 ± 0.19**	12.02 ± 0.44	12.85 ± 0.30*	12.94 ± 0.45*	13.54 ± 0.60**	13.21 ± 0.36**	74.6	30.2
Liver Weight Relative[d] (mg/g)	41.50 ± 0.58**	43.95 ± 1.40	46.82 ± 1.16**	47.72 ± 0.71**	48.75 ± 1.09**	49.09 ± 0.71**	39.8	22.4

Statistical significance for a dosed group indicates a significant pairwise test compared to the vehicle control group. Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at p ≤ 0.05; **p ≤ 0.01.
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 = 5	66 mg/kg n = 5	132 mg/kg n = 5	264 mg/kg n = 5	527 mg/kg n = 5	1,054 mg/kg n = 5	BMD_{1Std} (mg/kg)	BMD_{L1Std} (mg/kg)
A/G Ratio	1.38 ± 0.03**	1.41 ± 0.03	1.33 ± 0.02	1.31 ± 0.02	1.24 ± 0.01**	1.28 ± 0.03*	146.2	85.8
Cholesterol (mg/dL)	101.0 ± 5.8**	105.6 ± 4.6	111.2 ± 6.2	122.2 ± 3.6*	142.2 ± 2.5**	146.4 ± 6.5**	89.3	55.9
HDL Cholesterol (mg/dL)	46.0 ± 2.9**	50.0 ± 2.3	51.8 ± 2.5	54.0 ± 1.9*	64.8 ± 1.7**	69.8 ± 4.2**	161.7	68.1
Bile Salts/Acids (µmol/L)	57.4 ± 8.8**	53.9 ± 4.1	34.5 ± 4.3	40.8 ± 6.3	32.3 ± 5.6*	32.1 ± 4.3*	72.9	49.7

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 ≤ 0.05; **p ≤ 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; 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.

Hormones and Enzymes

Total thyroxine concentration was significantly decreased in dose groups ≥132 mg/kg and had a negative trend with a BMD (BMD_L) of 67.0 (15.6) mg/kg (Table 4). Serum cholinesterase activity was significantly decreased in all dosed groups by 28%–60%, beginning with the 66 mg/kg group; a BMD_L was not reportable because the BMD was below the lower limit of extrapolation (<22.0 mg/kg). Testing lower doses in future studies will therefore be necessary to calculate a BMD associated with decreased cholinesterase in the context of IDDP exposure.

Table 4. Hormones and Enzymes Summary

Endpoint	0 mg/kg[a,b] n = 5	66 mg/kg n = 5	132 mg/kg n = 5	264 mg/kg n = 5	527 mg/kg n = 5	1,054 mg/kg n = 5	BMD_{1Std} (mg/kg)	BMD_{L1Std} (mg/kg)
Total Thyroxine (µg/dL)	5.12 ± 0.23**	4.45 ± 0.21	4.23 ± 0.23*	3.64 ± 0.44**	4.21 ± 0.27*	3.31 ± 0.36**	67.0	15.6
Cholinesterase (IU/L)	277.4 ± 16.6**	201.0 ± 5.9**	172.4 ± 8.0**	148.4 ± 8.7**	141.2 ± 4.2**	110.2 ± 9.8**	<22.0[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 $p \leq 0.05$; **$p \leq 0.01$.
Benchmark response set at 1 standard deviation from the mean.
BMD = benchmark dose; BMD_L = benchmark dose lower confidence limit; 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]<22.0 = 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 <22.0 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
Cholinesterase	<22.0[a]	NR	66	ND	DOWN
Liver Weight Relative	39.8	22.4	132	66	UP
Total Thyroxine	67.0	15.6	132	66	DOWN
Bile Salts/Acids	72.9	49.7	527	264	DOWN
Liver Weight Absolute	74.6	30.2	132	66	UP
Cholesterol	89.3	55.9	264	132	UP
A/G Ratio	146.2	85.8	527	264	DOWN
HDL Cholesterol	161.7	68.1	264	132	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 = ratio of albumin to globulin; HDL = high-density lipoprotein.
[a]<22.0 = 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 IDDP 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.

No gene sets had estimated BMD median values <22.0 mg/kg. The most sensitive GO biological processes were monosaccharide biosynthetic process (GO:0046364), negative regulation of protein import into nucleus (GO:0042308), negative regulation of protein import (GO:1904590), and oxidative demethylation (GO:0070989) with BMDs ($BMDL_{S}$) of 28.8 (14.6), 32.1 (13.7), 32.1 (13.7), and 32.2 (20.1) 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:0046364 monosaccharide biosynthetic process	5/33	15%	*Pck2*; *Sds*; *Gulo*; *Atf3*; *Per2*	28.8	14.6–87.4	3	2
GO:0042308 negative regulation of protein import into nucleus	3/55	5%	*Tmsb4x*; *Ppm1b*; *Pde2a*	32.1	13.7–109.8	1	2
GO:1904590 negative regulation of protein import	3/55	5%	*Tmsb4x*; *Ppm1b*; *Pde2a*	32.1	13.7–109.8	1	2
GO:0070989 oxidative demethylation	3/12	25%	*Cyp3a18*; *Cyp1a2*; *Cyp3a23/3a1*	32.2	20.1–60.0	2	1
GO:0051782 negative regulation of cell division	4/15	27%	*Aspm*; *Chmp4c*; *Txnip*; *Aurkb*	33.1	15.1–98.7	3	1

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:0046823 negative regulation of nucleocytoplasmic transport	4/65	6%	*Tmsb4x*; *Sox4*; *Ppm1b*; *Pde2a*	36.2	15.9–130.7	1	3
GO:0090317 negative regulation of intracellular protein transport	5/35	14%	*Tmsb4x*; *Sirt4*; *Ppm1b*; *Nol3*; *Pde2a*	40.3	14.3–151.6	3	2
GO:1901222 regulation of NF-kappaB import into nucleus	3/35	9%	*Tmsb4x*; *Ppm1b*; *Nol3*	40.3	18.2–109.8	2	1
GO:0050710 negative regulation of cytokine secretion	3/45	7%	*Cidea*; *Tmsb4x*; *Tnfrsf9*	40.3	18.2–109.8	1	2
GO:0060416 response to growth hormone	3/30	10%	*Ugt2b1*; *Orm1*; *Igfbp3*	41.4	17.6–117.3	2	1

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.
[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.
GO process description version: https://doi.org/10.22427/NTP-DATA-002-00600-0002-000-0.[24]
GO:0046364 monosaccharide biosynthetic process: The chemical reactions and pathways resulting in the formation of monosaccharides, polyhydric alcohols containing either an aldehyde or a keto group and 3 or more carbon atoms.
GO:0042308 negative regulation of protein import into nucleus: Any process that stops, prevents, or reduces the frequency, rate, or extent of the movement of proteins from the cytoplasm into the nucleus.
GO:1904590 negative regulation of protein import: Any process that stops, prevents, or reduces the frequency, rate, or extent of protein import.
GO:0070989 oxidative demethylation: The process of removing one or more methyl groups from a molecule, involving the oxidation (i.e., electron loss) of one or more atoms in the substrate.
GO:0051782 negative regulation of cell division: Any process that stops, prevents, or reduces the frequency, rate, or extent of cell division.
GO:0046823 negative regulation of nucleocytoplasmic transport: Any process that stops, prevents, or reduces the frequency, rate, or extent of the directed movement of substances between the cytoplasm and the nucleus.
GO:0090317 negative regulation of intracellular protein transport: Any process that decreases the frequency, rate, or extent of the directed movement of proteins within cells.
GO:1901222 regulation of NIK/NF-kappaB signaling: Any process that modulates the frequency, rate or extent of NIK/NF-kappaB signaling.
GO:0050710 negative regulation of cytokine secretion: Any process that stops, prevents, or reduces the frequency, rate, or extent of the regulated release of cytokines from a cell.
GO:0060416 response to growth hormone: 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 growth hormone stimulus. Growth hormone is a peptide hormone that binds to the growth hormone receptor and stimulates growth.

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 <22.0 mg/kg. Six genes exhibited an increase in expression: *Cidea* (cell death-inducing DFFA-like effector a), *Ugt2b17* (UDP glucuronosyltransferase family 2 member B17), *Cyp3a23/3a1* (cytochrome P450, family 3, subfamily a, polypeptide 23-polypeptide 1), *Cryl1* (crystallin, lambda 1), *Abcc3* (ATP binding cassette subfamily C member 3), and *Akr7a3* (aldo-keto reductase family 7 member A3). Four genes exhibited a decrease in expression: *Sds* (serine dehydratase), *G6pc* (glucose-6-phosphatase, catalytic subunit), *C6* (complement C6), and *Slc6a6* (solute carrier family 6 member 6).

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
Sds	25044	1369864_a_at	<22.0[b] (NR)	2.6	DOWN
G6pc	25634	1370725_a_at,1386944_a_at	<22.0 (NR)	3.4	DOWN
Cidea	291541	1389179_at	<22.0 (NR)	3.0	UP
C6	24237	1384580_at	<22.0 (NR)	2.5	DOWN
Ugt2b17	286954	1370698_at	<22.0 (NR)	2.5	UP
Cyp3a23/3a1	25642	1387118_at	<22.0 (NR)	2.6	UP
Cryl1	290277	1376051_at	<22.0 (NR)	3.5	UP
Abcc3	140668	1369698_at	<22.0 (NR)	10.7	UP
Akr7a3	26760	1368121_at	<22.0 (NR)	3.8	UP
Slc6a6	29464	1368778_at,1374531_at	<22.0 (NR)	14.5	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]<22.0 = 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]
***Sds*:** Human Entrez Gene Summary (Human *SDS*): This gene encodes one of three enzymes that are involved in metabolizing serine and glycine. L-serine dehydratase converts L-serine to pyruvate and ammonia and requires pyridoxal phosphate as a cofactor. The encoded protein can also metabolize threonine to NH_4^+ and 2-ketobutyrate. The encoded protein is found predominantly in the liver. [provided by RefSeq, Jul 2008].
***G6pc*:** Human Uniprot function (Human *G6PC1*): Hydrolyzes glucose-6-phosphate to glucose in the endoplasmic reticulum. Forms with the glucose-6-phosphate transporter (SLC37A4/G6PT), the complex responsible for glucose production through glycogenolysis and gluconeogenesis. Thus, it is the key enzyme in homeostatic regulation of blood glucose levels.

Cidea**:** Human Uniprot function (Human *CIDEA*): Acts as a CEBPB coactivator in mammary epithelial cells to control the expression of a subset of CEBPB downstream target genes, including ID2, IGF1, PRLR, SOCS1, SOCS3, XDH, but not casein. By interacting with CEBPB, strengthens the association of CEBPB with the XDH promoter, increases histone acetylation and dissociates HDAC1 from the promoter (by similarity). Binds to lipid droplets and regulates their enlargement, thereby restricting lipolysis and favoring storage. At focal contact sites between lipid droplets, promotes directional net neutral lipid transfer from the smaller to larger lipid droplets. The transfer direction may be driven by the internal pressure difference between the contacting lipid droplet pair and occurs at a lower rate than that promoted by CIDEC. When overexpressed, induces apoptosis. The physiological significance of its role in apoptosis is unclear. {ECO0000250, ECO0000269|PubMed19843876}.
C6**:** Human Uniprot function (Human *C6*): Constituent of the membrane attack complex (MAC) that plays a key role in the innate and adaptive immune response by forming pores in the plasma membrane of target cells.
Ugt2b17**:** Human Uniprot function (Human *UGT2B17*): UDP-glucuronosyltransferase (UGT) that catalyzes phase II biotransformation reactions in which lipophilic substrates are conjugated with glucuronic acid to increase the metabolite's water solubility, thereby facilitating excretion into either the urine or bile (PubMed8798464, PubMed16595710, PubMed18719240, PubMed19022937, PubMed23288867). Catalyzes the glucuronidation of endogenous steroid hormones such as androgens (epitestosterone, androsterone) and estrogens (estradiol, epiestradiol) (PubMed8798464, PubMed16595710, PubMed18719240, PubMed19022937, PubMed23288867). {ECO0000269|PubMed16595710, ECO0000269|PubMed18719240, ECO0000269|PubMed19022937, ECO0000269|PubMed23288867, ECO0000269|PubMed8798464}.
Cyp3a23/3a1**:** Human Uniprot function (Human *CYP3A4*): A cytochrome P450 monooxygenase involved in the metabolism of sterols, steroid hormones, retinoids and fatty acids (PubMed10681376, PubMed11093772, PubMed11555828, PubMed14559847, PubMed12865317, PubMed15373842, PubMed15764715, PubMed20702771, PubMed19965576, PubMed21490593, PubMed21576599). Mechanistically, uses molecular oxygen inserting one oxygen atom into a substrate, and reducing the second into a water molecule, with two electrons provided by NADPH via cytochrome P450 reductase (NADPH–hemoprotein reductase). Catalyzes the hydroxylation of carbon-hydrogen bonds (PubMed2732228, PubMed14559847, PubMed12865317, PubMed15373842, PubMed15764715, PubMed21576599, PubMed21490593). Exhibits high catalytic activity for the formation of hydroxyestrogens from estrone (E1) and 17beta-estradiol (E2), namely 2-hydroxy E1 and E2, as well as D-ring hydroxylated E1 and E2 at the C-16 position (PubMed11555828, PubMed14559847, PubMed12865317). Plays a role in the metabolism of androgens, particularly in oxidative deactivation of testosterone (PubMed2732228, PubMed15373842, PubMed15764715, PubMed22773874). Metabolizes testosterone to less biologically active 2beta- and 6beta-hydroxytestosterones (PubMed2732228, PubMed15373842, PubMed15764715). Contributes to the formation of hydroxycholesterols (oxysterols), particularly A-ring hydroxylated cholesterol at the C-4beta position, and side chain hydroxylated cholesterol at the C-25 position, likely contributing to cholesterol degradation and bile acid biosynthesis (PubMed21576599). Catalyzes bisallylic hydroxylation of polyunsaturated fatty acids (PUFA) (PubMed9435160). Catalyzes the epoxidation of double bonds of PUFA with a preference for the last double bond (PubMed19965576). Metabolizes endocannabinoid arachidonoylethanolamide (anandamide) to 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid ethanolamides (EpETrE-EAs), potentially modulating endocannabinoid system signaling (PubMed20702771). Plays a role in the metabolism of retinoids. Displays high catalytic activity for oxidation of all-trans-retinol to all-trans-retinal, a rate-limiting step for the biosynthesis of all-trans-retinoic acid (atRA) (PubMed10681376). Further metabolizes atRA toward 4-hydroxyretinoate and may play a role in hepatic atRA clearance (PubMed11093772). Responsible for oxidative metabolism of xenobiotics. Acts as a 2-exo-monooxygenase for plant lipid 1,8-cineole (eucalyptol) (PubMed11159812). Metabolizes the majority of the administered drugs. Catalyzes sulfoxidation of the anthelmintics albendazole and fenbendazole (PubMed10759686). Hydroxylates antimalarial drug quinine (PubMed8968357). Acts as a 1,4-cineole 2-exo-monooxygenase (PubMed11695850). {ECO:0000269|PubMed10681376, ECO:0000269|PubMed10759686, ECO:0000269|PubMed11093772, ECO:0000269|PubMed11159812, ECO:0000269|PubMed11555828, ECO:0000269|PubMed11695850, ECO:0000269|PubMed12865317, ECO:0000269|PubMed14559847, ECO:0000269|PubMed15373842, ECO:0000269|PubMed15764715, ECO:0000269|PubMed19965576, ECO:0000269|PubMed20702771, ECO:0000269|PubMed21490593, ECO:0000269|PubMed21576599, ECO:0000269|PubMed22773874, ECO:0000269|PubMed2732228, ECO:0000269|PubMed8968357, ECO:0000269|PubMed9435160}.
Cryl1**:** Human Entrez Gene Summary (Human *CRYL1*): The uronate cycle functions as an alternative glucose metabolic pathway, accounting for about 5% of daily glucose catabolism. The product of this gene catalyzes the dehydrogenation of L-gulonate into dehydro-L-gulonate in the uronate cycle. The enzyme requires NAD(H) as a coenzyme and is inhibited by inorganic phosphate. A similar gene in the rabbit is thought to serve a structural role in the lens of the eye. [provided by RefSeq, Jul 2008].
Abcc3**:** Human Uniprot function (Human *ABCC3*): May act as an inducible transporter in the biliary and intestinal excretion of organic anions. Acts as an alternative route for the export of bile acids and glucuronides from cholestatic hepatocytes (by similarity). {ECO0000250}.
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 liver against the toxic and carcinogenic effects of AFB1, a potent hepatocarcinogen. {ECO0000269|PubMed18416522}.
Slc6a6**:** Human Uniprot function (Human *SLC6A6*): Sodium-dependent taurine and beta-alanine transporter. Chloride ions are necessary for optimal uptake. {ECO0000269|PubMed31345061, ECO0000269|PubMed31903486, ECO0000269|PubMed8382624}.

Summary

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

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 the lowest-observed-effect level for the study, cholinesterase inhibition appeared to be the most sensitive apical measure; the estimated benchmark dose (BMD) value was below the lower limit of extrapolation (<22.0 mg/kg). Further studies are warranted to assess cholinesterase effects at doses <22.0 mg/kg to obtain an accurate point of departure. The most sensitive apical endpoints for which a BMD could be determined were an increase in relative liver weight and a decrease in total thyroxine concentration with a BMD and benchmark dose lower confidence limit (BMD_L) of 39.8 (22.4) and 67.0 (15.6) mg/kg, respectively. The next most sensitive apical endpoints observed were a decrease in serum bile salt/acid concentration and an increase in absolute liver weight with BMDs (BMD_Ls) of 72.9 (49.7) and 74.6 (30.2) mg/kg, respectively.

Gene set-level transcriptional changes in the liver following IDDP exposure were estimated to occur at a BMD (BMD_L) as low as 28.8 (14.6) mg/kg, which corresponded to monosaccharide biosynthetic process (GO:0046364). The top 10 most sensitive individual genes, similar to cholinesterase, exhibited changes in expression at dose levels below which a reliable estimate of potency could be achieved (<22.0 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:0046364, with a BMD (BMD_L) of 28.8 (14.6) mg/kg. Individual gene transcriptional changes provided potency estimates <22.0 mg/kg, while apical endpoints provided potency estimates slightly higher than GO:0046364. 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 IDDP 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	Array ID
173	Corn Oil	0	0	Yes	041-052014-MW_(Rat230_2).CEL
174	Corn Oil	0	0	Yes	001-051914-MW_(Rat230_2).CEL
184	Corn Oil	0	0	Yes	073-052714-JAP_(Rat230_2).CEL
210	Corn Oil	0	0	Yes	009-051914-MW_(Rat230_2).CEL
215	Corn Oil	0	0	Yes	037-052014-MW_(Rat230_2).CEL
150	IDDP	0.169	66	Yes	NA
163	IDDP	0.169	66	Yes	NA
188	IDDP	0.169	66	Yes	007-051914-MW_(Rat230_2).CEL
226	IDDP	0.169	66	Yes	044-052114-MW_(Rat230_2).CEL
227	IDDP	0.169	66	Yes	079-052714-JAP_(Rat230_2).CEL
152	IDDP	0.338	132	Yes	008-051914-MW_(Rat230_2).CEL
153	IDDP	0.338	132	Yes	NA
170	IDDP	0.338	132	Yes	NA
222	IDDP	0.338	132	Yes	045-052114-MW_(Rat230_2).CEL
228	IDDP	0.338	132	Yes	080-052714-JAP_(Rat230_2).CEL
147	IDDP	0.675	264	Yes	010-051914-MW_(Rat230_2).CEL
156	IDDP	0.675	264	Yes	NA
158	IDDP	0.675	264	Yes	046-052114-MW_(Rat230_2).CEL
177	IDDP	0.675	264	Yes	081-052714-JAP_(Rat230_2).CEL
181	IDDP	0.675	264	Yes	NA
167	IDDP	1.35	527	Yes	011-051914-MW_(Rat230_2).CEL
172	IDDP	1.35	527	Yes	NA
189	IDDP	1.35	527	Yes	NA
224	IDDP	1.35	527	Yes	047-052114-MW_(Rat230_2).CEL
229	IDDP	1.35	527	Yes	082-052714-JAP_(Rat230_2).CEL
154	IDDP	2.7	1,054	Yes	012-051914-MW_(Rat230_2).CEL
157	IDDP	2.7	1,054	Yes	NA
180	IDDP	2.7	1,054	Yes	048-052114-MW_(Rat230_2).CEL
185	IDDP	2.7	1,054	Yes	NA
195	IDDP	2.7	1,054	Yes	083-052714-JAP_(Rat230_2).CEL

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	66 mg/kg n = 5	132 mg/kg n = 5	264 mg/kg n = 5	527 mg/kg n = 5	1,054 mg/kg n = 5
0	258.9 ± 3.6	256.1 ± 6.6	258.5 ± 2.3	257.2 ± 5.7	259.5 ± 4.3	262.0 ± 2.2
4	276.3 ± 4.6	273.6 ± 6.3	274.6 ± 2.5	271.2 ± 7.9	277.3 ± 7.1	269.1 ± 6.3

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.

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

Endpoint	0 mg/kg n = 5	66 mg/kg n = 5	132 mg/kg n = 5	264 mg/kg n = 5	527 mg/kg n = 5	1,054 mg/kg n = 5
Terminal Body Weight (g)	276.3 ± 4.6	273.6 ± 6.3	274.6 ± 2.5	271.2 ± 7.9	277.3 ± 7.1	269.1 ± 6.3
Brain Weight Absolute (g)	1.73 ± 0.05	1.78 ± 0.02	1.79 ± 0.03	1.82 ± 0.02	1.83 ± 0.02	1.73 ± 0.03
Brain Weight Relative[d] (mg/g)	6.28 ± 0.23	6.51 ± 0.19	6.51 ± 0.13	6.74 ± 0.22	6.60 ± 0.13	6.44 ± 0.16
Liver Weight Absolute (g)	11.46 ± 0.19**	12.02 ± 0.44	12.85 ± 0.30*	12.94 ± 0.45*	13.54 ± 0.60**	13.21 ± 0.36**
Liver Weight Relative (mg/g)	41.50 ± 0.58**	43.95 ± 1.40	46.82 ± 1.16**	47.72 ± 0.71**	48.75 ± 1.09**	49.09 ± 0.71**

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 = 5	66 mg/kg n = 5	132 mg/kg n = 5	264 mg/kg n = 5	527 mg/kg n = 5	1,054 mg/kg n = 5
Urea Nitrogen (mg/dL)	12.0 ± 0.8	11.0 ± 0.5	11.6 ± 1.0	13.0 ± 0.7	10.4 ± 0.9	12.2 ± 0.9
Creatinine (mg/dL)	0.29 ± 0.01	0.28 ± 0.01	0.27 ± 0.01	0.28 ± 0.01	0.26 ± 0.01	0.29 ± 0.01
Total Protein (g/dL)	5.94 ± 0.13	5.88 ± 0.12	6.02 ± 0.06	5.90 ± 0.14	6.10 ± 0.09	6.06 ± 0.14
Globulin (g/dL)	2.50 ± 0.07**	2.44 ± 0.07	2.58 ± 0.04	2.56 ± 0.07	2.72 ± 0.04	2.66 ± 0.09
A/G Ratio	1.38 ± 0.03**	1.41 ± 0.03	1.33 ± 0.02	1.31 ± 0.02	1.24 ± 0.01**	1.28 ± 0.03*
Albumin (g/dL)	3.44 ± 0.07	3.44 ± 0.06	3.44 ± 0.04	3.34 ± 0.07	3.38 ± 0.06	3.40 ± 0.05
Cholesterol (mg/dL)	101.0 ± 5.8**	105.6 ± 4.6	111.2 ± 6.2	122.2 ± 3.6*	142.2 ± 2.5**	146.4 ± 6.5**
Triglyceride (mg/dL)	54.0 ± 6.0	52.8 ± 7.9	53.0 ± 6.6	54.8 ± 4.6	52.6 ± 2.2	42.2 ± 3.6
LDL Cholesterol (mg/dL)	21.8 ± 0.7	23.0 ± 1.1	24.2 ± 1.0	23.8 ± 1.1	24.4 ± 0.7	22.8 ± 0.5
HDL Cholesterol (mg/dL)	46.0 ± 2.9**	50.0 ± 2.3	51.8 ± 2.5	54.0 ± 1.9*	64.8 ± 1.7**	69.8 ± 4.2**
Alanine Aminotransferase (IU/L)	61.40 ± 4.46	71.60 ± 6.03	63.00 ± 3.78	85.20 ± 12.40	61.40 ± 2.62	69.20 ± 2.31
Aspartate Aminotransferase (U/L)	107.40 ± 10.21	105.40 ± 10.47	84.20 ± 3.76	107.20 ± 17.94	81.20 ± 8.77	89.00 ± 4.89
Sorbitol Dehydrogenase (IU/L)	10.4 ± 0.7	10.2 ± 0.7	9.7 ± 0.8	15.8 ± 3.3	10.8 ± 0.9	10.7 ± 0.8
Bile Salts/Acids (µmol/L)	57.4 ± 8.8**	53.9 ± 4.1	34.5 ± 4.3	40.8 ± 6.3	32.3 ± 5.6*	32.1 ± 4.3*

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 $p \leq 0.05$; **$p \leq 0.01$.
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	66 mg/kg n = 5	132 mg/kg n = 5	264 mg/kg n = 5	527 mg/kg n = 5	1,054 mg/kg n = 5
Total Thyroxine (µg/dL)	5.12 ± 0.23**	4.45 ± 0.21	4.23 ± 0.23*	3.64 ± 0.44**	4.21 ± 0.27*	3.31 ± 0.36**
Cholinesterase (IU/L)	277.4 ± 16.6**	201.0 ± 5.9**	172.4 ± 8.0**	148.4 ± 8.7**	141.2 ± 4.2**	110.2 ± 9.8**

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

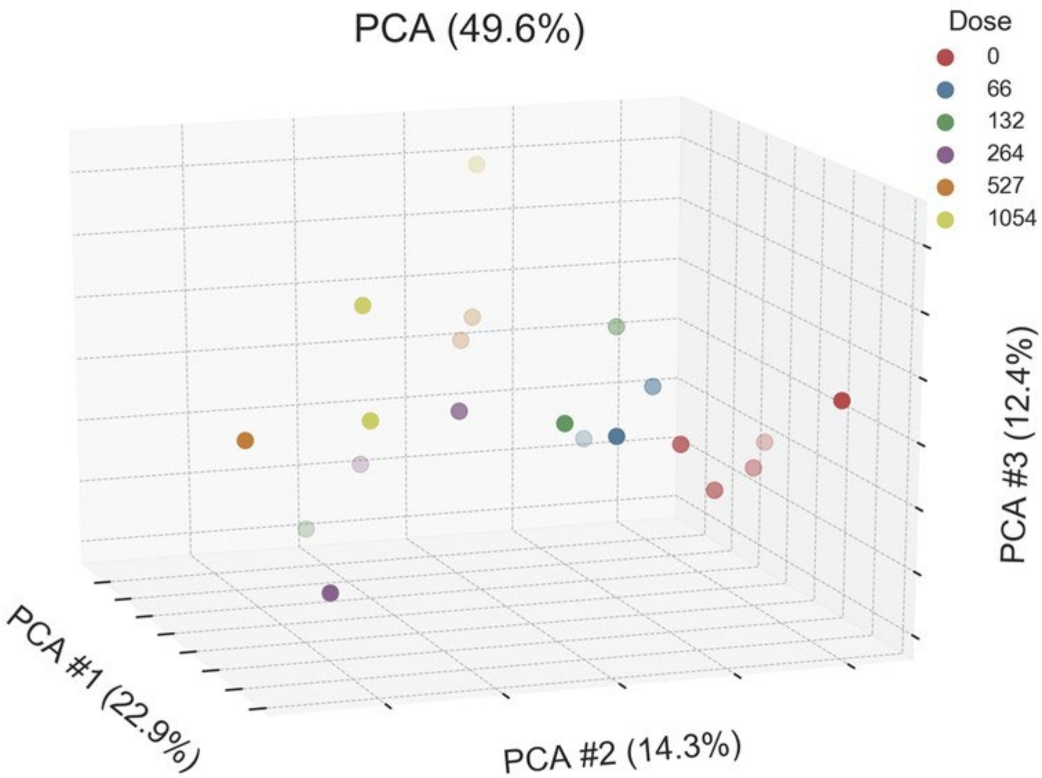

Figure C-1. A Principal Component Analysis of the Robust Multi-array Average-normalized Data

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. Lighter color (fogging) indicates a data point is farther back on the z-plane [principal component (PC) #1].

C.2. Additional Data Analysis

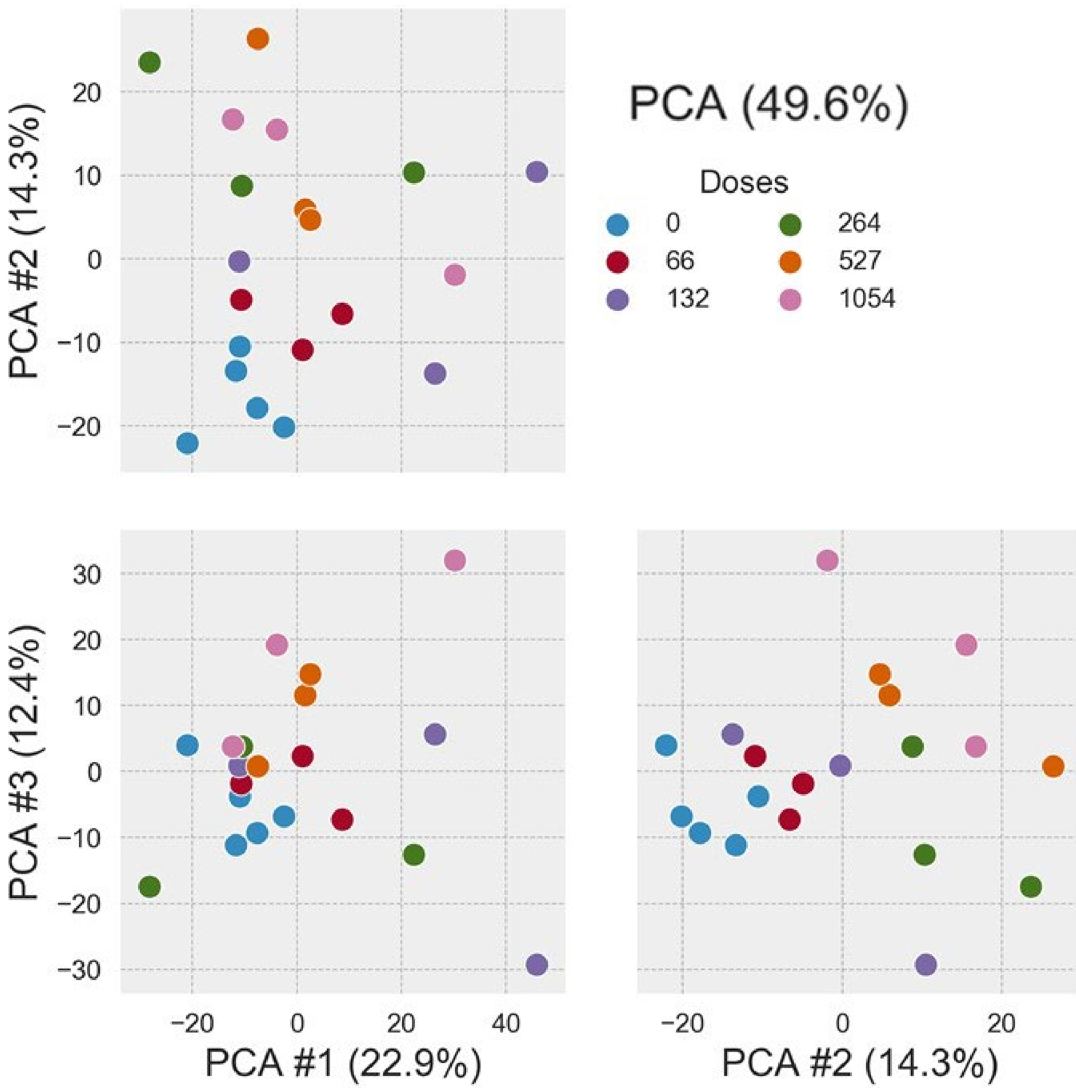

Figure C-2. An Alternative View of the Principal Component Analysis of the Robust Multi-array Average-normalized Data

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.

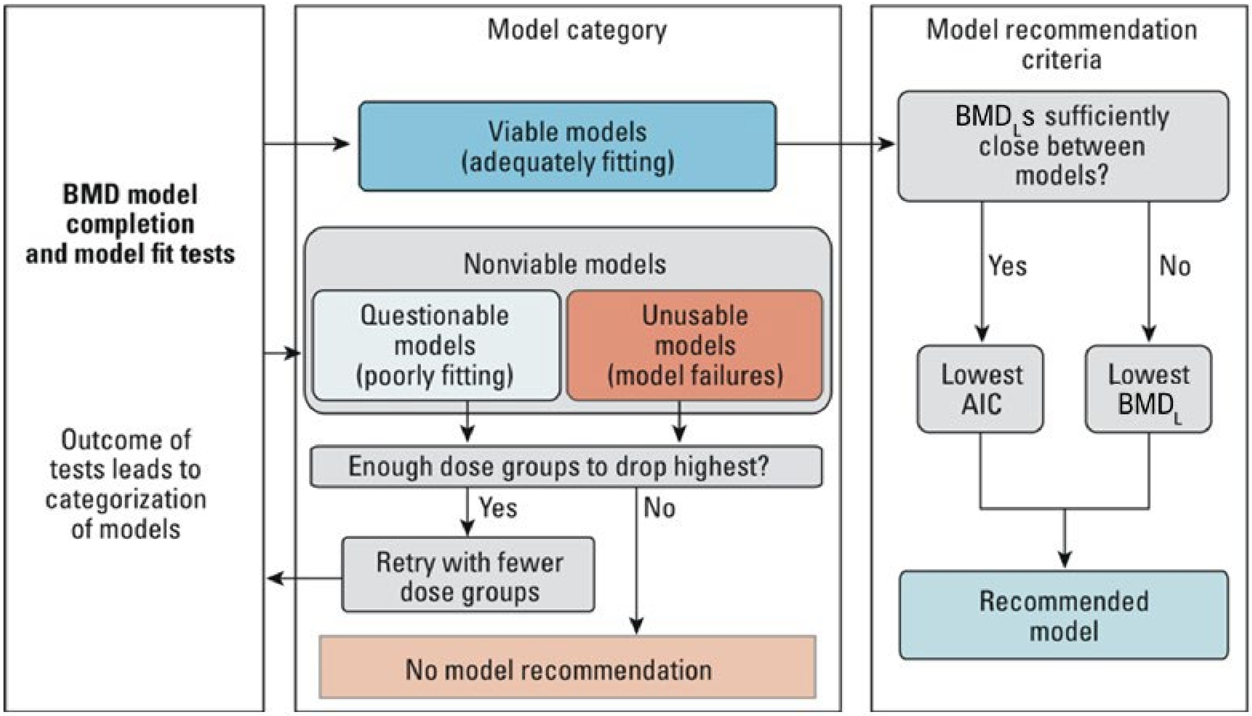

Figure D-1. Benchmark Dose Model Recommendation/Selection Methodology for Automated Benchmark Dose Execution of Apical Endpoints

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

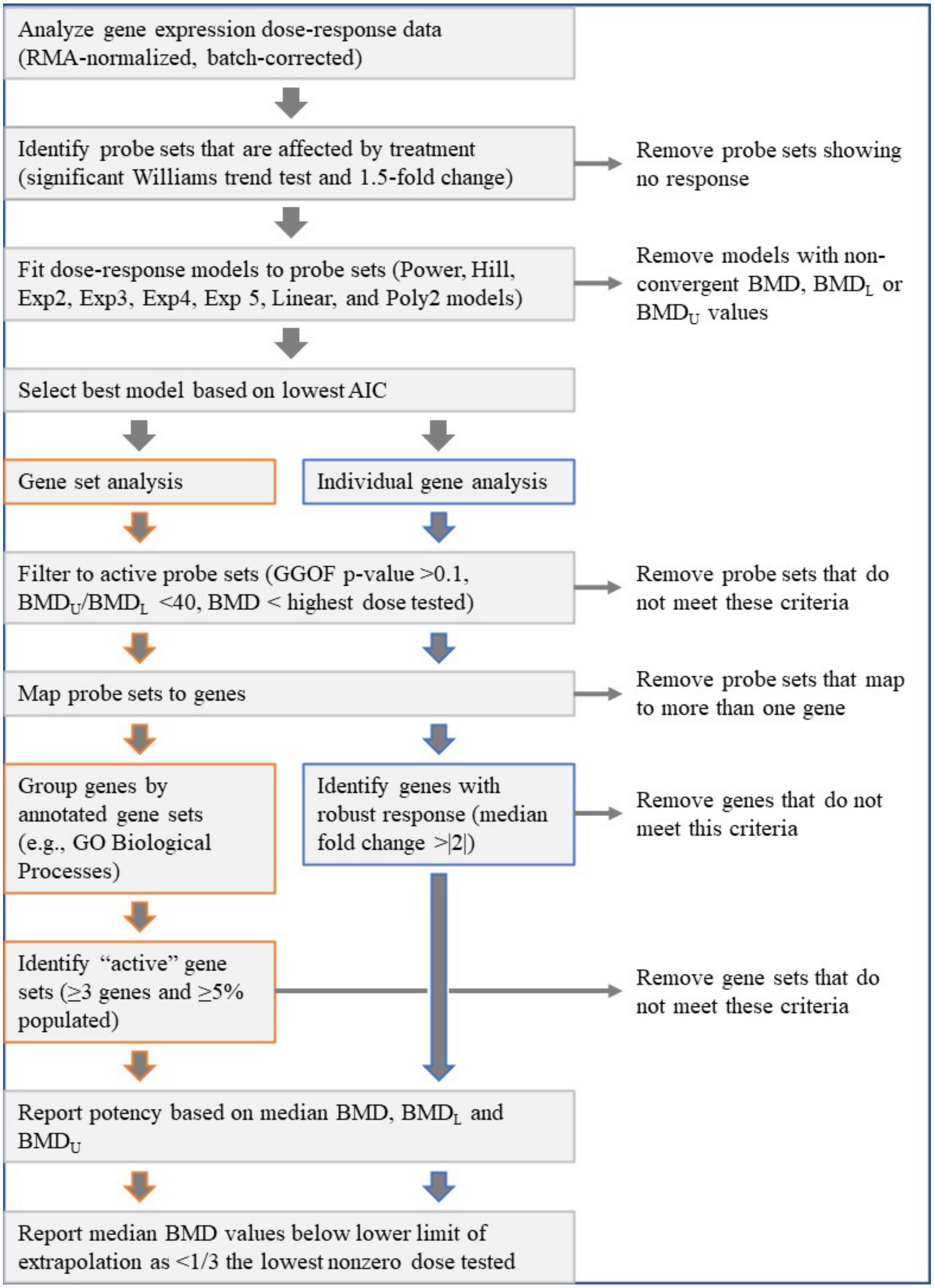

Figure D-2. Benchmark Dose Model Recommendation/Selection Methodology for Benchmark Dose Execution of Gene Sets with Expression Changes Enacted by Chemical Exposure

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

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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-04.[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
C20299_I04_Mean_Body_Weight_Summary.pdf

I05 – Clinical Observations Summary
C20299_I05_Clinical_Observations_Summary.pdf

PA06 – Organ Weights Summary
C20299_PA06_Organ_Weights_Summary.pdf

PA41 – Clinical Chemistry Summary
C20299_PA41_Clinical_Chemistry_Summary.pdf

R07 – Hormone Summary
C20299_R07_Hormone_Summary.pdf

F.4. Individual Animal Data

Individual Animal Body Weight Data
C20299_Individual_Animal_Body_Weight_Data.xlsx

Individual Animal Clinical Chemistry Data
C20299_Individual_Animal_Clinical_Chemistry_Data.xlsx

Individual Animal Clinical Observations Data
C20299_Individual_Animal_Clinical_Observations_Data.xlsx

Individual Animal Hormone Data
C20299_Individual_Animal_Hormone_Data.xlsx

Individual Animal Organ Weight Data
C20299_Individual_Animal_Organ_Weight_Data.xlsx

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