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Transcriptomic analyses of livers from mice exposed to 1,4-dioxane for up to 90 days to assess potential mode(s) of action underlying liver tumor development

G.A. Chappell [a,*], M.M. Heintz [a], L.C. Haws [b]

[a] ToxStrategies, Inc., Asheville, NC, United States
[b] ToxStrategies, Inc., Austin, TX, United States

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ABSTRACT

1,4-Dioxane is a volatile organic compound with industrial and commercial applications as a solvent and in the manufacture of other chemicals. 1,4-Dioxane has been demonstrated to induce liver tumors in chronic rodent bioassays conducted at very high doses. The available evidence for 1,4-dioxane-induced liver tumors in rodents aligns with a threshold-dependent mode of action (MOA), with the underlying mechanism being less clear in the mouse than in rats. To gain a better understanding of the underlying molecular mechanisms related to liver tumor development in mice orally exposed to 1,4-dioxane, transcriptomics analysis was conducted on liver tissue collected from a 90-day drinking water study in female B6D2F$_1$/Crl mice (Lafranconi et al., 2020). Using tissue samples from female mice exposed to 1,4-dioxane in the drinking water at concentrations of 0, 40, 200, 600, 2,000 or 6,000 ppm for 7, 28, and 90 days, transcriptomic analyses demonstrate minimal treatment effects on global gene expression at concentrations below 600 ppm. At higher concentrations, genes involved in phase II metabolism and mitotic cell cycle checkpoints were significantly upregulated. There was an overall lack of enrichment of genes related to DNA damage response. The increase in mitotic signaling is most prevalent in the livers of mice exposed to 1,4-dioxane at the highest concentrations for 90 days. This finding aligns with phenotypic changes reported by Lafranconi et al. (2020) after 90-days of exposure to 6,000 ppm 1,4-dioxane in the same tissues. The transcriptomics analysis further supports overarching study findings demonstrating a non-mutagenic, threshold-based, mitogenic MOA for 1,4-dioxane-induced liver tumors.

1. Introduction

1,4-Dioxane is a volatile organic compound currently used in industrial processes as a solvent, in the manufacture of other chemicals, and as a laboratory reagent (ATSDR, 2012). Chronic exposure to high levels of 1,4-dioxane via the inhalation or oral routes has been observed to cause liver tumors in laboratory rodents (Argus et al., 1973; Argus MF, Arcos JC, 1965; International Center for Medical Research. et al., 1988; Kano et al., 2009; Kasai et al., 2009; Kociba et al., 1974; NCI, 1978). In recent years, investigators have put forth a hypothesized MOA for 1,4-dioxane-induced mouse liver tumors, with hepatic cytotoxicity and subsequent regenerative hyperplasia proposed as key events (KE) for tumor development, subsequent to metabolic saturation and consequential accumulation of the parent compound in the blood (Dourson et al., 2014, 2017). However, questions remain as to whether there is sufficient information to understand early events in the development of hepatic tumors in mice exposed to 1,4-dioxane, and to support an initiating event of hepatotoxicity.

To further investigate the MOA related to 1,4-dioxane hepatocarcinogenicity in rodents, specifically mice, female B6D2F$_1$/Crl mice were exposed to 0, 40, 200, 600, 2,000 or 6,000 ppm (approximately 0, 7.2, 37.3, 116, 364, and 979 mg/kg bw/day) 1,4-dioxane in drinking water for 7, 28, or 90 days (Lafranconi et al., 2020). The B6D2F$_1$ mouse, which has been shown to be particularly susceptible to the development of liver tumors (Yamate et al., 1990; Katagiri et al., 1998), was selected to match as closely as possible the strain used in a previous study that demonstrated increased liver tumors at 66 mg/kg bw/day 1,4-dioxane (Kano et al., 2009). In the in-life portion of the present study reported by Lafranconi et al. (2020), the threshold for metabolic clearance was determined to be between 2000 and 6000 ppm, with pathological changes observed in the liver only after

* Corresponding author.
E-mail address: gchappell@toxstrategies.com (G.A. Chappell).

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90 days of exposure to the highest concentration (6000 ppm). The liver pathology was characterized as glycogen-like vacuolation, centrilobular hypertrophy, increased centrilobular GST-P staining, apoptosis, and a pan-lobular increase in cell proliferation (Lafranconi et al., 2020). These findings were concluded to demonstrate an early mitogenic response to 1,4-dioxane following sub-chronic (90 days) exposure to concentrations that exceeded the metabolic clearance threshold (Lafranconi et al., 2020). Mitogenesis is well-recognized as a nongenotoxic MOA for cancer (Cohen and Ellwein, 1990; U.S. EPA, 2005) with species differences (Elcombe et al., 2014), and is especially relevant to liver tumors in sensitive strains of mice (Maronpot, 2009).

As transcriptomic data can provide additional and/or supporting information regarding underlying mechanisms of effects associated with specific exposure scenarios (Gao et al., 2015; Dean et al., 2017; Joseph, 2017; Mulas et al., 2017), and can potentially be integrated into mode of action (MOA) analysis and human health risk or hazard assessments (Chepelev et al., 2015; Moffat et al., 2015; Johnson et al., 2020; LaRocca et al., 2020), whole transcriptome analyses were conducted on liver tissues from the 90-day drinking water study (7, 28, or 90 days of exposure). Transcriptomic signatures can also demonstrate adaptive, transient, and/or beneficial reactive responses to exposure. Considering the existing 1,4-dioxane evidence base, we hypothesized that genes related to xenobiotic metabolism, cell death, and cell proliferation would be altered by 1,4-dioxane exposure. Further, we sought to identify any additional molecular signaling alterations related to the liver effects seen in 1,4-dioxane-exposed mice. To address the question of genotoxicity, the presence of mRNA-level responses that may indicate enrichment of DNA damage and/or response pathways was specifically investigated. Gene set enrichment analysis and dose–response modeling were conducted to understand alterations in biological and disease processes across treatment groups. The transcriptomic signatures in the livers of exposed mice were also considered in relation to phenotypic data (i.e., apical endpoints) as determined by histopathological and immunohistochemical analyses of sections from the same liver tissue blocks, which demonstrated a significant increase in single-cell apoptosis and proliferation after 90 days of exposure, and an overall lack of significant treatment effect in the liver at concentrations of 1,4-dioxane below 6000 ppm (Lafranconi et al., 2020). The transcriptomic alterations were considered together with the phenotypic data reported by Lafranconi et al, (2020) to inform the MOA underlying the liver effects observed in female B6C2F$_1$/Crl mice. This information is important for understanding the relevance of the findings and dose–response observed in sensitive strains of mice for assessing human health risks where potential exposure occurs with much lower dosages, such as via ingestion of contaminated drinking water.

2. Materials and methods

2.1. Animal husbandry and exposure conditions

The in-life study method details are described in Lafranconi et al. (2020). Briefly, the subchronic toxicity of 1,4-dioxane was evaluated in a 90-day study in female B6D2F$_1$(BDF1)/Crl mice (Charles River Laboratories, Inc. [Raleigh, NC] aged between 5 and 8 weeks) exposed continuously to 0, 40, 200, 600, 2000, or 6000 ppm 1,4-dioxane in drinking water for 7, 28, or 90 days. The targeted mg/kg/day dose levels were 0, 10, 50, 150, 500, and 1500 mg/kg/day. The mouse strain and route of exposure (drinking water) were chosen to enable comparison to the results of the cancer bioassay findings reported by Kano et al. (Kano et al., 2009). Daily dosages at various time points were estimated using drinking water concentrations, body weights and average water consumption per group. Female mice were fed *ad libitum* LabDiet Certified Rodent Diet #5002 (PMI Nutrition International; St. Louis, MO). Animal care followed applicable animal welfare standards including the U.S. Department of Agriculture's Animal Welfare Act (9) Code of Federal Regulations (CFR) Parts 1, 2 and 3, National Research Council Guide for the Care and Use of Laboratory Animals. Washington, DC (NRC, 2011), and the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (AVMA, 2013). Non-fasted mice were euthanized by CO_2 anesthesia and decapitation at 7, 28, and 90 days of exposure. Liver tissues were fixed in neutral, phosphate-buffered 10% formalin and embedded in paraffin.

2.2. RNA sequencing

Formalin-fixed paraffin embedded (FFPE) liver samples from each mouse (n = 5 per treatment group; i.e., each duration and concentration) were microtomed to obtain a single 4–6 μm liver section mounted on a glass slide (uncovered), yielding a total of 90 samples for RNA sequencing. Slides were shipped to BioSpyder Technologies (Carlsbad, CA) where the unstained liver sections were evenly scraped from the slides and processed according to the TempO-Seq protocol, as previously described (Yeakley et al., 2017). DNA libraries created from each liver sample were sequenced using a HiSeq 2500 Ultra-High-Throughput Sequencing System (Illumina, San Diego, CA). RNA sequencing data are publicly available at NCBI's Gene Expression Omnibus[1] (GEO series accession number GSE154899).

2.2.1. Data processing and analysis

Sequencing data were analyzed using multiple packages in the R software environment, version 4.0.2 (cran.r-project.org/). The number of sequenced reads per probe were extracted from the sequencing output files; a traditional alignment step was not required because TempO-Seq uses gene-specific probe sequences. The DESeq2 R package (version 1.28.1) (Love et al., 2014) was used to normalize data to account for sample-to-sample variation in sequencing depth. Samples with below-optimal sequencing depth or low representation of expressed genes were not included in the comparative analysis. This was characterized by a total number of sequence reads >2 standard deviations below the mean sequenced reads per sample (5,635,830 and 8,839,173 across two sequencing runs), or a total number of genes sequenced >2 standard deviations below the mean number of genes sequenced per sample (16,132 and 17,350 across two sequencing runs). Application of these criteria resulted in the removal of five samples from the total 90 samples that were sequenced. Removal of low-count probes was not conducted because it is not necessary when using the DESeq2 package, owing to the application of shrunken fold-changes and independent filtering to stabilize low-count probes (Love et al., 2014).

2.2.2. Identification of differentially expressed genes

Significant differentially expressed genes (DEGs) were identified for each concentration of 1,4-dioxane within DESeq2 based upon estimated variance-mean dependence in the TempO-Seq count and a model using the negative binomial distribution. DEGs for each concentration compared to controls within the same timepoint were determined using a Wald statistical test and betaPrior set to "false" within DESeq2. Genes were considered to be significant DEGs if one of their corresponding probes had a false discovery rate (FDR) <10% following adjustment for multiple testing using the Benjamini and Hochberg (BH) procedure (Love et al., 2014).

2.2.3. Biological pathway enrichment analysis across concentrations of 1,4-dioxane

Biological pathways associated with gene expression profiles were

[1] https://www.ncbi.nlm.nih.gov/geo/

identified by pathway enrichment analysis. Mouse gene identifiers were converted to human identifiers using the biomaRt R package (v2.44.1) based on the Ensembl genome database (http://uswest.ensemble.org/index.html). The gene expression data were then queried for enrichment among gene sets in collections available in the Molecular Signatures Database (MSigDB) (http://software.broadinstitute.org/gsea/msigdb/index.jsp). The Canonical Pathways sub-collections were used (c2.cp.v6.2), which include gene sets from the following pathway databases: BioCarta online maps of metabolic and signaling pathways (BIOCARTA) (Nishimura, 2001), the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata et al., 1999), the Pathway Interaction Database (PID) (Schaefer et al., 2009), and the Reactome database of reactions, pathways, and biological processes (REACTOME) (Croft et al., 2011).

Enrichment of gene sets and pathways was determined using two methods: the gene set enrichment analysis (GSEA) statistical method and a hypergeometric test. The GSEA method follows the analysis platform made available by the Broad Institute (http://software.broadinstitute.org/gsea/index.jsp); the second employed a simpler hypergeometric test (Falcon and Gentleman, 2008). The GSEA method (Subramanian et al., 2005) determines whether sets of genes (e.g., the members of a molecular signaling pathway) are significantly concordant between various defined groups (in the case presented herein, different doses and timepoints) based on a ranking metric (in this case, the Wald statistic for expression differences between the 1,4-dioxane concentrations and control mice). The GSEA method was applied within Platform for Integrative Analysis of Omics data (PIANO) R package (v2.4.0) (Väremo et al., 2013), with geneSetStat = "gsea" and significance calculated using permutation-based nominal P values based on weighted Kolmogorov-Smirnov test enrichment scores, adjusted for multiple hypothesis testing by calculating FDRs using the BH method (Subramanian et al., 2005). The second method, a hypergeometric test, considers only significant DEGs (i.e., FDR $<10\%$ by DESeq2 analysis) for overrepresentation among genes sets listed in the Canonical pathways sub-collections using the Fisher combined probability test function in the PIANO R package (using "runGSAhyper"). No fold-change criteria were set. For both analyses, a minimum of 5 and a maximum of 500 genes was set for the gene set size (number of member genes represented in the dataset tested, i.e., the results of the sequencing experiment presented herein) criteria for inclusion in the analysis. Gene sets with an FDR $<10\%$ were considered to be significantly enriched.

2.2.4. Investigation of DNA damage response

To further investigate enrichment of gene sets relevant torel DNA damage response and/or repair, a collection of gene sets was curated by searching through all gene sets in the MSigDB collections (v6.2) using key words related to DNA damage response. A total of 89 gene sets that are related to DNA damage and/or response were identified and then tested for enrichment among significant DEGs (i.e., FDR $<10\%$ by DESeq2 analysis) using a hypergeometric test for overrepresentation, using all genes among these 89 gene sets as the background (i.e., the gene "universe"). No fold-change criteria were set for the DEGs tested for enrichment. This targeted approach was conducted separately from the gene set enrichment analysis using the broader Canonical Pathways gene sets as a means to specifically evaluate enrichment of DNA damage-related gene sets. Some overlap exists in the gene sets from the Canonical Pathways collection and the curated list of DNA damage-specific list of 89 gene sets. A minimum of 5 and a maximum of 500 genes was set for the gene set size (number of member genes represented in the dataset tested, i.e., the results of the sequencing experiment presented herein) criteria for inclusion in the analysis. Gene sets with an FDR $< 10\%$ were considered to be significantly enriched.

Additionally, high-throughput screening (HTS) data available via the US EPA's ToxCast downloadable data (invitroDBv3.2 database summary files[2]) were reviewed for 1,4-dioxane in a battery of nine assays (plus relevant viability or baseline assays) that are related DNA damage/repair (Hsieh et al., 2019).

2.3. Benchmark dose analysis

Dose-response modeling was conducted in using BMDExpress software (v2.2) (Phillips et al., 2019) using normalized expression data from DESeq2 without transformation. A Williams trend test (P value cutoff = 0.05) was employed to identify genes perturbed by 1,4-dioxane exposure. Fold-change filters and correction for multiple tests were not applied. Benchmark dose (BMD) analysis was conducted with linear, power, hill, 2° and 3° polynomial, and exponential models 2 to 5. The models were run assuming constant variance and a benchmark response (BMR) of 1 standard deviation. Functional classification of dose-responsive genes (genes with BMD P < 0.1) was conducted using the Gene Ontology (GO) and REACTOME gene sets available within BMDExpress. Genes were filtered from the analysis according to the default parameters within BMDExpress, as follows: genes with BMD/BMDL > 20, BMDU/BMDL > 40, BMDs above the highest dose (6000 ppm)), and/or genes with a BMD $>$ 10-fold below the lowest positive dose were removed from functional classification analysis. No filters for minimum or maximum number of genes per gene set were used. Benchmark doses for the gene sets were also estimated. Additional parameters for the BMD modeling and pathway analyses can be found in Supplemental Materials.

3. Results

3.1. In-life summary

The results of the in-life portion of the study are described in Lafranconi et al. (2020). Briefly, there was no treatment-related effect on clinical signs, clinical chemistry parameters, body weights, or survival at any dose or timepoint in 1,4-dioxane-treated groups compared to controls. Liver weights were slightly increased in the 6000 ppm group at all timepoints; no changes were observed at lower concentrations. Histopathological analysis revealed minimal to mild vacuolation consistent with glycogen deposition in the centrilobular regions of the liver in animals exposed to 1,4-dioxane at concentrations ≥600 ppm after 7 days of exposure, which was nearly completely resolved by day 28. Minimal to mild centrilobular hypertrophy and centrilobular apoptosis was evident in the 2000 ppm and 6000 ppm groups at 28 and 90 days of exposure. There were no consistent treatment-related changes in hepatocellular proliferation in any dose group at 7 or 28 days according to immunohistochemical staining for bromodeoxyuridine (BrdU) incorporation, while there was a treatment-related increase at 90-days in the 6000 ppm group. The increase in BrdU incorporation corresponded with increased relative liver weights and blood levels of 1,4-dioxane (Lafranconi et al., 2020).

3.2. Transcriptomic changes associated with exposure to 1,4-dioxane

RNA sequencing was performed on liver samples to examine exposure effects of 1,4-dioxane on the hepatic gene expression of female mice compared to time-matched control mice. All sample libraries passed quality control measures necessary to be sequenced. As already noted in the *Materials and Methods*, following sequencings, five samples were removed from the analysis due to low sequencing depth or low gene diversity, from the 200 and 600 ppm groups across all three timepoints. There was an overall lack of transcriptomic response in the 40 and 200 ppm concentration groups, with the number of DEGs for

[2] https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data (downloaded August 16th, 2019)

these two concentrations across the three timepoints ranging from 0 to 22 (Table 1, Supplemental Table S1). At 600 ppm, an increase in the transcriptomic response was observed at both the 7 and 90 day timepoints, but not at 28 days. At 6000 ppm, the number of DEGs exhibited a similar pattern as the 600 ppm concentration, with a higher response at 7 and 90 days relative to 28 days (Table 1, Fig. 1). Approximately half of the DEGs for the 600 ppm group were also differentially expressed in the 6000 ppm group at 90 days, while at 7 days there was less overlap in the same genes being differentially expressed in the 600 vs. the 6000 ppm groups. At 2000 ppm, the transcriptomic response was similar across timepoints. The lower number of DEGs at 2000 ppm compared to 600 ppm at days 7 and 90 was an unexpected finding, and is without evidence of spurious origin. The overall relatively low number of DEGs across all experimental groups and timepoints likely contributed to this variability. The virtual lack of transcriptomic response following exposure to 1,4-dioxane concentrations below 600 ppm at all timepoints supports a conclusion that there is a threshold concentration for hepatic transcriptomic response to 1,4-dioxane in female mice somewhere in the range of 200 to 600 ppm. It is noted that the lowest dose tested in the Kano et al. (2009) bioassay was 500 ppm and the lowest dose in the NCI (1978) drinking water bioassay in mice was 5000 ppm (NCI, 1978; Kano et al., 2009).

3.3. Gene set enrichment analysis

3.3.1. Analysis by dose group relative to time-matched controls

The results of both gene set enrichment analysis methods were evaluated to further understand 1,4-dioxane treatment-related effects. Although the top-most significantly enriched pathways were similar across the two methods used for pathway enrichment analysis (Supplemental Tables S2 and S3), the hypergeometric method was determined to be less informative for the objective of the present study due to the minimal changes in gene expression at the lower concentrations (40–200 ppm), resulting in a complete lack of gene set enrichment at those concentrations. Thus, the results discussed below focus on the pre-ranked GSEA method for enrichment analysis. Due to the minimal treatment effect of 1,4-dioxane at any dose or timepoint on gene expression changes, liberal criteria were applied to identify DEGs and enriched signaling pathways. The full set of results for the hypergeometric test can be found in Supplemental Table S3. Pathway enrichment analysis of significantly differentially expressed genes at 40 ppm and 200 ppm 1,4-dioxane yielded very few significantly enriched gene sets/pathways using the pre-ranked GSEA test (Table 2, Supplemental Table S2).

The decreased regulation of complement and coagulation cascades, mitochondrial β-oxidation and several other fatty acid metabolism pathways observed in the 600 ppm group is consistent with other transcriptomic analyses of liver tissues from primate and mouse studies in which animals were treated with nuclear receptor agonists that induce mitosis and DNA synthesis (e.g., fibrates (Cariello et al., 2005; Lu et al., 2011; de la Rosa Rodriguez et al., 2018) (Table 2).

At the 2000 and 6000 ppm concentrations, significant enrichment of xenobiotic metabolism pathways was evident, which increased in significance with increasing time and dose. Examples of enriched up-regulated gene sets associated with phase II metabolism, specific to glutathione conjugation, include KEGG “glutathione metabolism” and REACTOME “glutathione conjugation” (Table 2). The enrichment of these gene sets was driven by altered genes that encode glutathione transferase isoforms. Additionally, similar to the pathway alterations observed at 600 ppm, complement and coagulation cascade pathways were enriched in the negative direction (down-regulated) at 2000 and 6000 ppm 1,4-dioxane due to decreased expression of genes encoding proteolytic subunits in the complement system and gene members of the serpin family (serine protease inhibitors) relative to controls (Table 2). The complement cascade is a part of the innate immune system and deficiency of certain serpins (e.g., Serpina1) has been associated with liver damage (Law et al., 2006), which is consistent with the reported increase in cell death in the highest 1,4-dioxane dose groups (Lafranconi et al., 2020). Down-regulation of extracellular matrix regulators is also related to the loss of genes related to clotting factors. The significant decrease in expression of lipid metabolism-related gene sets in the 2000 and 6000 ppm may be related to liver injury in these high 1,4-dioxane dose-groups following saturation of metabolism and accumulation of the parent compound (as described in Lafranconi et al., 2020).

At the 90-day timepoint, mitotic cell cycle and DNA synthesis pathways were significantly enriched in the 6000 ppm treatment group: aurora B kinase signaling, mitotic phase transition and checkpoint signaling, and general cell cycle (e.g., Reactome “Cell cycle, mitotic”) (Fig. 2, Table 2), indicating a mitogenic proliferative response that was not observed at earlier time points. Enriched tubulin folding pathways share many of the same gene members as the cell cycle gene sets (Fig. 2). This is consistent with biomarkers of proliferative liver response in the same tissues, as reported in Lafranconi et al. (2020). Specifically, a treatment-related pan-lobular increase in hepatocellular proliferation was observed at 90-days in animals exposed to 6,000 ppm, as measured by BrdU incorporation. The increase in BrdU incorporation corresponded with an increase in relative liver weight as well as blood levels of 1,4-dioxane. There were no consistent, treatment-related changes in hepatocellular proliferation at 7 or 28 days in any dose group (Lafranconi et al., 2020).

3.3.2. Targeted analysis of DNA damage response

According to the targeted analysis (hypergeometric test for over-representation) of changes in expression in genes included in a curated list of 89 gene sets related to DNA damage response and repair (Supplemental Table S4), there was no enrichment. A hypergeometric test was necessary for this assessment, due to the nature of the evaluation using a focused list of gene sets (Supplemental Table S5). Additionally, 1,4-dioxane was inactive in the battery of HTS assays used to identify compounds with genotoxic potential (Supplemental Table S6). It should be noted that challenges exist in testing volatile chemicals (such as 1,4-dioxane) in HTS assays, as these *in vitro* assays involve the use of open vessels with incubations carried out at temperatures ranging from 4 °C to 37 °C. In such conditions, a substance with a high vapor pressure can potentially volatilize during the course of the assay, thereby influencing the concentration of the test substance in the system. While 1,4-dioxane has a molecular weight less than 140 g/mol (88.11 g/mol), which indicates volatility, its vapor pressure and log octanol/water partition coefficients (38.1 mmHg and −0.27, respectively) are within suitable boundaries for ToxCast/Tox21 assays (Tice et al., 2013; Richard et al., 2016). Overall, these results are consistent with other findings indicating that 1,4-dioxane does not cause direct DNA damage in the liver *in vivo* in mice, nor does it cause changes in *in vitro* assays designed to detect DNA damaging agents (as reviewed in (EPA, 2010; ATSDR, 2012)). These findings support a non-mutagenic MOA.

3.3.3. Benchmark dose modeling

The dose–response for individual genes were analyzed using BMD modeling, and functional characterization of the dose-responsive genes was analyzed and visualized. The BMD results confirmed pathway enrichment results obtained for single dose groups. For example, similar to what was found at 90 days in the ≥600 ppm 1,4-dioxane groups, the REACTOME gene sets “glutathione conjugation” and “Phase II – Conjugation of compounds” were significantly enriched at 90 days with median BMD_{1SD} values of 1548 and 1652 ppm, respectively, and median BMDLs of 1236 and 1251 ppm, respectively (Table 3, Fig. 3). In addition, “innate immune system”, a part of the complement and coagulation cascade pathway, was also significantly enriched in the negative direction with a median $BMD_{1SD} > 3200$ ppm. Cell cycle and mitosis gene sets (median $BMD_{1SD} > 3400$ ppm or

Table 1
Number of differentially expressed genes for each dose and length of exposure compared to time-matched control groups (shown as total DEG (Up-regulated [↑], Down-regulated [↓])). Full DESeq2 results can be found in Supplemental Table S1.

Exposure Duration (days)	1,4-Dioxane Concentration (ppm)				
	40	200	600	2000	6000
7	0 (0)	2 (↑0, ↓2)	411 (↑165, ↓246)	20 (↑6, ↓14)	415 (↑180, ↓235)
28	1 (↑0, ↓1)	1 (↑0, ↓1)	1 (↑0, ↓1)	49 (↑21, ↓28)	232 (↑87, ↓145)
90	5 (↑1, ↓4)	22 (↑11, ↓11)	323 (↑165, ↓158)	33 (↑25, ↓8)	727 (↑352, ↓375)

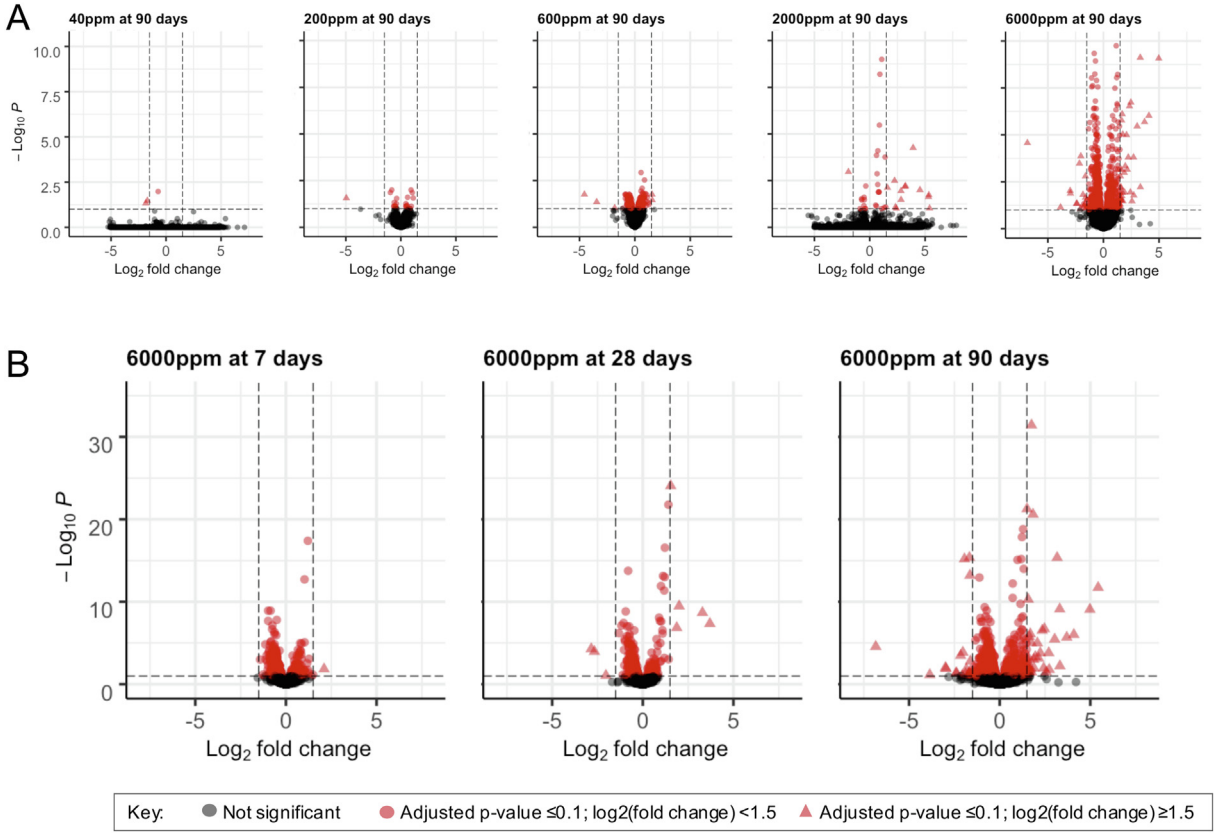

Fig. 1. Volcano plots showing differentially expressed genes across concentrations at the 90-day timepoint (A), and across all three timepoints at the 6000 ppm concentration (B). Red points represent probes with an adjusted p-value ≤ 0.1; circles represent probes within a log2 (fold change) < 1.5, and red triangles represent probes with a log2 (fold change) ≥ 1.5. A: y-axis is scaled for all plots from 0 to 10 (resulting in some points cut off the plot for the 6000 ppm concentration). B: y-axis for all plots is scaled from 0 to 35. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

higher, BMDLs > 2200 ppm or higher) along with a single "DNA repair" gene set (median BMD_{1SD} > 4000 ppm) were enriched among dose-responsive genes at 90 days. Individual genes within the "DNA repair" gene set that were identified as dose-responsive are mainly histone encoding genes and DNA polymerase genes involved in DNA synthesis (Supplemental Table S7). An exception is the DNA repair gene *Rad51*, which was found to have a significant dose-responsive trend via BMDExpress (p = 0.0217 by Williams Trent Test) at 90 days. However, this gene had generally low expression in all treatment groups and was not significantly differentially expressed at any individual dose at any timepoint relative to time-respective controls according to DESeq2 analysis (adjusted p-value ≥ 0.1 for all probes for all concentrations and timepoints; Supplemental Table S1).

Similar to the gene sets that were determined as significantly enriched at 90 days according to the GSEA analysis at individual doses/timepoints, phase II metabolism, cell cycle and mitosis gene sets were up-regulated with comparable BMD values at 28 days (Fig. 3). The "DNA Repair" gene set was not significantly enriched according to BMD modeling at 28 days. Moreover, "fatty acid metabolism" and "immune system" were down-regulated at 28 days (median BMD_{1SD} 3385 and 3367 ppm, respectively) but with less statistical significance compared to the results at 90 days (i.e., lower Fisher's test p-values). Among the dose-responsive genes at 7 days, phase II metabolism gene sets were enriched, with much higher median BMD_{1SD} values than those determined at 28 and 90 days. Although the gene ontology used in the BMDExpress software (REACTOME) was not an exact match to that of the GSEA analysis, the REACTOME gene sets were included in both analyses. Further, many similar gene sets exist across different ontologies, enabling a reasonable comparison of biological signals within the two analyses.

At 7 days, the top-most enriched gene sets among dose-responsive genes were related to signal transduction and were down-regulated, with median BMDs > 2000 ppm. This may represent an early stress response and/or cytotoxicity. "Glucuronidation" and "Phase II – Conjugation of compounds" were enriched, and up-regulated (median BMDs 1092 and 2301 ppm, respectively). The "DNA repair" gene set

Table 2
Top most significantly enriched pathways for each treatment group according to the GSEA method (Subramanian et al., 2005). The top five most significantly enriched pathways for each direction of change are shown in the table; in cases where five gene sets were not significantly enriched, only those with an adjusted p-value < 0.1 are shown. Full results are presented in Supplemental Table S2.

1,4-Dioxane (ppm)	Duration (days)	Overall Direction	Gene set	Adjusted p-value
40	7	Up	None	NA
		Down	None	NA
	28	Up	REACTOME DEGRADATION OF THE EXTRACELLULAR MATRIX	0.023524
			REACTOME HS GAG DEGRADATION	0.094038
		Down	None	NA
	90	Up	None	0.000913
		Down	REACTOME SRP DEPENDENT COTRANSLATIONAL PROTEIN TARGETING TO MEMBRANE	0.0015971
			REACTOME UNFOLDED PROTEIN RESPONSE	0.0019783
			KEGG TERPENOID BACKBONE BIOSYNTHESIS	0.0024729
			REACTOME TRANSLATION	0.0031942
			REACTOME CHOLESTEROL BIOSYNTHESIS	0.0032972
200	7	Up	REACTOME TRANSLATION	< 0.0001
			REACTOME PEPTIDE CHAIN ELONGATION	< 0.0001
			REACTOME 3 UTR MEDIATED TRANSLATIONAL REGULATION	< 0.0001
			REACTOME SRP DEPENDENT COTRANSLATIONAL PROTEIN TARGETING TO MEMBRANE	< 0.0001
			KEGG RIBOSOME	< 0.0001
		Down	KEGG BIOSYNTHESIS OF UNSATURATED FATTY ACIDS	0.019492
			KEGG PEROXISOME	0.021777
			REACTOME SULFUR AMINO ACID METABOLISM	0.022062
			KEGG ARGININE AND PROLINE METABOLISM	0.028247
			REACTOME PYRUVATE METABOLISM AND CITRIC ACID TCA CYCLE	0.029404
	28	Up	None	NA
		Down	None	NA
	90	Up	None	NA
		Down	None	NA
600	7	Up	REACTOME 3 UTR MEDIATED TRANSLATIONAL REGULATION	< 0.0001
			REACTOME PEPTIDE CHAIN ELONGATION	< 0.0001
			REACTOME NONSENSE MEDIATED DECAY ENHANCED BY THE EXON JUNCTION COMPLEX	< 0.0001
			KEGG RIBOSOME	< 0.0001
			REACTOME INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	< 0.0001
		Down	KEGG PROPANOATE METABOLISM	< 0.0001
			REACTOME FORMATION OF FIBRIN CLOT CLOTTING CASCADE	< 0.0001
			REACTOME COMMON PATHWAY	< 0.0001
			KEGG FATTY ACID METABOLISM	< 0.0001
			KEGG BUTANOATE METABOLISM	< 0.0001
	28	Up	None	NA
		Down	REACTOME TRNA AMINOACYLATION	0.066645
			PID PLK1 PATHWAY	0.098824
	90	Up	None	
		Down	REACTOME HEPARAN SULFATE HEPARIN HS GAG METABOLISM	0.026099
			REACTOME A TETRASACCHARIDE LINKER SEQUENCE IS REQUIRED FOR GAG SYNTHESIS	0.036539
			REACTOME HS GAG BIOSYNTHESIS	0.095041
2000	7	Up	REACTOME INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	< 0.0001
			REACTOME INFLUENZA LIFE CYCLE	< 0.0001
			REACTOME PEPTIDE CHAIN ELONGATION	< 0.0001
			REACTOME TRANSLATION	< 0.0001
			REACTOME SRP DEPENDENT COTRANSLATIONAL PROTEIN TARGETING TO MEMBRANE	< 0.0001
		Down	KEGG VALINE LEUCINE AND ISOLEUCINE DEGRADATION	< 0.0001
			KEGG TRYPTOPHAN METABOLISM	< 0.0001
			KEGG PPAR SIGNALING PATHWAY	< 0.0001
			KEGG FATTY ACID METABOLISM	< 0.0001
			KEGG PROPANOATE METABOLISM	< 0.0001
	28	Up	REACTOME PEPTIDE CHAIN ELONGATION	< 0.0001
			KEGG RIBOSOME	0.0021614
			REACTOME INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	0.0023879
			REACTOME 3 UTR MEDIATED TRANSLATIONAL REGULATION	0.012228
			REACTOME CELL DEATH SIGNALLING VIA NRAGE NRIF AND NADE	0.014006
		Down	KEGG BIOSYNTHESIS OF UNSATURATED FATTY ACIDS	< 0.0001
			REACTOME FATTY ACYL COA BIOSYNTHESIS	0.020023
			REACTOME POST TRANSLATIONAL PROTEIN MODIFICATION	0.021946
			KEGG STEROID HORMONE BIOSYNTHESIS	0.022519
			REACTOME METABOLISM OF AMINO ACIDS AND DERIVATIVES	0.023419
	90	Up	REACTOME FORMATION OF TUBULIN FOLDING INTERMEDIATES BY CCT TRIC	0.045617
			BIOCARTA P53 PATHWAY	0.059268
			SIG REGULATION OF THE ACTIN CYTOSKELETON BY RHO GTPASES	0.068946
			REACTOME GLUTATHIONE CONJUGATION	0.079748
			REACTOME POST CHAPERONIN TUBULIN FOLDING PATHWAY	0.085827
		Down	REACTOME DEGRADATION OF THE EXTRACELLULAR MATRIX	< 0.0001
			BIOCARTA INTRINSIC PATHWAY	< 0.0001
			KEGG COMPLEMENT AND COAGULATION CASCADES	0.0053303
			REACTOME LIPID DIGESTION MOBILIZATION AND TRANSPORT	0.015464

(*continued on next page*)

Table 2 (*continued*)

1,4-Dioxane (ppm)	Duration (days)	Overall Direction	Gene set	Adjusted p-value
			REACTOME FORMATION OF FIBRIN CLOT CLOTTING CASCADE	0.016814
6000	7	Up	BIOCARTA EIF PATHWAY	< 0.0001
			KEGG RIBOSOME	< 0.0001
			REACTOME PEPTIDE CHAIN ELONGATION	< 0.0001
			REACTOME INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	< 0.0001
			REACTOME INFLUENZA LIFE CYCLE	< 0.0001
		Down	KEGG COMPLEMENT AND COAGULATION CASCADES	< 0.0001
			BIOCARTA COMP PATHWAY	< 0.0001
			NABA ECM REGULATORS	0.0010723
			REACTOME FORMATION OF FIBRIN CLOT CLOTTING CASCADE	0.0012178
			BIOCARTA CLASSIC PATHWAY	0.0013393
	28	Up	REACTOME GLUTATHIONE CONJUGATION	0.05646
		Down	KEGG COMPLEMENT AND COAGULATION CASCADES	< 0.0001
			KEGG ARGININE AND PROLINE METABOLISM	0.0027586
			NABA ECM REGULATORS	0.0036022
			BIOCARTA INTRINSIC PATHWAY	0.0036782
			REACTOME FORMATION OF FIBRIN CLOT CLOTTING CASCADE	0.004578
	90	Up	PID AURORA B PATHWAY	0.00041562
			REACTOME FORMATION OF TUBULIN FOLDING INTERMEDIATES BY CCT TRIC	0.00083123
			REACTOME GLUTATHIONE CONJUGATION	0.017352
			REACTOME POST CHAPERONIN TUBULIN FOLDING PATHWAY	0.026444
			REACTOME PREFOLDIN MEDIATED TRANSFER OF SUBSTRATE TO CCT TRIC	0.043203
		Down	PID HNF3A PATHWAY	< 0.0001
			KEGG PANTOTHENATE AND COA BIOSYNTHESIS	< 0.0001
			REACTOME LIPID DIGESTION MOBILIZATION AND TRANSPORT	0.0014751

that was enriched at 90 days was also enriched at 7 days (BMD median of 3506 ppm). The histone and ubiquination genes underlying the enrichment of this gene set are involved in DNA synthesis and potentially cell proliferation.

Overall, BMD analysis confirmed the increase in phase II xenobiotic metabolism and a decrease in complement cascade and lipid metabolism pathways that was observed via analysis at each individual dose, as well as a significant increase mitotic cell cycle and cellular proliferation at concentrations above 2000 ppm at 90 days (Fig. 3). The BMD_{1SD} and BMDLs were well above 600 ppm for some pathways that were significant at 600 ppm according to gene set enrichment analysis comparing each dose relative to the controls. This may be explained by the fact that BMD modeling analysis accounts for variability across the whole experiment, as well as the general dose–response curve information, as opposed to specifically comparing one dose group to the time-matched controls.

4. Discussion

Mechanistic data provide important information for human health risk assessment, in particular with respect to providing an understanding of the underlying mode/mechanisms of an adverse outcome. Such mechanistic data can inform the MOA of a chemical via the identification of specific key molecular or cellular events. Specifically, transcriptomic analysis can contribute to understanding drug- or chemical-induced liver toxicity by identifying biomarkers of effect or exposure, expression signatures, and/or changes in signaling (Merrick and Bruno, 2004; Cui and Paules, 2010). The identification of a MOA for a carcinogen is important for the selection of the risk assessment approach under current regulatory paradigms. Specifically, a mutagenic vs. a non-mutagenic MOA have historically been subject to linear low-dose extrapolation vs. a threshold approach, respectively, for risk assessment (U.S. EPA, 2005). In the case of 1,4-dioxane, several groups, including regulatory agencies, have applied a threshold approach (NICNAS, 1998; TNO/RIVM, 1999; Stickney et al., 2003; Health Canada, 2005), while others have applied a non-threshold approach (OEHHA, 2002; U.S. EPA, 2013). Previously, a MOA for rodent liver tumors was hypothesized that included metabolic saturation followed by cytotoxicity-induced regenerative repair (Dourson et al., 2014, 2017). Biomarker analyses and histological examinations were conducted on the same liver tissues discussed herein, and reported by Lafranconi et al (2020). Collectively, these analyses demonstrated saturated metabolism of 1,4-dioxane in mice, as well as increased proliferation following 90 days of oral exposure via drinking water, at concentrations ≥2000 ppm. These results provide additional mechanistic information for 1,4-dioxane, informing potential key events in a MOA for liver cancer in a sensitive strain of mouse. The transcriptomic information also adds insights as to molecular events that explain these biomarker and histopathology findings. Moreover, the transcriptomic analyses serve to identify potential key events for further examination that were not visible with the more conventional histopathological observations.

As described in the *Materials and Methods*, gene expression data from the livers of 1,4-dioxane-exposed mice were analyzed for individual gene changes, gene set enrichment using two different statistical methods, and BMD modeling for individual genes, as well as functional classification of dose-responsive genes. The results demonstrate a generally low response to 1,4-dioxane in the livers of mice and the mRNA level. Overall, gene set enrichment demonstrated up-regulation of phase II metabolism in a dose–response manner. After 90 days of exposure, an increase in cell cycle signaling was evident in the highest concentration treatment group. Changes in individual genes that did not converge into gene set enrichment, and a general loss of signal transduction at the pathway level at the 7-day timepoint likely represents a non-specific adaptive and/or general stress response. Such changes were mitigated after 28 days of exposure, potentially related to the up-regulation of Phase II metabolism and, thus, detoxification. Importantly, transcriptomic profiling conducted to specifically query the enrichment of DNA damage response gene sets demonstrated a lack of DNA damage response at the mRNA level. The few enriched gene sets related to up-regulation of DNA damage response at 6000 ppm (i.e., p53 signaling pathways) according to the more lenient GSEA enrichment analysis and BMD functional classification analysis may be related to apical endpoints reported in Lafranconi et al. (2020): up-regulation of signaling pathways for cell cycle are potentially related to the reported increased BrdU labeling, and enrichment of cell death signaling potentially related to the increase in apoptosis as evi-

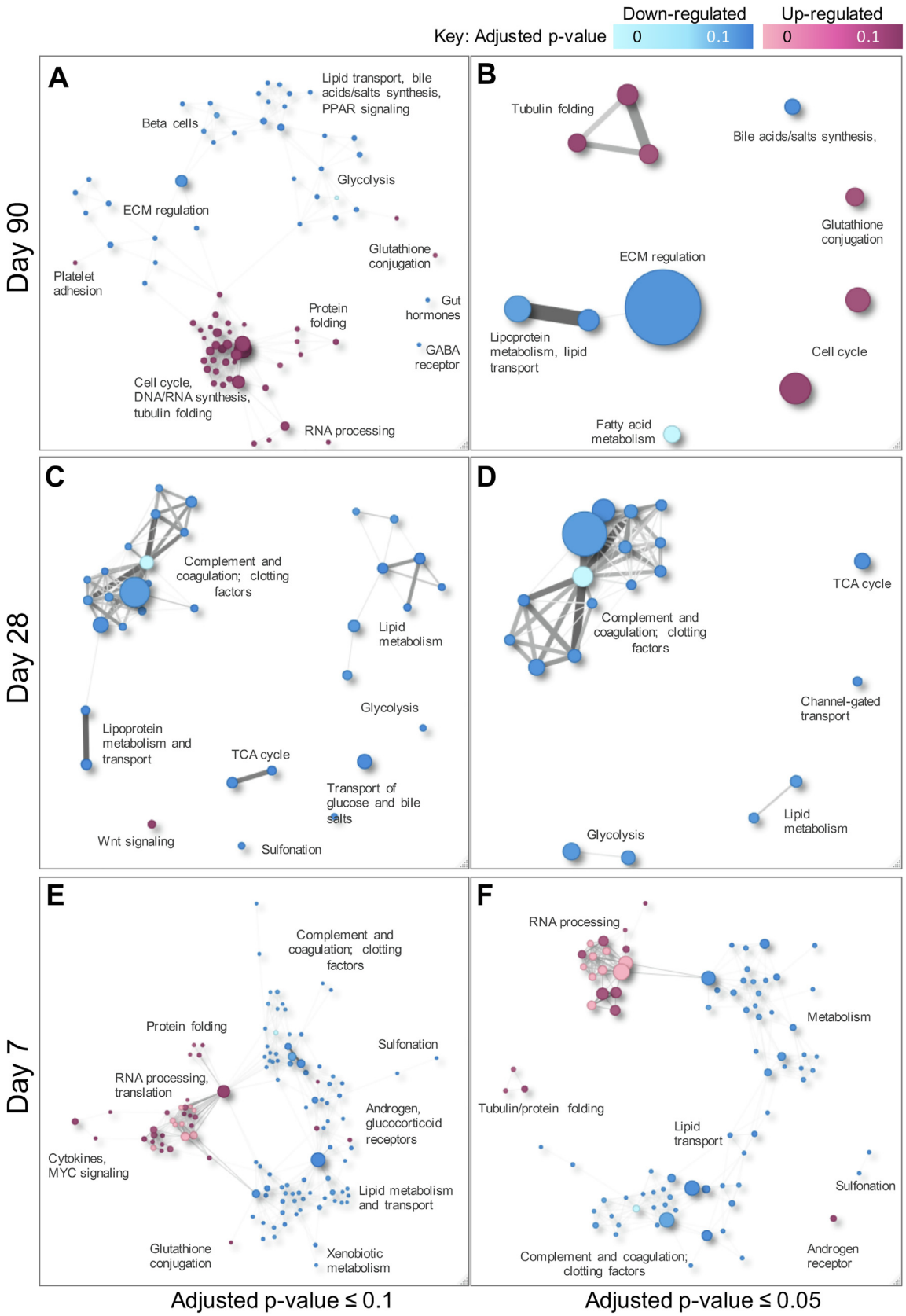

Fig. 2. Network plots showing enriched gene sets at 6000 ppm relative to controls. (A/C/E: adjusted p-value ≤ 0.1, B/D/F: adjust p-value ≤ 0.05). Node size is scaled on number of member genes within the gene set, and node color is scaled according to significance (lighter blue/pink node color indicates more highly significant relevant to darker blue/pink node color). Nodes are spatially organized according to likeness, according to common individual genes within the gene sets. Lines connecting nodes represents common members, with thickness of the line scaled according to number of common gene members. Color of the nodes represents statistical significance as noted in the color bar key. For visualization, general descriptive categories are denoted for gene sets with common genes and, thus, similar functionality, as opposed to listing all actual gene set names. Select individual gene set of highest statistically significant enrichment are shown. Full results for all dose groups and timepoints are in Supplemental Table S2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
BMD modeling results for select up-regulated enriched gene sets related to xenobiotic metabolism and cell cycle. Full results are presented in Supplemental Table S7.

Gene set	Exposure Duration (days)	Median BMD_{1SD} (ppm)	Median BMDL (ppm)	Fisher's exact two-tail test p-value
Glutathione Conjugation	7	2305	1819	$7.46x10^{-4}$
	28	1682	1399	$6.75x10^{-5}$
	90	1548	1236	$6.16x10^{-4}$
Phase II - Conjugation of compounds	7	2301	1696	$4.11x10^{-5}$
	28	1903	1401	$9.28x10^{-9}$
	90	1652	1251	$3.84x10^{-2}$
Cell Cycle	7	3521	2333	NS
	28	5455	2523	NS
	90	3874	2243	$4.54x10^{-3}$
Cell Cycle Checkpoints	7	3628	2428	NS
	28	5455	2849	NS
	90	3474	2265	$6.56x10^{-2}$
Cell Cycle, Mitotic	7	3521	2333	NS
	28	3639	2523	$2.56x10^{-2}$
	90	3414	2242	$9.44x10^{-3}$

NS, not significant for enrichment among dose-responsive genes.

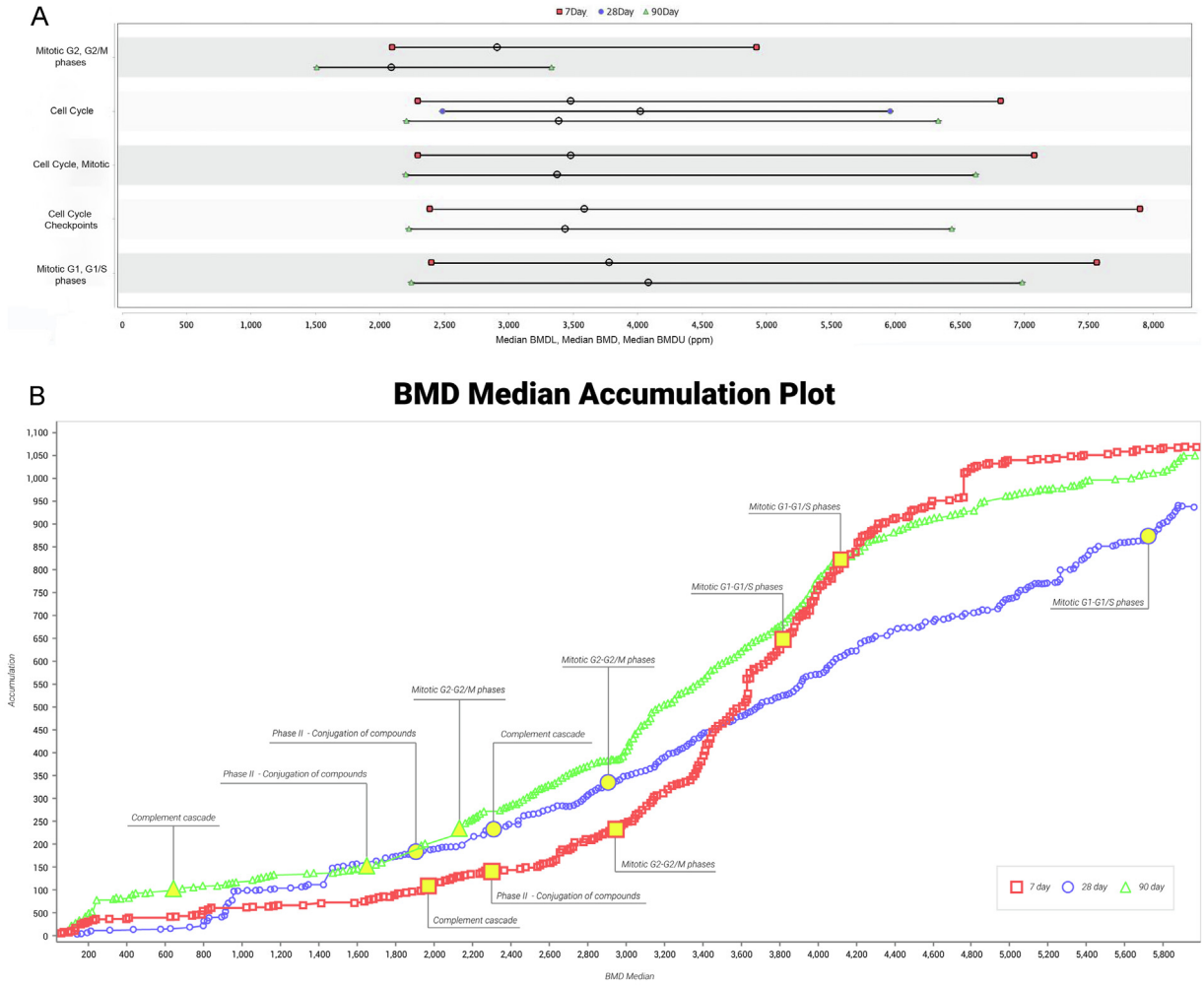

Fig. 3. BMDExpress analysis visualizations. A: Range plots for selected gene sets related to cell cycle. Data are shown for gene sets/timepoints with significant enrichment. B: Accumulation plot for all three timepoints, with select gene sets discussed herein annotated by text.

denced by Caspase 3 staining. While p53 signaling is known to be activated by DNA damage, it also can be activated by non-genotoxicants (Catizone et al., 2019). The individual genes driving the enrichment of p53-relevant pathways in the GSEA and BMDExpress analyses were regulators of apoptosis (e.g., *Bax*) and cytokines, without alteration to DNA repair enzymes nor the p53 gene itself. No individual genes for DNA damage repair enzymes were differentially expressed compared to controls at any dose or timepoint according to the DESeq2 analysis. This indicated that changes in cell cycle occurred in the high concentration group independent of DNA damage. This finding is corroborated by an overall negative profile for 1,4-dioxane in a set of HTS assays within the ToxCast/Tox21 database that are indicators of

DNA damage and/or repair (Hsieh et al., 2019). This finding aligns with the proposed MOA for 1,4-dioxane rodent hepatotoxicity involving cytotoxicity and subsequent regenerative hyperplasia (Dourson et al., 2017), as well as with the mitogenic response reported for the same liver tissues evaluated herein (Lafranconi et al., 2020)

Transcriptomics data can provide important information for proposing potential key events for an alternative MOA or supporting existing key events in established MOAs. Signaling on the molecular level can demonstrate or inform underlying mechanisms of toxicity. Transcriptomic responses following relatively short exposures that are transient may represent a non-specific adaptive and/or stress response (Dean et al., 2017). For example, after 7 days of exposure to 1,4-dioxane in the present study, there were many more DEGs than following 28 days of exposure at the 600 and 6000 ppm concentrations. However, there were very few enriched gene sets at the 7-day timepoint, for any exposure concentration. This indicates that the altered genes are not members of a cohesive signaling pathway and may represent a transient response to the exposure scenario. After 28 days of exposure, the majority of the DEGs at the 7-day timepoint had returned to levels similar to the time-matched controls for the 600 and 6000 ppm groups. While the 2000 ppm group had overall more DEGs at 28 days compared to either 7 and 90 days, most of the DEGs at 7 days were not differentially expressed at 28 days. Following a sub-chronic exposure duration of 90 days, transcriptomic response was increased at the 600 and 6000 ppm concentrations. A 28-day “sub-acute” timepoint has been used to identify liver chemical carcinogenicity signatures in experimental animals (Waters et al., 2003), while 90-day exposures have been suggested to accentuate gene expression changes related to the carcinogenic activity of chemicals (Auerbach et al., 2010). Notably, transcriptomic analysis in target tissue following exposure durations of 14 days or less in *in vivo* models has been shown to be predictive of non-DNA-reactive mechanisms in hepatic tumors (Fielden et al., 2007). In the present study, the transcriptomic profiles at three different exposure durations were absent of a gene expression signal for DNA damage response or repair.

In addition to the pathway level enrichment of phase II metabolism and an increase in mitotic cell cycle at high concentrations and later timepoints, reduced expression of genes involved in coagulation and complement cascade, as well as extra-cellular matrix regulation, was a significant and transient signal in the present study; this signal was normalized at 90 days at all concentrations except for 6000 ppm. Although the significance of this finding is not fully known, downregulation of coagulation cascade proteins in the livers of mice with hyperplasia-mediated liver regeneration has been previously demonstrated (Tatsumi et al., 2009).

Although alterations to nuclear receptors involved in xenobiotic metabolism represents a known molecular initiating event for some cases of chemically-induced hepatotoxicity and/or hepatocarcinogenicity, in particular those with increased proliferation, the only general nuclear receptor gene set included in the analysis presented herein (“BIOCARTA_NUCLEARRS_PATHWAY”) was not significantly enriched. Thus, individual CYP-encoding genes that are considered indicators of several common nuclear receptors known to play a role in rodent liver pathogenesis (aryl hydrocarbon receptor [AhR], constitutive androstane receptor [CAR], peroxisome proliferator-activated receptor [PPAR], and pregnane X receptor [PXR]) were reviewed for treatment effect. The CYP-encoding genes were not differentially expressed in any dose group or timepoint, with the exception of the PXR-related *Cyp3a11* (human homolog *CYP3A4,* Li et al., 2009), which was significantly up-regulated at 90 days in the 600 and 6000 ppm dose groups (Supplemental Table S1). The biological plausibility that PXR may be affected by 1,4-dioxane in mouse livers is supported by the fact that PXR regulates phase II conjugating enzymes. However; *Cyp3a11* was only significantly up-regulated at the highest dose at 90 days, while phase II metabolism pathways were up-regulated at early timepoints as well as at the 600 ppm concentration, indicating that the two expression changes may not be dependent upon one another. PXR, among other xenobiotic-metabolizing nuclear receptors, is known to be differentially expressed across species, leading to species-specific liver effects in rodents (Luisier et al., 2014; Yamada et al., 2015). While this result suggests the possibility that 1,4-dioxane exposure affects the PXR, further investigation beyond *Cyp3a11* mRNA level is necessary to confirm such a molecular event.

It should be noted that in the present study, due to the minimal treatment effect of 1,4-dioxane on gene expression at any dose or timepoint, liberal criteria were applied to identify DEGs and enriched signaling pathways. For example, no fold-change criterion was set for the identification of DEGs, and the use of the full complement of genes ranked by the Wald statistic for gene set enrichment rather than filtered by a significance cut-off was the approach emphasized herein (GSEA method as opposed to the hypergeometric test, with the exception of the DNA damage response analysis). These liberal criteria enabled identification of minimally altered genes and signaling networks and demonstrated that changes to signaling pathways were limited. Trends in changes to signaling pathways related to mechanisms of hepatoxicity and/or carcinogenesis were subtle and specific to high dose groups. This highlights the overall low effect of 1,4-dioxane on gene expression in the livers of mice, particularly at concentrations below 600 ppm. The results indicate that the threshold concentration for hepatic transcriptomic response to 1,4-dioxane in female mice, whether it be transient and/or adaptive or related to pathology, exists somewhere in the range of 600–2000 ppm.

In summary, the transcriptomic response in livers of mice exposed to 1,4-dioxane in a drinking water study demonstrates minimal treatment effects on global gene expression at concentrations below 600 ppm, with an increase in phase II metabolism and cellular cycle signaling in the absence of a significant increase in DNA damage response signaling at the mRNA level at 600 ppm and above. These findings align with the phenotypic findings of histopathological and biochemical analysis of the same liver tissues, and support the non-mutagenic, threshold-based mitogenic MOA for mouse liver tumors proposed by Lafranconi et al. (2020) based on all the study findings.

CRediT authorship contribution statement

G.A. Chappell: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing - original draft, Writing - review & editing. **M.M. Heintz:** Formal analysis, Writing - original draft. **L.C. Haws:** Funding acquisition, Supervision, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2021.01.003.

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