Materials and Methods for the In Vivo Repeat Dose Biological Potency Studies of 1,2-Dichlorobenzene (CASRN 95-50-1) in Female Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats and B6D2F1/Crl Mice (Whole-body Inhalation Studies)

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About This Document

Researchers in the Division of Translational Toxicology at the National Institute of Environmental Health Sciences conducted short-term in vivo transcriptomic studies of 1,2-dichlorobenzene (1,2-DCB, CASRN 95-50-1). The primary purpose of these short-term studies was to characterize the biological potency of 1,2-DCB via inhalation exposure. 1,2-DCB is a chlorinated aromatic hydrocarbon that is used in industrial applications, including as a solvent for waxes, resins, and paints; a precursor in the manufacture of agrochemicals and dyestuffs; and an ingredient in the production of disinfectants and deodorants.

This document details the Materials and Methods used to conduct the short-term studies and obtain the data posted on the CEBS data page.[1] These studies employed mathematical model-based approaches to identify and report the potency of dose-responsive effects and do not provide interpretative conclusions. Previous external peer review[2] determined that the study design, analysis methods, and presentation of results for these types of studies were appropriate. The full report on these in vivo, repeat-dose biological potency studies of 1,2-DCB is in preparation, with anticipated publication in Spring 2026.

Materials and Methods

Chemistry

Procurement and Characterization of 1,2-Dichlorobenzene

1,2-Dichlorobenzene (1,2-DCB) was obtained from Oakwood Products, Inc. (Estill, SC) in a single lot (216310R29M). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at Battelle (Columbus, OH). Reports on analyses performed in support of the 1,2-DCB studies are on file at the National Institute of Environmental Health Sciences (NIEHS).

The identity and purity of lot 216310R29M, a clear, colorless liquid at room temperature, was evaluated using gas chromatography (GC) with mass spectrometry (MS) detection. The MS spectrum was consistent with the National Institute of Standards and Technology library spectrum for 1,2-DCB. The overall purity of the test article was estimated at approximately 100%.

Bulk 1,2-DCB was stored in the original shipping container at room temperature. Reanalysis of the bulk chemical was performed by the analytical chemistry laboratory within 30 days of study termination and no degradation was detected.

Vapor Generation and Exposure System

1,2-DCB was pumped from a stainless-steel reservoir into a heated glass vaporizer column filled with glass beads and wrapped with heat tape. A waste collection flask was connected to the bottom of the column for collection of residual 1,2-DCB not completely vaporized within the vaporizer column. Preheated nitrogen entered the column from below, vaporized 1,2-DCB, and carried the vapor from the generator cabinet located in the control room to the distribution manifold located in the exposure room through a heated Teflon® transport line. The nitrogen-test article mixture was diluted with heated air before it entered the distribution manifold. Concentration in the manifold was determined by the chemical pump rate, dilution air flow rate, nitrogen flow rate, and special modifications to the distribution manifold.

Individual heated Teflon delivery lines carried the vapor from the exposure valves in the distribution cabinet to the chamber inlets. The exposure valves diverted vapor delivery to the manifold exhaust until the generation system was stable, and exposures were ready to proceed. The rate of 1,2-DCB vapor delivery to each chamber was controlled by precision metering valves at the manifold. When the exposure started, the exposure valves actuated, directing the vapor into the chamber inlet, where it was diluted with conditioned air to achieve the desired exposure concentration. Conditioned air was a temperature-controlled and filtered mix of air derived from each exposure chamber's wet and dry air duct supplies.

The exposure system consisted of seven exposure chambers with target test article concentrations of 0 (control group), 1, 10, 30, 100, 250, and 500 ppm. The inhalation exposure chamber (Lab Products, Inc., Seaford, DE) was designed so that uniform vapor concentrations could be maintained throughout the chamber with catch pans in place. The total active mixing volume of each chamber was 1.7 m^3. A small particle detector (Model 3022A; TSI, Inc., Shoreview, MN)

was used in the exposure chambers, both with and without animals, to ensure vapor (not aerosol) was produced. No particle counts above the minimum resolvable level were detected.

Vapor Concentration Monitoring

Exposure chamber and room concentrations of 1,2-DCB were monitored using an online GC equipped with a flame ionization detector (FID). All chambers were sampled at approximately twice per hour during exposure through Teflon tubing connected to each exposure chamber's relative-humidity sampling lines at a location close to the GC/FID. The samples flowed into a 16-port Hastelloy®-C stream-select valve that directed a continuous stream of sampled atmosphere to a 6-port Hastelloy-C gas-sampling valve with a 1 mL sample loop. Valves were mounted in a dedicated valve oven to maintain temperature. A vacuum regulator maintained a constant vacuum in the sample loop to compensate for variations in sample line pressure. An in-line flow meter between the vacuum regulator and GC allowed for digital measurement of sample flow. The concentration measurements and relative standard deviations were all within the acceptance criteria of 10% for all exposure chambers and below the limit of detection for the control chambers and exposure rooms in both studies.

Chamber Atmosphere Characterization

Buildup and decay rates for chamber vapor concentrations were determined prior to (without animals) and during (with animals) the studies. The time to achieve 90% of the target concentration after the beginning of vapor generation (T_{90}) and the time for the chamber concentration to decay to 10% of the target concentration after vapor generation was terminated (T_{10}) were estimated from the concentration versus time curves. At a chamber airflow rate of 15 ft^3/min, the theoretical T_{90} value was 9.2 minutes. The estimated values in both rat and mouse studies ranged from 8 to 11 minutes, and a value of 12 minutes was used for the studies. Estimated T_{10} for both studies ranged from 10 to 13 minutes.

Prior to the studies, the persistence of 1,2-DCB was monitored in the 250 and 500 ppm chambers after exposure without animals present. Although T_{10} values were acceptable, the concentration of 1,2-DCB never reached 0 ppm in the ≥10 ppm chambers and required ≥144 minutes to reach 1% of the starting concentration (T_1) without animals present. During the studies and with animals present, T_1 was 79 (500 ppm rat chamber) and 192 minutes (250 ppm mouse chamber). The reason for the prolonged T_1 values is unknown, but the prolonged T_1 values were considered to have no effect on study findings given the low levels compared to the target chamber concentrations.

The uniformity of 1,2-DCB vapor concentration was evaluated in all exposure chambers without animals present and repeated during the studies with animals present in the lowest (1 ppm) and highest (500 or 250 ppm for rats and mice, respectively) exposure concentration chambers. Concentrations were measured at 12 chamber positions, one in the front and one in the back, for each of the six possible animal cage positions per chamber. Chamber concentration uniformity was maintained throughout the studies.

To measure the stability and purity of 1,2-DCB in the generation and delivery system, samples of the test atmosphere were collected from the distribution line, generator reservoir, and the highest, lowest, and control exposure concentration chambers for both species at the beginning and end of the exposure day. Exposure atmosphere samples were collected with sorbent gas-sampling

tubes in series with a silica gel sorbent tube. Samples were extracted with methanol for analysis. In addition, analysis was performed on a second set of samples collected from the same locations and exposure times and extracted in acetone to determine whether any impurities in 1,2-DCB were obscured by methanol. No impurity peaks were present in any samples. 1,2-DCB was not detected in the silica gel samples, demonstrating 100% capture of the inhalation exposure atmosphere onto sorbent media. The stability and purity of 1,2-DCB were maintained throughout the exposure system.

Study Design for Rats

Female Sprague Dawley (Hsd:Sprague Dawley® SD®) rats were obtained from Inotiv (Envigo, at time of procurement, Indianapolis, IN). Sprague Dawley rats were employed to represent the Division of Translational Toxicology (DTT)'s typical rat strain of choice. Females were chosen instead of males to reduce the overall study size and limit the complexity of comparisons between the mouse and rat data sets. On receipt, the rats were 6 weeks of age. Animals were quarantined for 9 days and then randomly assigned to one of seven exposure groups. The rats in each exposure group were exposed to 1,2-DCB via whole-body inhalation for 6 hours plus T_{90} per day for 5 consecutive days (study days 0–4) at exposure concentrations of 0, 1, 10, 30, 100, 250, or 500 ppm. There were 5 core female rats in each exposed group and 10 in the 0 ppm group; an additional 3 rats were added to each group for internal concentration assessment. Immediately following the final exposure on study day 4, blood, lung, and liver samples were collected from the internal concentration assessment animals once chamber concentrations were at or below the regulatory limit of 12 ppm without additional health and safety considerations. Euthanasia, blood/serum collection, and tissue sample collection for all core animals were completed on study day 5, the day following the final exposure. In addition, blood, lung, and liver samples were collected from three core animals in each group on study day 5 for internal concentration assessment.

Study Design for Mice

Female B6D2F1/Crl mice were obtained from Charles River Laboratory (Raleigh, NC). Given previously observed carcinogenic effects in the lung in female mice and in the liver in male and female mice with a close structural analog (1,4-dichlorobenzene),[3] these studies employed the same strain/sex in which these effects were observed to allow for phenotypic anchoring of the biological interpretation of the data generated in the proposed studies. Females were chosen instead of males to reduce the overall study size and limit the complexity of comparisons between the mouse and rat data sets. On receipt, the mice were 8 weeks of age. Animals were quarantined for 9 days and then randomly assigned to one of six exposure groups. The mice in each exposure group were exposed to 1,2-DCB via whole-body inhalation for 6 hours plus T_{90} per day for 5 consecutive days (study days 0–4) at exposure concentrations of 0, 1, 10, 30, 100, or 250 ppm. There were 5 core female mice in the 1, 10, 30, and 100 ppm groups, 8 in the 250 ppm group, and 10 in the 0 ppm group; for internal concentration assessment, an additional 3 female mice were added to the 0, 1, 10, 30, and 100 ppm groups, and an additional 5 female mice were added to the 250 ppm group. Immediately following the final exposure on study day 4, blood, lung, and liver samples were collected from the internal concentration assessment animals once chamber concentrations fell below the regulatory limit of 12 ppm without additional health and safety considerations. Euthanasia, blood/serum collection, and tissue

sample collection for all surviving core animals were completed on study day 5, the day following the final exposure. In addition, blood, lung, and liver samples were collected from up to three core females in each group on study day 5 for internal concentration assessment.

Exposure Concentration Selection Rationale

The exposure concentrations evaluated in these studies were based on data in the published literature. In addition, a pilot study was conducted during the prestudy exposure engineering phase to confirm concentrations would be well tolerated for the duration of the planned study. Informed by the pilot exposure in a small number of animals, the highest concentrations were selected to be 500 ppm and 250 ppm for rats and mice, respectively. The lowest concentration of 1 ppm was selected to be tenfold less than the second lowest nonzero exposure concentration to produce a no-effect level at the transcriptome level.

Clinical Examinations and Sample Collection

Clinical Observations

All animals were observed twice daily for signs of mortality or moribundity, except for the day of receipt (rats) and at removal (rats and mice) when animals were observed once. Clinical observations were performed once prior to exposure on study day 0 and at study termination.

Body and Organ Weights

Animals were weighed during quarantine for randomization, on the first day of exposure (study day 0), daily thereafter (prior to exposure), and on the day of necropsy (study day 4 or 5). During necropsy for all core animals, the heart, liver, kidneys, and lungs were removed, and organ weights were recorded; bilateral organs were weighed separately.

Clinical Pathology

Animals were anesthetized with a 70% carbon dioxide (CO_2)/30% oxygen (O_2) mixture and bled in random order 1 day after the final day of exposure. After blood collection, animals were euthanized by exsanguination (rats) or 100% CO_2 followed by exsanguination (mice). Blood samples were collected within approximately a 2-hour window. Blood was taken via retro orbital plexus (rats) or retro orbital sinus (mice) and collected into tubes containing tripotassium ethylenediaminetetraacetic acid (K_3 EDTA) for hematology analysis (rats only) and into serum collection tubes without anticoagulant for clinical chemistry (rats and mice). The following hematology parameters were measured on a Sysmex XN-2000V (Sysmex America, Lincolnshire, IL) for rats: erythrocyte count, hemoglobin, hematocrit, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, white blood cell count and differential, reticulocyte count, and platelet count. Manual hematocrit was determined using a microcentrifuge and capillary reader. Blood smears were prepared, and qualitative evaluation of cellular morphology was performed per study protocol. The following clinical chemistry parameters were measured on a Roche cobas® c501 Chemistry Analyzer (Roche Diagnostics, Indianapolis, IN) for rats and mice: alanine aminotransferase, albumin, alkaline phosphatase, aspartate aminotransferase, total bile acids, total bilirubin, direct bilirubin, cholesterol, creatine kinase, creatinine, glucose, sorbitol dehydrogenase, total protein, triglycerides, and blood urea

nitrogen. Globulin, albumin/globulin ratio, and indirect bilirubin were calculated based on direct measurements (e.g., indirect bilirubin = total bilirubin − direct bilirubin).

Internal Concentration Assessment

An assessment was conducted to determine systemic exposure and tissue distribution and evaluate whether the test chemical had bioaccumulative properties (i.e., whether the half-life was >24 hours). Blood, lung, and liver samples were collected from the internal concentration assessment animals immediately following the last exposure on study day 4 (once chamber concentrations were at or below the regulatory limit of 12 ppm without additional health and safety considerations) and on study day 5 (approximately 18 hours following the last exposure) from core animals designated for internal concentration assessment. On study day 4, blood was collected via cardiac puncture for all surviving rats and mice (up to 3/exposure group) while animals were anesthetized with CO_2/O_2 (70%/30%). On study day 5, blood samples from designated core animals (3/exposure group) were taken via retro orbital plexus (rats) or retro orbital sinus (mice) while animals were anesthetized with CO_2/O_2 (70%/30%). Blood samples were collected within a 2-hour window. Blood was collected into tubes containing K_3 EDTA and three aliquots of 100 µL were transferred into headspace vials and kept on wet ice. Internal standard, $^{13}C_6$ 1,2-DCB (added as a mixture of $^{13}C_6$ 1,2-DCB and $^{13}C_6$ 1,4-DCB), was added to aliquoted samples and then stored frozen (−85°C to −60°C). All samples were frozen within 1 hour of collection. After blood collection, animals were euthanized by exsanguination and lung and liver tissues were collected (following organ weight measurements for core animals designated for internal concentration assessment) from each animal within 1 hour of each other. Up to three aliquots of approximately 100 mg (rats) or 50 mg (mice) lung and liver tissue were collected from each animal and flash frozen. Samples were stored frozen (−85°C to −60°C) until analysis.

Transcriptomics

Sample Collection for Transcriptomics

Within approximately 20 minutes of euthanasia, tissue samples were collected following organ weight measurements in the following order: lung, heart, liver (left lobe), kidney, and ovary (no ovary weights were taken) from all remaining animals on study day 5 for transcriptomic analysis. Two samples of lung, heart, liver, and kidney tissue (approximately 5 mm^3) were collected and placed into cryotubes containing RNA*later*™. The ovaries were collected whole bilaterally (one sample) and placed into cryotubes containing RNA*later*. The tissue samples were stored at 2°C to 8°C for approximately 55 hours for rats or 72 hours for mice. The RNA*later* was then removed and the samples were stored in a −85°C to −60°C freezer until processed for RNA isolation.

RNA Isolation, Library Creation, and Sequencing

RNA isolation was performed on tissue samples preserved in RNA*later*. Tissues were homogenized in QIAzol lysis buffer (Qiagen Inc., Valencia, CA) using the TissueLyser II bead-beating system followed by RNA extraction using the RNeasy 96 QIAcube HT kits (Qiagen Inc., Valencia, CA) with a DNA digestion step. The concentration and purity of all isolated samples were determined from absorbency readings taken at 260 and 280 nm using a NanoDrop ND-

2000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The readings accurately determined the concentration of each sample while ensuring that an acceptable purity (A_{260}/A_{280} ratio) between 1.80 and 2.20 was achieved. Further quality control (QC) evaluation of each RNA sample was performed using the RNA 6000 Nano kit and analyzed with a 2100 Bioanalyzer (Agilent Technology, Foster City, CA), which evaluates the RNA Integrity Number (RIN). RIN must be >3.0 to meet acceptable quality for TempO-Seq analysis. All samples were divided into at least two aliquots. One aliquot was used for BioSpyder TempO-Seq S1500+ analysis. Any tissue samples remaining after RNA isolation were stored at −85ºC to −60ºC until submitted frozen to the National Toxicology Program (NTP) Frozen Tissue Bank.

Isolated RNA was utilized for analysis using either the Rat S1500+ v1.2 TempO-Seq or Mouse S1500+ v1.2 TempO-Seq (BioSpyder, Carlsbad, CA) platform with a minimum of 500 mapped read counts/transcript and approximately 1.5 million counts/sample. Work instructions developed by Battelle using the BioSpyder User Guide[4] were followed, including the optimized overnight annealing procedure, outlined as follows. Two microliters of each diluted RNA sample (50 ng/µL) was hybridized with the S1500+ surrogate detector oligo pool mix (2 µL per sample) in a 384-well plate using the following thermocycler settings: 70°C for 10 minutes, followed by a gradual decrease to 45°C over 50 minutes, held at 45°C for 16 hours, and ending with a decrease to 25°C. The plates were held at 25°C for no longer than 8 hours. After annealing, the annealed RNA was transferred to 96-well plates for nuclease digestion (24 µL nuclease mix addition followed by 90 minutes at 37°C), followed by ligation (24 µL ligation mix addition followed by 60 minutes at 37°C) and heat denaturation (at 80°C for 15 minutes). For amplification, polymerase chain reaction (PCR) Pre-Mix and Primers were transferred from the BioSpyder S1500+ surrogate kit 96-well plates into a 384-well plate. Ten microliters of each ligated product were then added to the 384-well plate, sealed, and centrifuged briefly before 30 cycles of amplification.

All steps during the TempO-Seq S1500+ surrogate assay were performed using a QuantStudio 6 Flex System. The amplification step produced well-specific barcoded primer pairs that allowed identification of each well after being combined into a single sequencing library. Five microliters of amplified libraries were pooled together and purified using NucleoSpin gel and a PCR clean-up kit (Macherey-Nagel Inc., Allentown, PA). Once cleaned, the library concentration was determined by quantitative PCR using a KAPA Library Quantification Kit (Roche Sequencing, Indianapolis, IN), and the library was diluted to a final concentration of 400 pM. A PhiX control library (Illumina, San Diego, CA) was spiked into the final library as a system control. The final library was loaded onto an Illumina next generation sequencing cartridge, NovaSeq 6000 S1 Reagent Kit v1.5 (100 cycles) (Illumina, San Diego, CA), along with the BioSpyder-provided custom sequencing primer. Processing of sequencing data was conducted using Illumina's BCL2FASTQ software employing default parameter settings.

Sequence Data Processing

FASTQ files of TempO-Seq reads were aligned to the probe sequences from the target platform using Bowtie version 1.3.1[5] with the following parameters: -v 3 -k 1 -m 1 --best --strata. This configuration allows up to three mismatches and reports the single best alignment. After alignment, the total sequenced reads, the percentage of reads aligning to the platform manifest, the alignment rate, and the percentage of expressed probes (≥5 reads per probe) were calculated for each sample.

Sequencing Quality Checks and Outlier Removal

Each sample was evaluated for quality using the following metrics: sequencing depth, alignment to the platform manifest, number of aligned reads, % of probes with at least five reads, average per base quality, and per base N content. Samples were flagged for values below the following thresholds for the QC metrics: sequencing depth <300 K, total alignment rate <40%, unique alignment rate <30%, number of aligned reads <300 K, or percentage of probes with at least five reads <50%. FastQC was run on all samples to ensure adequate per base quality and per base N content, where N represents bases that could not be identified. Nine samples were flagged after applying the per-sample QC metrics. In addition, principal component, hierarchical cluster, and inter-replicate correlation analyses were used collectively to identify outlier samples. These analyses confirmed the nine flagged samples as outliers, identified additional outliers, and discovered a set of mouse kidney samples with potential tissue contamination. A total of 2 rat and 21 mouse samples were removed before downstream analysis, with 198 rat and 164 mouse samples available for downstream analysis.

The processing of samples from the study of 1,2-DCB was conducted in parallel with one other chemical that was studied under a similar protocol, therefore allowing for a more powerful collective assessment of the data. Specifically, during RNA isolation and extraction, the samples from both studies were distributed over twelve 96-well plates (i.e., three plates per species per chemical). Prior to amplification and library generation, the samples were randomized over five 384-well plates (i.e., one plate per tissue). The sample layout on the plate avoided the edge wells to preclude edge-well effects, which can affect downstream sequencing results. The final sample counts used for benchmark dose (BMD) analysis of the transcriptomics data are shown in Table 1.

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

	0 ppm	1 ppm	10 ppm	30 ppm	100 ppm	250 ppm	500 ppm
Rats							
Heart	10	5	5	5	5	5	5
Kidney	9	5	5	5	5	5	5
Liver	10	5	5	5	5	5	5
Lung	10	5	5	5	5	5	4
Ovary	10	5	5	5	5	5	5
Mice							
Heart	10	5	5	5	5	7	NA
Kidney	5	4	4	3	2	6	NA
Liver	9	2	4	5	5	6	NA
Lung	10	4	5	5	5	6	NA
Ovary	10	5	5	5	5	7	NA

NA = not applicable.

Data Normalization

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

Data Analysis

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

Two approaches were employed to assess the significance of pairwise comparisons between exposed and 0 ppm 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[6; 7] and Dunnett.[8] Clinical pathology data, which typically have skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley[9] and Dunn.[10] The Jonckheere test[11] 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[12] were examined by NIEHS staff. Values from animals suspected of illness from causes other than experimental exposure and values that the laboratory indicated as inadequate because of measurement problems were eliminated from the analysis.

A no-observed-effect level (NOEL) was identified as the highest exposure concentration not showing a significant ($p \leq 0.05$) pairwise difference relative to the 0 ppm group. A lowest-observed-effect level (LOEL) was identified as the lowest exposure concentration demonstrating a significant ($p \leq 0.05$) pairwise difference relative to the 0 ppm group. Throughout the results section for apical endpoints, interpretation of BMDs is made in relationship to NOEL and LOEL values for specific endpoints, as defined here, and are not meant to reflect an overall study NOEL or LOEL.

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

Data files for apical endpoints, including clinical pathology, organ weights, and body weights, were created using nontransformed individual animal data. With the exception of body weights (study day 0, terminal, and study day 5) and body weight gain, to be included in the data file, an endpoint had to show a significant trend and at least one significant pairwise response. The expression data files were then loaded into BMDExpress 3.2.0119 using a "generic" platform annotation. BMD modeling was conducted with ToxicR Laplace model averaging, applying a benchmark response (BMR) of 1 standard deviation. Constant variance was assumed in the BMD modeling. Results from the BMD analysis were subsequently subjected to a defined category

analysis in which modeled responses were excluded if they did not meet the following criteria: $R^2 > 0.6$, BMD/benchmark dose lower confidence limits (BMD_L) < 10, and BMD < highest dose. Some endpoints that met the initial statistical criteria for inclusion (i.e., significant trend and at least one significant pairwise response) did not yield a BMD result because no viable model was obtained.

Benchmark Dose Analysis of Transcriptomics Data

The BMD analysis of the transcriptomic data was performed in a manner consistent with the guidance provided in the 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*.[13]

Dose-response modeling of transcriptional effects was carried out using BMDExpress 3.20.0095, a robust, interactive, and user-friendly update of BMDExpress software[14] that can be downloaded at no cost (https://github.com/auerbachs/BMDExpress-3/releases). The initial mapped read counts from the TempO-Seq data from each well were counts per million normalized. The values were log2 transformed and imported into BMDExpress. The platform selection in BMDExpress was S1500_Plus_Rat for rats (date: October 31, 2024) and S1500_Plus_Mouse for mice (date: October 31, 2024). Before importing the data into BMDExpress, all detection oligos (DO) with "0" count values in any sample were excluded.

In BMDExpress, the data underwent a twofold prefiltering process. First, a Williams trend test[6; 7] was performed with nominal p value < 0.05 with 10,000 permutations. The DOs that passed the Williams trend test were then subject to the Curve Fit Prefilter for which the Hill, Power, Linear, Exponential 3, and Exponential 5 models were selected. A BMR factor of 2 standard deviations was used and the variance setting was constant.

The BMD analysis on the transcripts that passed the Curve Fit Prefilter was conducted using the ToxicR MAP/Laplace Bayesian MA fitting approach, implemented in BMDExpress. All continuous models (Hill, Power, Exponential 3, Exponential 5) were utilized. The parameters were set as follows: BMR type as relative deviation; BMR factor at 25%; variance as constant; and a Step Function Threshold at 0.5.

Gene set analysis (a.k.a. Functional Classification) was carried out using Gene Ontology biological process (GO BP) gene sets. For the GO analysis, the following settings were selected: (1) remove BMD > highest dose from category descriptive statistics; (2) remove BMD with R^2 < the cutoff of 0.6; (3) remove DOs with step function lower than first dose; (4) minimum number of genes in gene set of 40; (5) maximum number of genes in gene set of 500; (5) under DO to gene conversion, identify conflicting probe sets with correlation cutoff for conflicting probe sets of 0.5. Individual gene functional classification was conducted similarly to the GO gene set analysis, except that the maximum and minimum gene set size requirements were omitted.

Active GO BP terms were identified using criteria requiring at least three genes and being at least 5% populated. BMD, BMD_L, and benchmark dose upper confidence limit (BMD_U) values at the 5% level (i.e., 5th percentile) were reported as potency metrics for the active GO BP gene

sets. BMD, BMD_L, and BMD_U values for the individual gene analyses reflected the average of DOs that met the fit criteria and corresponded to that gene.

Empirical False Discovery Rate Determination for Genomic Dose-response Modeling

Synthetic null data were generated using the probe-filtered (i.e., "no 0") 0 ppm data from each tissue from the 1,2-DCB rat and mouse studies. The synthetic null data were generated using the Synthetic Null Data application (SanNDGen; https://rstudio.niehs.nih.gov/sandgen/), which employed the normal distribution method previously described for generating synthetic data.[15] In short, for each set of tissue/species, 0 ppm data were used to generate a distribution for each probe. This distribution was resampled to generate 1,000 values for each probe. These values were distributed into synthetic samples, which were then organized into 20 different experiments paralleling the distribution of samples in the experimental study (i.e., 10 samples in the 0 ppm group and 5 for each exposed group). Each of the 20 synthetic null experiments was processed through BMDExpress using the identical parameters used to analyze the experimental data. The resultant data were then used to determine the empirical false discovery rates, which are reported as percentages of possible genes and GO BPs.

Data Accessibility

Primary and analyzed data used in this study are available to the public at https://doi.org/10.22427/NIEHS-DATA-NIEHS-12.[1]

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