The Power of Resolution: Contextualized Understanding of Biological Responses to Liver Injury Chemicals Using High-throughput Transcriptomics and Benchmark Concentration Modeling

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ABSTRACT

Prediction of human response to chemical exposures is a major challenge in both pharmaceutical and toxicological research. Transcriptomics has been a powerful tool to explore chemical-biological interactions, however, limited throughput, high-costs, and complexity of transcriptomic interpretations have yielded numerous studies lacking sufficient experimental context for predictive application. To address these challenges, we have utilized a novel high-throughput transcriptomics (HTT) platform, TempO-Seq, to apply the interpretive power of concentration-response modeling with exposures to 24 reference compounds in both differentiated and non-differentiated human HepaRG cell cultures. Our goals were to (1) explore transcriptomic characteristics distinguishing liver injury compounds, (2) assess impacts of differentiation state of HepaRG cells on baseline and compound-induced responses (eg, metabolically-activated), and (3) identify and resolve reference biological-response pathways through benchmark concentration (BMC) modeling. Study data revealed the predictive utility of this approach to identify human liver injury compounds by their respective BMCs in relation to human internal exposure plasma concentrations, and effectively distinguished drug analogs with varied associations of human liver injury (eg, withdrawn therapeutics trovafloxacin and troglitazone). Impacts of cellular differentiation state (proliferated vs differentiated) were revealed on baseline drug metabolizing enzyme expression, hepatic receptor signaling, and responsiveness to metabolically-activated toxicants (eg, cyclophosphamide, benzo(a)pyrene, and aflatoxin B1). Finally, concentration-response modeling enabled efficient identification and resolution of plausibly-relevant biological-response pathways through their respective pathway-level BMCs. Taken together, these findings revealed HTT paired with differentiated *in vitro* liver models as an effective tool to model, explore, and interpret toxicological and pharmacological interactions.

Key words: high-throughput sequencing; HepaRG cells; S1500+ geneset; benchmark concentration analysis; toxicogenomics; liver injury.

Genomics has transformed our understanding of cellular and molecular pathways (Spellman *et al.*, 1998; Waters and Fostel, 2004). However, low throughputs have precluded thorough characterization of concentration-response and chronological effects, which are essential for mechanistic and predictive toxicology research (Aardema and MacGregor, 2002; Fielden and Zacharewski, 2001). Numerous transcriptomic studies have attempted to characterize liver injury in response to chemical exposures at the molecular level, but the complexities of biological-response dynamics (eg, concentration-dependence, temporal progression, and varied rates of hepatic metabolism to activate and/or detoxify chemicals) have largely confounded our ability to sufficiently capture these dynamics for predictive toxicology research.

Liver injury in humans persists as a leading cause of failed therapeutics (Kullak-Ublick *et al.*, 2017; Stevens and Baker, 2009), whereas chronic liver injury continues to be a prevalent cause of death in the USA (Health, 2017), and is linked to environmental factors (Al-Eryani *et al.*, 2015; Ramos-Lopez *et al.*, 2015; Stine and Chalasani, 2017). The importance of liver function in chemical metabolism has led to a revolution in the field of *in vitro* liver models toward more physiologically-relevant systems better suited to model the complexities of liver tissue and responses to chemical exposures (Grego *et al.*, 2017; Ramaiahgari *et al.*, 2014, 2017). Moreover, our understanding of the progression of cellular responses from adaptive, nontoxic responses toward chemical-induced injury remains largely unexplored.

The National Toxicology Program has pioneered the development of a novel transcriptomic gene set, the human Sentinel 1500+ (S1500+) (Mav *et al.*, 2018). The gene set was designed to leverage the sensitivity and throughput of *in vitro* systems to enable concentration-response modeling of transcriptomic biological-response space (House *et al.*, 2017; Merrick *et al.*, 2015). We have applied this gene set, which targets approximately 2700 unique human transcripts, using an emerging targeted Next Generation sequencing platform, Tempo-Seq (Yeakley *et al.*, 2017), to generate high-throughput transcriptomics (HTT) data. In this study, we investigated biological responses to a broad range of exposure concentrations with a reference set of 24 compounds over 3 independent experimental runs. Two culture configurations of HepaRG cells were assessed to explore the impact of “differentiated” (2D-DIFF) versus non-differentiated (PROLIF, proliferated during compound exposures) cultures. The goal was to assess the predictive utility of this focused set of approximately 2700 “sentinel” transcripts, paired with differentiated *in vitro* liver models, morphological assessments, and cytotoxicity assays. We investigated the utility to quantitatively identify liver injury compounds (eg, drug analogs), modeled metabolically-activated/attenuated responses to classical hepatic receptor modulators, characterized differences between 2D-DIFF and PROLIF HepaRG culture models, and identified/resolved biological-response pathways in context with human therapeutic or environmental exposures. The results revealed numerous examples where the power of resolution afforded in transcriptomic concentration-response modeling enabled contextualized characterization and interpretation of hepatic responses to chemical exposures.

MATERIALS AND METHODS

Cell culture. Cryopreserved HepaRG cells (Lonza, Catalog: NSHPRG) were thawed, and approximately 20 000 cells per well were seeded onto collagen(I)-coated 384-well plates (Corning,

Catalog: 356667) 10 days prior to compound exposures for recovery/differentiation with differentiated cultures (2D-DIFF). For proliferated/non-differentiated cultures (PROLIF), 2000 cells per well were seeded 3 days prior to chemical exposure. Cells were plated using Williams E medium (ThermoFisher, Catalog: A1217601) supplemented with plating additive (Lonza, Catalog: MHTAP), and 24 h later exchanged with William’s E medium supplemented with maintenance additive (Lonza, Catalog: MHPIT) for the duration of cultures.

Compound exposure. Compounds were procured from MRI Global (Kansas City, Missouri). Detailed information of the compounds is provided in [Supplementary Data 1](#). Chemicals were blinded to the laboratory personnel involved in compound exposures. Briefly, 500X concentrations of each compound were prepared in DMSO, with the exception of phenobarbital and acetaminophen, which were prepared in exposure medium due to aqueous solubility incompatible with dissolution in DMSO. Stock concentrations (500X) were serially diluted in 96-well plates for a total of 10 concentrations (half-log spacing). From these stocks, 2 μ l of each compound was stamped in a separate 96-well plate containing maintenance medium (Lonza, Catalog: MHPIT) using Vialflo semi-automated pipettor (Integra Biosciences, Catalog: 6031). Compound mixtures were subsequently mixed thoroughly, and 50 μ l was transferred to the culture plates containing cells. Media from each exposed culture well were exchanged after the initial 48 h of treatment, spent culture media were removed for lactate dehydrogenase (LDH) assays, and fresh exposure medium was added to the plates, as described above. The total chemical exposure period was 96 h prior to cell lysis for Tempo-Seq analysis. Three independent technical replicates per treatment group, and 3 independent experimental runs were performed in this study.

Cell morphology and cytotoxicity assessments. Phase contrast cell morphology photomicrographs from each cell culture well in the study were acquired pre- and post-exposures in 24 h intervals using Incucyte automated imager (Essen Biosciences, Catalog: 4647). Cell morphology photomicrographs are available at the link provided in [Supplementary Data 6](#) (<https://doi.org/10.22427/NTP-DATA-002-00058-0001-0000-6>, Accessed March 22, 2019). Briefly, plates were transferred to an Incucyte automated imager with environmental controls (37°C, 5% CO₂). The imager can capture photomicrographs from six 384-well plates in a single imaging batch. Cell viability was assessed by measuring LDH release from damaged cell membranes. Briefly, 20 μ l of the exposure media from each culture well were collected at 48 and 96 h post exposure and LDH release was measured using CytoTox-ONE reagent following the manufacturer’s protocol (Promega, Catalog: G7890).

Tempo-Seq analysis. At the conclusion of the 96 h compound exposure period, exposure media were removed and cells were washed once with 50 μ l phosphate-buffered saline (ThermoFisher, Catalog: 10010023) followed by addition of 20 μ l of 1X Tempo-Seq lysis buffer (Biospyder) to culture plates. Plates were then incubated for 15 min at room temperature and frozen at –80°C. Tempo-Seq analysis was performed as described previously, targeted for an sequencing depth of approximately 500 mapped read counts per transcript (Yeakley *et al.*, 2017).

BMDExpress 2.2 analysis of gene- and pathway-level benchmark concentrations. BMDExpress 2.2 is a robust, interactive and user-friendly update of BMDExpress software (Yang *et al.*, 2007), and

can be freely downloaded (Auerbach, 2017). This application was used to rapidly analyze over 26 million data points within this transcriptomics dataset. For this, our initial mapped read counts generated with the TempO-Seq data from each well were normalized by dividing by the total mapped read counts for each respective assay well, then multiplied by 10^6 (CPM normalized). Next, these data were floored with a threshold mapped read count of 5, log₂ transformed, and viewed in Principal Component Analysis analysis to remove distant outliers. These data were then annotated with exposure concentrations, and 2 forms of these data were created: (1) unfiltered for overt cytotoxicity and (2) filtered to remove overt cytotoxicity. This pre-processing code is available at: <https://github.com/NIEHS/TGMX0001>. These data were then imported into BMDEExpress 2.2 and analyzed for benchmark concentration (BMC) estimations. Here, transcript expression data were pre-filtered using the William's Trend Test with either a 2-fold cutoff (gene-level BMC thresholding for liver injury potential) or 1.5-fold cutoff for pathway-level analyses. Next, these pre-filtered data were fit to 8 different mathematical models (Hill, Power, Linear, Polynomial 2, Exponential 2, Exponential 3, Exponential 4, and Exponential 5) to determine the best fitting BMC value for each transcript using a 1 SD threshold from vehicle control levels within a given dataset (3 independent experimental runs/datasets for each culture model system). Data with fit P-values less than .0001 were excluded, as were data with highly uncertain BMC values (BMDU/BMDL > 40). These gene-level BMCs were automatically graphed in accumulation plots referenced throughout the manuscript to reflect the distribution and accumulation of BMCs across the range of concentrations examined. In these accumulation plots, the y-axis (ordinate) represents an integer-increasing number of BMCs (best fitting), and the x-axis represents the range of exposure concentrations examined, with each data point reflecting respective derived median BMCs, in nanomolar units. Gene-level BMCs (2-fold cutoff) were analyzed for the liver injury classification method (unfiltered for cytotoxicity). For pathway-level analyses, gene-level BMCs (1.5-fold cutoff, filtered to remove overt cytotoxicity) were functionally classified to identify biological-response pathways annotated within Gene Ontology (GO), Signaling Pathways (Reactome), CPDB, Hallmark, and other previously published pathway sets using BMDEExpress 2.2. Pathway-level BMCs were further filtered with a 3-genes passed all filter requirement, BMDL/BMDU < 40, and a pathway population of at least 10% to further reduce the "noise" within the data. These pathway-level outputs were then plotted automatically in BMDEExpress 2.2, along with other data exploration tools to interact with these data. Input and output files from these analyses are provided in data available at the link provided in [Supplementary Data 6](#). Data quality assessments included analysis of the total numbers of differentially expressed genes (DEGs), which were largely consistent across the 3 independent runs ([Supplementary Data 9](#)). Chemical treatment groups that induced stronger responses (higher numbers of DEGs) showed a greater overlap of DEGs compared to groups with weaker transcriptional responses. Evaluation of connectivity (measured based on Pearson Correlation Coefficient [PCC] of maximum gene fold-change) showed a better concordance across individual runs, suggesting a reproducible rank pattern of transcriptional responses.

Ingenuity pathway analysis. Normalized data (Williams Trend Test, $p < .05$ and fold change above 1.5-fold) from BMDEExpress 2.2 were exported and data for each chemical were uploaded to Ingenuity Pathway Analysis (IPA) software (Build: 470319M,

Qiagen). A 2-fold cutoff was used for IPA analysis. Concentration-related pathway comparisons were performed on IPA pathways with a significance threshold of $p < .05$. These differentially regulated pathways, with $-\log(p\text{-value})$, were exported into text files and transcriptional heatmaps were generated using Multiple Array Viewer (MEV) software (Howe et al., 2010).

Identification of a BMC threshold to quantitatively identify liver injury compounds. High-throughput transcriptomics data were fit to nonlinear regression models using BMDEExpress 2.2 as described above. For this analysis, gene-level HTT data were alternatively filtered with a 2-fold cutoff with no exclusions based on indications of cytotoxicity to enable identification of the ranges of compound exposures transitioning into higher, more injurious ranges. Based on these uniquely applied filters for compound-induced liver injury estimations, along with those described above (eg, CPM normalization, floor equal to 5 mapped read counts, log₂ transformation, outlier removal, >1 SD from vehicle control, BMDL/BMDU < 40), gene-level accumulation plots were generated and analyzed. An apparent relationship between the number of derived BMCs (ie, total BMCs as a marker of the propensity for BMC accumulation) and qualitative associations with liver injury was evident within BMC accumulation plots. A novel approach was developed to interpret these observations. First, the total number of BMCs was tabulated from accumulation plots for each of the 24 compounds across the 3 independent experimental runs with both 2D-DIFF and PROLIF culture modes ([Supplementary Data 2](#)). Next, BMC totals for chemicals rarely associated with liver injury, comprised of: potassium chloride, sucrose, aspirin, levofloxacin, phenobarbital, and caffeine, were analyzed across experimental runs (a total of 18 individual BMCs) for normality (Shapiro-Wilk W Goodness of Fit Test, JMP 13, SAS), and a standard deviation was calculated. A threshold of 3 SD from the mean BMC of these chemicals was determined to be 105 BMCs (BMC No.105) with 2D-DIFF cultures, which was subsequently used as a quantitative threshold to classify chemicals as having potential to cause liver injury and quantify the potencies at which liver injury potential may be of greater concern. A similar threshold of BMC No.59 was derived from PROLIF cultures.

RESULTS

High-throughput Transcriptomics as a Sensitive Marker to Quantitatively Distinguish Liver Injury Compounds

In this HTT study utilizing the S1500+ gene set with cryopreserved/differentiated HepaRG cultures (Jackson et al., 2016), our goal was to leverage the highly differentiated yet elastic properties of HepaRG (Cerec et al., 2007) to predict human responses to chemical exposures. To this end, transcriptomic responses were assayed from cultures exposed to 24 reference compounds ([Supplementary Data 1](#)) in both differentiated (2D-DIFF) and non-differentiated (PROLIF) HepaRG cultures, along with cell morphology photomicrographs captured from each culture well (pre-/post-exposures), and cytotoxicity (LDH leakage, 48 and 96 h). Transcriptomic data were analyzed by various approaches, including BMDEExpress 2.2 (Auerbach, 2017) to explore concentration-response relationships and profile responses to classical liver injury compounds, and those rarely associated with liver injury. Gene-level accumulation plots of identified BMCs (concentrations reflecting a statistically-significant concentration-related deviation of individual transcripts from baseline) are shown in [Figure 1A](#) (2D-DIFF). Here, BMCs are plotted sequentially (integers) as they accumulate at

each respective BMC potency along the range of exposure concentrations examined. These accumulation profiles readily distinguished liver injury compounds (eg, aflatoxin B1, tamoxifen, benzo(a)pyrene, chlorpromazine, troglitazone and trovafloxacin) from compounds rarely associated with liver injury (eg, caffeine, sucrose, and potassium chloride), based on their respective propensities to alter gene expression. This BMC visualization readily distinguished human clinical sensitivities to liver injury when comparing antibacterial drug analogs trovafloxacin (withdrawn from market) (Dalvie et al., 1997; Lubasch et al., 2000) to levofloxacin (prescribed and rarely associated with liver injury) (Figure 1B). Here, marked numbers of BMCs were revealed with trovafloxacin exposures ($\mu = 764$), whereas levofloxacin produced few BMCs ($\mu = 36$) up to 300 μM exposures. This was consistent with trends observed with higher-frequency liver injury compounds across the evaluated set of 24 reference chemicals. Given the therapeutic target of trovafloxacin and levofloxacin is antibacterial treatment of pneumonia (Noreddin and Elkhatab, 2010), these responses appear to be off-target effects on human liver transcription rather than blocking bacterial DNA gyrase and topoisomerase IV activities. Moreover, relating observed BMC totals to human peak plasma level concentrations (trovafloxacin: C_{max} approximately 5 μM , levofloxacin C_{max} approximately 13 μM), 5 μM trovafloxacin exposure produced approximately 218 gene-level BMCs (Figure 1B), whereas 13 μM levofloxacin produced on average 13 BMCs, consistent with the rarity of clinical reports of liver injury with levofloxacin at therapeutic concentrations (Gulen et al., 2015). A second drug analog comparison, troglitazone versus rosiglitazone, is shown in Figure 1C (Smith, 2003; Tanne, 2007). Although more subtle gene-level contrast was observed, HTT distinguished higher concentrations of troglitazone (withdrawn liver injury compound with C_{max} approximately 6.4 μM) from structurally-similar analog rosiglitazone (C_{max} approximately 1.7 μM). Accounting for their respective peak plasma levels, rosiglitazone would be identified to have lower potential for liver injury using this approach, which was further evident in observed cytotoxicity response with 300 μM troglitazone that was not observed with rosiglitazone after 96 h exposures (Figure 1D, Supplementary Data 3). Interestingly, both PPAR γ agonists produced relatively few BMCs (< 100) at concentrations approximating their respective C_{max} .

Gene-level accumulation plots were exported from BMDEpress 2.2, and further analyzed using a novel method to quantitatively derive a threshold of liver injury concern from BMC accumulation plots within this reference chemical dataset (Supplementary Data 1). First, BMC accumulation totals with 2D-DIFF HepaRG were compiled for 6 low-risk compounds (caffeine, sucrose, KCl, levofloxacin, phenobarbital, and aspirin), which have been rarely associated with liver injury, to define a threshold number of 105 BMCs (BMC No.105), as depicted by the horizontal line in Figure 1A. This value represented 3 SD above the mean BMC total for these low-risk compounds across 3 independent experimental runs, which corresponds to more than 99.7% of this normal distribution. Applying this threshold metric of transcriptomic response, compounds were ranked by their BMC totals to qualitatively identify liver injury compounds exceeding BMC No.105, and to quantitatively estimate their respective potencies at each respective threshold-crossing BMC (Supplementary Data 2). Fourteen out of the 24 evaluated compounds produced ≥ 105 BMCs within the range of concentrations examined. Established hepatotoxic compounds were largely distinguished from compounds rarely associated with hepatotoxicity, supporting the qualitative utility of this

approach. Moreover, derivations of BMC No.105 potencies, in context with therapeutic/environmental human plasma concentrations, enabled estimations of the likelihood for human liver injury (Sipes et al., 2017). For this, we calculated an index for compound-induced liver injury as the ratio of $10XC_{\text{max}}$ divided by each respective BMC No.105 value. These potency-driven translation metrics identified acetaminophen, troglitazone, ritonavir, menadione, rifampicin, and trovafloxacin as higher probability liver injury compounds based on their established therapeutic or environmental exposure ranges (Supplementary Data 2). Drugs such as omeprazole, tamoxifen, and chlorpromazine, or environmental compounds benzo(a)pyrene and aflatoxin B1 showed potential for liver injury in terms of the total number of BMCs observed with this approach. However, typical internal human plasma exposure levels produced $10XC_{\text{max}}/\text{BMC No.105}$ values less than 1. These would be interpreted to require higher than typical exposure levels, or enhanced individual susceptibility to result in to human liver injury. A $100XC_{\text{max}}$ factor translates to each of these compounds being classified as liver injury compounds with ratios greater than 1. Cyclophosphamide, valproic acid (VPA) and dimethylnitrosamine (DMN), which have known associations to liver injury, were not accurately identified with this approach. However, for cyclophosphamide and VPA, the maximum *in vitro* exposure level assessed in this study was 300 μM , which was below the typical 10X safety factor recommended for *in vitro* regulatory studies that account for uncertainty and concentrating effects in the liver (Prueksaritanont et al., 2013). For DMN, the semi-volatile nature of this compound may have impacted the exposure levels required to better model *in vivo* exposures.

Multiplexed LDH leakage assays from spent culture media (48 h, 96 h) and cell morphology evaluations (photomicrographs) from each culture well for 2D-DIFF and PROLIF models are provided (Supplementary Data 3, 4, and 6). No concentration-related LDH leakage was observed for any of the compounds rarely associated with liver injury in 2D-DIFF cultures after 96 h exposure (Figure 1D). Withdrawn drugs, troglitazone and trovafloxacin produced marked LDH release, whereas their respective comparator analogs rosiglitazone and levofloxacin showed no compound-related LDH leakage (Figure 1D) in the range of concentrations examined. Exposures to aflatoxin B1, B(a)P, tamoxifen, chlorpromazine, ritonavir, and menadione each led to detectable cytotoxicity, and displayed varied potencies (Figure 1D). Qualitative comparisons of these data with HTT-derived and ranked BMC totals revealed LDH leakage was specific to the highest ranked compounds after 48 h exposure. Intermediate, lower-incidence liver injury compounds such as rosiglitazone, rifampicin, omeprazole, CDCA, diphenhydramine were not cytotoxic after 48 h exposures, but with repeated exposures were moderately (approximately 2-fold) cytotoxic with rifampicin, omeprazole, and diphenhydramine (Supplementary Data 3). CDCA and rosiglitazone produced no clear LDH leakage under the conditions examined, but morphological signs of cellular stress/death were evident at the top exposure levels (Supplementary Data 4). Quantitatively, HTT-derived potencies for liver injury predictions appeared more potently sensitive than LDH leakage and cell morphology Lowest Observed Effect Level (LOELs). BMC No.105 concentrations were approximately 7- to 13-fold lower than LDH leakage potencies and morphology LOEL's, respectively (Supplementary Data 4).

Impact of Culture Configuration on Hepatocyte Functionality

Expression of drug metabolism enzymes is a well-established hallmark of hepatocyte differentiation (Olsavsky Goyak et al.,

Figure 1. Concentration-response accumulation plots of S1500+ (2978 transcripts) in 2D-DIFF HepaRG cultures using BMDExpress 2.2. A, Gene-level accumulation plots of 24 test chemicals used in the study. B, Gene-level accumulation plots of trovafoxacin and levofloxacin. C, Gene level-accumulation plots of troglitazone and rosiglitazone. Read counts from the TempO-Seq high-throughput transcriptomic analysis of 2978 transcripts were normalized, and benchmark concentration accumulation plots were derived using BMDExpress 2.2. X-axis represents the range of exposure concentrations examined, with each data point reflecting respective derived benchmark concentrations, in nanomolar units, and y-axis represents benchmark concentration accumulation (integer accumulation for each BMC). Data shown from one of 3 independent experimental runs and were representative of trends for each run. D, Lactate dehydrogenase (LDH) leakage data of 2D-DIFF HepaRG cells exposed to various chemicals for 96 h. X-axis represents concentration of the compound in micromolar units, and y-axis represents relative fluorescence units (RFU). Data are representative of 3 independent runs.

2010). Comparisons of metabolism-related transcriptomic profiles between untreated 2D-DIFF and PROLIF HepaRG (Figure 2A) for major drug metabolizing enzymes (eg, CYP3A4, CYP2C9, and CYP2C19) revealed constitutively elevated transcripts in 2D-DIFF versus PROLIF mode, consistent with their respective differentiation states and historically-observed functional activities. For example, CYP1A1, an isoform more highly expressed in

fetal liver (Baxter et al., 2015), was markedly higher (ie, 47X) in PROLIF versus 2D-DIFF cultures (Figure 2A). Analogous to adult and fetal liver tissue, CYP1A1 mRNA expression was further inducible in both culture modes by classical aryl hydrocarbon receptor (AhR) agonists including omeprazole (OMP) (Figure 2B). Fold-inducibility by OMP was somewhat lower with PROLIF versus 2D-DIFF (31- vs 54-fold), but PROLIF achieved maximum

Figure 2. Cell culture configuration impacts on hepatocyte functionality. A, Hierarchical clustering of genes associated with hepatocyte differentiation and xenobiotic metabolism pathways in 2D-DIFF and PROLIF HepaRG cultures; heatmap and clustering of genes were performed on Partek Genomics Suite 6.6. B, Omeprazole induced CYP1A1 gene induction in 2D-DIFF (blue) and PROLIF (black); x-axis represents concentration of omeprazole in micromolar, and y-axis represents average mapped read counts (TempO-Seq) of 3 replicates, and its standard deviation (SD). C, Omeprazole induced CYP3A4 gene induction in 2D-DIFF (blue) and PROLIF (black); x-axis represents concentration of omeprazole in nanomolar, and y-axis represents average mapped read count (TempO-Seq) of 3 replicates $\pm$ SD. Data are representative of 3 independent runs.

CYP1A1 mapped read count levels approximately 8X higher than 2D-DIFF cultures. These data were consistent with trends reported for adult versus developing human liver (Baxter *et al.*, 2015), and may reflect a lower proficiency for PROLIF HepaRG to metabolically clear OMP. Other metabolism-related transcripts such as PTGS1, GSTA2, SULT2A1, EPHX2, and NQO1 were also upregulated in proliferated mode, consistent with a less differentiated state (Figure 2A).

OMP is also bio-activated in human liver to a sulfone metabolite by CYP3A and CYP2C19. Here, OMP exposures with 2D-DIFF cultures elicited a markedly more efficacious CYP3A4 induction compared with PROLIF cultures, with approximately 12-fold lower CYP3A4 basal expression, and approximately 4X lower CYP3A4 inducibility (17.6-fold vs 65.6-fold). These findings were consistent with a diminished capacity of PROLIF cultures to bio-activate OMP to induce CYP3A metabolism and model this drug-induced feed-forward autoinduction mechanism. (Bertino *et al.*, 2012; Curi-Pedrosa *et al.*, 1994).

Metabolic activation is also known to play a fundamental role in chemotherapeutic effects of cyclophosphamide (El-Serafi *et al.*, 2015). Consistent with the trends observed with OMP, cyclophosphamide produced on average approximately 8X more BMCs (pathway-level as described below) in 2D-DIFF cultures compared to PROLIF (62.7 vs 7.67) across 3 experimental runs. Tabulated gene- and pathway-level BMC totals did not reveal this trend of enhanced sensitivity in 2D-DIFF cultures with other compounds requiring metabolic activation such as

acetaminophen, trovafloxacin, tamoxifen, diphenhydramine, or chlorpromazine. However, across the 14 chemicals crossing the liver injury estimation threshold, there was a clear shift (approximately 2X) in the average BMC No.105 (2D-DIFF) greater than each respective BMC No.59 (PROLIF) (Supplementary Data 2). LDH leakage and cell morphology LOELs revealed 2D-DIFF cultures modeled more pronounced responses to compounds associated with metabolic activation and liver injury (eg, chlorpromazine, diphenhydramine, menadione, omeprazole rifampicin, ritonavir, tamoxifen, troglitazone, and trovafloxacin). Interestingly, benzo(a)pyrene, activated by fetal liver enriched CYP1A1, showed a reversed trend in LDH leakage, which was more pronounced in PROLIF versus 2D-DIFF at the 48 h time point, and approximately 10X more potent for cell morphology LOEL (Supplementary Data 3 and 4).

Concentration-Response Modeling to Identify and Resolve Biological-Response Pathways

For HTT to effectively capture biologically-relevant responses to reference chemical exposures, *in vitro* cell culture systems must display adequate tissue-like functionality. A qualifying step for highly differentiated *in vitro* liver models is effective modeling of classical hepatic receptor signaling pathways controlling drug metabolism and clearance (Castell *et al.*, 2006; Zhang *et al.*, 2012). IPA revealed enriched activation of these prototypic reference pathways (Figure 3A) in response to prototype hepatic receptor agonists/activators with 2D-DIFF HepaRG. This was

Figure 3. Hepatic nuclear receptor pathway functionality in differentiated (2D-DIFF) HepaRG. A, Activation of classical nuclear receptor signaling pathways AhR, CAR, PXR, PPAR α , and FXR in 2D-DIFF HepaRG. $-\log(p\text{-value}) > 1.3$ (dotted line) represents significant activation (Ingenuity Pathway Analysis). B, Representative gene-level activation of hepatic nuclear receptors pathways with prototypical inducers omeprazole (AhR, CYP1A1); phenobarbital (CAR, CYP2B6); rifampicin (PXR, CYP3A4); fenofibric acid (PPAR α , CYP4A22); chenodeoxycholic acid (FXR, SLC51 β). Data are representative of 3 independent runs.

further confirmed by the induction of expression of sentinel transcripts CYP1A1 (AhR), CYP2B6 (CAR), CYP3A4 (PXR), SLC51 β (FXR), and CYP4A22 (PPAR α), analogous to liver tissue and primary human hepatocyte cultures (Figure 3B). Many of these reference transcripts are recommended for regulatory studies to predict clinical drug-drug interaction potential (Sipes et al., 2017). Comparisons of 2D-DIFF versus PROLIF culture mode (Figure 3B, Supplementary Data 5) revealed markedly higher dynamic range of responsiveness (fold-change) with CYP1A1 (AhR-OMP), CYP2B6 (CAR-PB), CYP3A4 (PXR-RIF), and SLC51 β (FXR-CDCA), with comparable responsiveness for CYP4A22 (PPAR α -FFA). These reference transcripts and pathway functionality responses indicate 2D-DIFF cultures better-supported toxicologically-important cell signaling pathways, which were consistent with the notable impacts of enhanced differentiation on baseline metabolism and cell morphology architecture.

Aflatoxin B1 and benzo(a)pyrene (B(a)P) are classical liver injury compounds associated with DNA damage, apoptosis, and cancer (Bedard and Massey, 2006; Li et al., 2001). These compounds were included to assess modeling of genotoxicity and liver injury via metabolic activation. IPA analysis of 2D-DIFF cultures revealed enriched pathways for cell cycle regulation, DNA damage, p53 signaling and cancer, consistent with their observed *in vivo* effects. Moreover, BMC analyses highlight plausibly-relevant BMCs consistent with a mechanism of genotoxicity with aflatoxin B1 and B(a)P (Figure 4A, Supplementary Data 7). Quantitative translation of these BMCs to human environmental exposures is challenging. However, estimates of aflatoxin B1 human serum concentrations range from 0.054 nM (non-contaminated diet, background liver injury rates) to 1.6 μM (exposures associated with liver injury) (Dhanasekaran et al., 2011), which bracket the observed BMCs in both culture modes. B(a)P was overall less potent than aflatoxin B1, consistent with their respective carcinogenic potencies (Wilson et al., 1995) and identified perturbation of KEGG “pathways in cancer” with mean BMCs of 290 nM (aflatoxin B1) versus 1.38 μM (B(a)P). PPAR α perturbation was also identified with these genotoxic agents (Figure 4C), and has been frequently associated with increased replicative DNA and liver tumors in mice (Peters et al., 1997), oxidative stress (Goel et al., 1986), cell cycle regulation and DNA damage (Peters et al., 1998; Tachibana et al., 2008).

However, the directionality of BMCs for genes within this pathway was not consistent with a classical PPAR α agonist mechanism. Coupled with the proximity of PPAR α BMCs to cellular stress/cytotoxicity, stress-induced attenuation of fatty acid metabolism may be operative (eg, suppression of CYP4A11 and FABP1). PXR and AhR pathway perturbations associated with metabolism of aflatoxin B1 and benzo(a)pyrene (Dohnal et al., 2014; Kim et al., 1998) were also identified (Figure 4C).

HTT data with exposures to aflatoxin B1 and B(a)P mirrored trends of cell morphology and LDH leakage data. In 2D-DIFF cultures, focal areas of cellular damage/repair (eg, stretched regions of epithelial cells in 2D-DIFF cultures) were observed (Figure 4D). Additionally, decreased numbers of attached cells were apparent with increasing concentrations of aflatoxin B1 and B(a)P, indicating cell stress/damage, cytotoxicity, and attenuated proliferation (PROLIF mode). These findings were consistent with identified biological-response pathways such as cell cycle, DNA damage, apoptosis, p53 modulation, and cancer (Figure 4B). LOELs of morphological changes for each culture well were documented (Supplementary Data 4), and generally far less potently sensitive indicators of compound-induced effects than HTT-derived BMCs (approximately 5–20X). BMCs derived from PROLIF cultures were somewhat more sensitive (approximately 2X for the 14 compounds with threshold BMCs) for these highlighted pathways, and similar to overall trends in BMC accumulation. These results indicate an enhanced sensitivity of the proliferating culture configuration of HepaRG cells to these reference DNA damaging agents. The overall pathway responsiveness was distinct between 2D-DIFF and PROLIF HepaRG models exposed to AFB1 and B(a)P. Hierarchical clustering of biological pathways largely separated the culture models, highlighting the differential activation of cell cycle and DNA damage check point, Nrf2 mediated oxidative stress pathway, interleukin signaling etc (Figure 4C).

Cyclophosphamide is a widely-prescribed chemotherapeutic agent requiring metabolic activation to a 4-hydroxy metabolite, which spontaneously tautomerizes and decomposes to the DNA alkylating phosphoramidate mustard, and toxic byproduct acrolein (Rodriguez-Antona and Ingelman-Sundberg, 2006). In this study, neither 2D-DIFF nor PROLIF HepaRG showed signs of cellular stress or cytotoxicity in the range of exposure

Figure 4. The power of concentration-response toxicogenomics. **A**, Heatmap of approximately 500 pathway (Ingenuity Pathway Analysis, IPA) responses of 24 chemicals calculated by comparing fold-change values (> 2-fold) to their respective vehicle controls. $-\log(p\text{-value})$ was displayed in the heatmap; $-\log(p\text{-value}) > 1.3$ ($p\text{-value} = .05$) was considered significantly altered pathway upon chemical exposure in 2D-DIFF HepaRG. Zoomed heatmap shows unsupervised clustering of compounds at their respective exposures that led to cell cycle and DNA damage responses. *Exposure concentrations with observed cytotoxicity were removed from the analysis. **B**, BMDExpress 2.2 visualization of GO biological processes in concentration-response. Stress signaling pathways associated with DNA damage and cell cycle with aflatoxin B1 and benzo(a)pyrene exposures are shown in the plot. X-axis represents concentrations of chemical in nanomolar, Y-axis represents accumulation rank of pathway-level benchmark concentrations. **C**, Hierarchical clustering of canonical pathway changes in 2D-DIFF and PROLIF HepaRG with aflatoxin B1 and benzo(a)pyrene exposures. $-\log(p\text{-value})$ are displayed in the heatmap; $-\log(p\text{-value}) > 1.3$ ($p\text{-value} = .05$) considered significantly altered. Zoomed heatmap shows unsupervised clustering of differentially regulated pathways in 2D-DIFF and PROLIF HepaRG. Data are representative of three independent runs. **D**, Representative photomicrographs of vehicle control cultures for 2D-DIFF (upper panel) and PROLIF (lower panel) HepaRG at the beginning and end of the compound exposure period, and with 1.5 μM aflatoxin B1. **E**, Accumulation plots from BMDExpress 2.2 showing cyclophosphamide induced Gene Ontology (GO) biological processes in 2D-DIFF and PROLIF HepaRG with increasing concentration. X-axis represents the range of exposure concentrations examined, with each data point reflecting respective derived median benchmark concentrations, in nanomolar units, and y-axis represents benchmark concentration accumulation (integer accumulation for each BMC).

concentrations examined (up to 300 μM , C_{max} approximately 120 μM) (Supplementary Data 3). However, BMC analysis (Figure 4E) revealed cyclophosphamide-induced perturbations in numerous cellular pathways with 2D-DIFF HepaRG that were

consistent with clinically-observed biological responses such as lipid/fatty acid metabolism, xenobiotic metabolism, CAR/PXR, cell cycle arrest, DNA damage/repair, p53 signaling, apoptosis, immune function, and hypoxia (El-Serafi et al., 2014). In

comparison to PROLIF cultures, far greater numbers of pathway-level BMCs were derived with 2D-DIFF cultures (Figure 4E), which was consistent with their respective competencies for CYP2B6-mediated metabolic activation, and counter to the relative sensitivities with aflatoxin B1 and B(a)P.

Menadione represents another liver injury compound that is associated with generation of reactive oxygen species and oxidative stress (Laux and Nel, 2001). Adverse outcomes in humans exposed to high concentrations of menadione include liver injury and glucose-6-phosphate dehydrogenase deficiency resulting in hemolytic anemia (Tetef et al., 1995). Pathway analysis using BMDExpress 2.2 revealed biological response processes related to these apical outcomes, with significantly perturbed GO Biological Processes including: cellular response to reactive oxygen species ($\text{BMC} = 8.77 \pm 0.832 \mu\text{M}$), response to oxidative stress ($\text{BMC} = 7.98 \pm 1.19 \mu\text{M}$), apoptotic process ($\text{BMC} = 8.68 \pm 2.59 \mu\text{M}$), signal transduction involved in DNA damage checkpoint ($\text{BMC} = 11.2 \pm 2.81 \mu\text{M}$), and glycolytic process through glucose-6-phosphate ($\text{BMC} = 4.26 \pm 0.389 \mu\text{M}$) (Figure 5A, Supplementary Data 7). IPA also identified similar pathway alterations (Figure 4A). Quantitative comparisons of pathway-level BMCs to reported therapeutic internal exposures (C_{max} approximately $0.2\text{--}7.4 \mu\text{M}$) indicate plausibly-relevant pathway potencies consistent with reports of human cancer patients undergoing clinical trials (Lim et al., 2005).

BMC Approach Distinguishes Drug Analogs and Associated Biological-Response Pathways

Comparison of drug analogs trovafloxacin versus levofloxacin, which have contrasting clinical adverse liver effects, revealed marked differences in the numbers and identities of significantly altered gene sets/pathways (BMC analysis), consistent with their respective therapeutic and associated off-target/unintended effects (Figure 5B, Supplementary Data 7). Anticipated pathways related to cellular injury were evident with trovafloxacin (withdrawn from the market due to liver injury), yet largely absent with lower-risk analog levofloxacin (Supplementary Data 7). A common progression across experimental runs emerged with increasing concentrations of trovafloxacin from seemingly benign pathways (eg, lipid hydroxylation) toward progressively more toxicological BMCs (oxidative stress, DNA damage, and cell death). PROLIF HepaRG produced far fewer BMCs than 2D-DIFF cultures in response to trovafloxacin exposures, but were significantly more potently sensitive (ie, approximately 2X left-shifted), analogous to the majority of liver injury compound exposures in this study. Comparisons of derived BMC potencies to human therapeutic exposure concentrations (trovafloxacin human C_{max} approximately $5.3 \mu\text{M}$) revealed responses with 2D-DIFF HepaRG largely on the order of this low micromolar range (Figure 5B). As the therapeutic action of these drug analogs is antibacterial, identification of the GO Biological Process pathway Antibacterial Humoral Response ($\text{BMC} = 5.11 \mu\text{M}$) may be relevant to therapeutic action of this class of drugs with known associations with immunomodulatory effects (Khan et al., 1998).

Troglitazone and rosiglitazone represent a second drug analog pair within this study. Both compounds are therapeutic targets for PPAR γ , with potential to exert direct effects on liver related to fatty acid metabolism/transport. As noted above, troglitazone was the more cytotoxic analog, consistent with its increased associations with liver injury (Lloyd et al., 2002; Racheck et al., 2009). IPA demonstrated highly similar pathway enrichment with these analogs (eg, PPAR signaling), except for the highest concentration of troglitazone, which was cytotoxic

(Supplementary Data 8). Surprisingly, in BMC analysis, although analogous pathways were observed, the relative potencies for identified PPAR γ pathways (2D-DIFF) showed troglitazone to be more potent (lower BMC) than rosiglitazone (Figure 6A). These relative potencies did not mirror their relative therapeutic target potencies observed in PPAR γ gene-reporter assays (troglitazone-500nM, rosiglitazone-18nM) (Camp et al., 2000). This apparent reversed order of pathway-level BMCs/potency could have been the result of differential sensitivities of troglitazone versus rosiglitazone to cellular stress/death (Figure 1A and Supplementary Data 3) (Lloyd et al., 2002). Figure 6B and Supplementary Data 7 highlight representative toxicological pathways (eg, DNA damage, apoptosis, response to toxic substances) consistent with the enhanced cytotoxicity of troglitazone, relative to rosiglitazone. Comparisons of these toxicologically-related BMCs to human C_{max} suggested most of these observed pathways would have relatively high likelihoods (Sipes et al., 2017) of human response with troglitazone ($\text{BMC}_{\text{Median}} < C_{\text{max}}$), and much lower potential for human response with rosiglitazone ($\text{BMC}_{\text{Median}} \gg C_{\text{max}}$, [approximately 60X]). Although confounding cellular stress/death may contribute to the differential potencies for PPAR γ , adipose is the classical therapeutic target tissue, and differential sensitivity of certain transcripts to PPAR γ agonists in liver, or the well-documented crosstalk between PPAR γ and other PPAR isoforms might also play a role (Larsen et al., 2003). Further analysis of individual transcripts making up various PPAR γ -annotated pathways (Figure 6C) revealed highly responsive transcripts FABP4 and ADIPOQ were, in fact, well-matched to the relative potencies between troglitazone and rosiglitazone observed in cell-based gene-reporter assays: rosiglitazone ($\text{EC}_{50} = 18 \text{ nM}$ versus $\text{BMC}_{\text{FABP4}} = 0.361 \text{ nM}$ and $\text{BMC}_{\text{ADIPOQ}} = 10.3 \text{ nM}$), and troglitazone ($\text{EC}_{50} = 550 \text{ nM}$ versus $\text{BMC}_{\text{FABP4}} = 13.1 \text{ nM}$ and $\text{BMC}_{\text{ADIPOQ}} = 489 \text{ nM}$) (Chen et al., 2017). These observations confirm our ability to identify biological-response pathways related to the therapeutic targets with these PPAR activators, while revealing important considerations for deriving median BMC values from historically-annotated gene sets with insufficient context (eg, concentration-response modeling) to resolve the true identities of biological-response pathways.

DISCUSSION

Biological systems, by design, are constantly interacting with xenobiotics (eg, nutrients, environmental chemicals, prescription drugs, etc), yet our understanding of biological responses to these exposures remains in its infancy. This is in large part due to our limited ability to characterize molecular-level dynamics and their quantitative relationships to human outcomes. Low throughputs of traditional 'omics' methods have impaired our ability to characterize and interpret biological responses to human exposures, and our continued use of de-differentiated *in vitro* systems poorly model the complexities of tissue functionality. In this report, we have addressed some of these challenges through integration of differentiated *in vitro* liver models with a novel HTT platform (TempO-Seq) and cellular health assessments. By leveraging the power of concentration-response modeling, we have identified liver injury compounds by their respective potencies (BMCs), characterized impacts of HepaRG differentiation state and identified/resolved chemically-altered biological-response pathways for improved toxicological interpretation.

A novel, data-driven approach was developed to identify liver injury compounds and their respective potencies of

Figure 5. High-throughput transcriptomic analyses showing pathway-level benchmark concentration (BMC) responses. A, Pathway analysis of menadione-associated Gene Ontology (GO) biological processes in 2D-DIFF HepaRG. B, Pathway analysis of trovafloxacin and levofloxacin exposure in 2D-DIFF and PROLIF HepaRG. Accumulation plots were generated from BMDEpress 2.2. X-axis represents the range of exposure concentrations examined, with each data point reflecting respective derived median BMCs, in nanomolar units, and y-axis represents BMC accumulation (integer accumulation for each BMC).

toxicological concern using gene-level BMC modeling. HTT data revealed a propensity to accumulate hundreds of BMCs with compounds historically associated with liver injury, which tracked with morphological changes and LDH leakage. Examples of this included drug analog comparisons where drugs withdrawn from the market were readily distinguished (Figs. 1B and 1C). Leveraging 6 reference compounds within the dataset that have been rarely associated with liver injury (caffeine, sucrose, KCl, levofloxacin, phenobarbital, and aspirin), a threshold was identified in both 2D-DIFF (BMC No.105) and PROLIF (BMC No.59) culture models to quantitatively interpret BMC accumulation profiles (Supplementary Data 2). Defining these thresholds enabled both qualitative estimation of liver injury potential, and approximation of the relative potencies for liver injury. Although *in vivo* BMCs for liver injury in humans are generally not known, the potency estimations here with 2D-DIFF cultures reasonably tracked with anticipated ranges for human liver injury. For example, the highest BMC No.105 was observed with acetaminophen (BMC = 845 μ M), which was consistent with the very high reported minimum lethal concentration in humans of 1056 μ M (Dollery, 1998). Withdrawn therapeutics troglitazone and trovafloxacin produced large numbers of BMCs overlapping with clinically-achieved plasma concentrations, whereas aflatoxin B1 or benzo(a)pyrene yielded very low BMC No.105 values consistent with highly potent liver toxicants. We have further extended this approach to calculate the likelihood of human liver injury (Sipes et al., 2017). Here we calculated chemical-induced liver injury indices as a ratio of 10X C_{max} divided by each respective BMC threshold (Supplementary Data 2). Using this approach, we identified trovafloxacin, troglitazone, acetaminophen, ritonavir, menadione, and rifampicin as higher likelihood liver injury compounds at typical human exposures. Extending this safety factor to 100X C_{max} identified all the evaluated human liver injury compounds in this study with the exception of DMN (semi-volatile), VPA (high C_{max}), and cyclophosphamide (high C_{max}). For these 3 compounds, *in vitro*

exposure concentrations were likely insufficient to apply this approach, given top concentrations examined were 300 μ M (DMSO soluble), and less than 10X C_{max} recommended in regulatory studies (Guidance, 2017). Further evaluation with larger sets of chemicals is warranted to qualify the utility of this approach.

Cellular differentiation and tissue-like functionality are critical aspects for predictive *in vitro* toxicology modeling. Normal biology for liver hepatocytes includes various hallmarks such as detoxification enzyme expression, nuclear receptor functionality, and polarized architectures with active transport. Leverage of HTT enabled broad characterization of molecular-level perturbations with a high degree of gene-level specificity. Direct comparison of differentiated (2D-DIFF) and non-differentiated (PROLIF) HepaRG was possible due to their unique ability (elasticity) to adapt to both highly differentiated and non-differentiated states. Our results revealed multiple examples of toxicologically-relevant impacts of varied differentiation state. As expected, expression of approximately 100 xenobiotic-metabolizing enzymes within the S1500+ gene set readily distinguished the 2 culture models (Figure 2A), with specific reference transcripts sensitive to hepatocyte differentiation tracking well with historical literature. Non-differentiated (PROLIF) cultures showed enrichment of developing/fetal liver transcripts CYP1A1 and SULT2A1, whereas 2D-DIFF cultures showed higher expression of CYP3A4, CYP2C9, SUTL1B1, NR1I3 (CAR), IGF1BP1 (Taub, 1996), prothrombin secreting F2, and complement C5 gene regulating lipid homeostasis (Supplementary Data 6—BMD analysis file) (Bavia et al., 2016). This elevated expression with 2D-DIFF corresponded well to the pronounced dependence on culture configuration for metabolically-activated cyclophosphamide (Figure 4E), and for omeprazole induction of CYP3A4 (Figure 2C). Moreover, the enhanced sensitivity of benzo(a)pyrene with PROLIF HepaRG was consistent with the elevated expression of CYP1A1. Interestingly, other metabolically-activated hepatotoxicants such as acetaminophen, aflatoxin B1, and

Figure 6. High-throughput transcriptomic analysis with BMDEExpress 2.2 identifies biological-response processes associated with chemical exposures. A, PPAR- γ pathway perturbation with exposures to troglitazone and rosiglitazone across concentration response in 3 independent runs. X-axis represents compound exposure concentrations in nanomolar units, and y-axis represents benchmark concentration accumulation (integer accumulation for each BMC). B, Toxicological pathways associated with troglitazone and rosiglitazone exposure in 2D-DIFF HepaRG across 3 independent runs. X-axis represents the range of exposure concentrations examined, with each data point reflecting respective derived median BMCs, in nanomolar units, and y-axis represents BMC accumulation (integer accumulation for each BMC). C, Expression levels of individual transcripts associated with PPAR γ pathway in troglitazone- and rosiglitazone-exposed 2D-DIFF HepaRG. X-axis represents the range of exposure concentrations examined, with each data point reflecting respective derived median BMCs, in nanomolar units, y-axis represents log₂ transformed mapped read count.

troglitazone appeared comparable or more potently sensitive in PROLIF cultures. These findings suggest that a sufficient degree of metabolic activation was operative in PROLIF cultures for these compounds, or that PROLIF cultures were inherently

sensitized analogous to proliferating/regenerating liver (Adachi et al., 1994). Duration of exposure (96 h) may also have contributed to these observations, given metabolic activation depends on metabolite accumulation (eg, troglitazone), and liver injury/

repair dynamics often require multiple weeks to detect liver enzyme leakage in humans. Acetaminophen, primarily metabolized by CYP2E1 to toxic metabolite NAPQI (Mazaleuskaya et al., 2015), showed little potency difference in estimated liver injury between the 2 culture models (Supplementary Data 6), consistent with their comparable mapped read counts of CYP2E1 expression. Aflatoxin B1, known to be activated by CYP3A metabolism to a genotoxic epoxide, was likely potentiated in proliferating HepaRG, which also contain 3 copies of the CYP3A gene locus (7q22) (Cerec et al., 2007). The mean BMC No.59 (PROLIF) was approximately 2-fold more potent with compounds exceeding these respective BMC thresholds BMC No.105 (2D-DIFF) values. LDH leakage assays further demonstrated PROLIF cultures to be more potently sensitive. Finally, in addition to the elevated basal expression of detoxification enzymes with 2D-DIFF HepaRG, enhanced modeling of major hepatic receptor signaling pathways (eg, CAR, PXR, PPAR) was also evident in response to classical liver enzyme inducers (Figure 3B, Supplementary Data 5). This was likely attributable to their superior cell-cell interaction networks in differentiated HepaRG, known to be critical for nuclear receptor function (Negishi, 2017).

A primary goal of this study was to employ the resolving power of concentration-response modeling to effectively identify reference biological-response pathways. Using both IPA and BMC modeling, we identified numerous examples of plausibly-relevant biological-response pathways that were consistent with reference chemical associations. Genotoxic agents altered DNA damage/repair, p53, apoptosis, and cell cycle pathways (Figure 4B); cyclophosphamide perturbed fatty acid metabolism, DNA damage, ER stress, immune response, and hypoxia (Figure 4E); menadione changed glucose-6-phosphate, oxidative stress, and apoptosis pathways; whereas both troglitazone and rosiglitazone altered PPAR γ signaling and lipid hydroxylation pathways at lower concentrations, generally progressing towards cell cycle, cellular stress, toxic response, and cellular death/apoptosis at higher concentrations (Figure 6). Although it is not possible to fully describe the pathway analyses here, the highlighted results demonstrate the effectiveness of this approach to screen chemicals for liver injury potential, while providing initial data to begin exploration of potential mechanisms of toxicity that leverage the discriminating context of concentration-response modeling. They also demonstrate challenges in applying historically-annotated gene sets across biological model systems. More research is needed to expand our understanding of cause-and-effect pathway relationships within differentiated models that build linkages between molecular-level responses and phenotypic outcomes.

Drug analog comparisons have been a highly effective means of conducting translational research in human pharmaceutical development. In this study, comparisons between trovafloxacin versus levofloxacin, and troglitazone versus rosiglitazone, highlighted the diagnostic utility of our HTT approach to distinguish chemically-similar, but biologically-distinct analogs. This suggests potential utility for read-across efforts. In each case, the identities and relative potencies of liver injury compounds were resolved, whereas gene and pathway perturbations revealed plausible associations to anticipated human pharmacology, off-target, and toxicological effects.

The impact of this investigation has multiple implications for the scientific community. First, the approach with HepaRG cells and HTT (S1500+) enables highly informative toxicology screening. Using a consistent genetic background for year-over-year screening, the method empowers highly efficient

surveying of concentration-response relationships that characterize biological-response space with sufficient specificity to apply gene-level interpretation. These interpretations include both qualitative and quantitative predictions of liver injury potential, and characterization of the progressions of pathway response through BMC modeling and IPA. Our confidence with these emerging approaches will continue to grow through expansion of reference chemical coverage, phenotypic anchoring (eg, pathology-type imaging over time) to explore cause-and-effect relationships, and coverage of additional tissue models with enhanced complexity.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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