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HYPHOTHESIS REVIEW

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Transcriptomic effect marker patterns of genotoxins – a comparative study with literature data

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Abstract

Microarray approaches are frequently used experimental tools which have proven their value for example in the characterization of the molecular mode of action of toxicologically relevant compounds. In a regulatory context, omics techniques are still not routinely used, amongst others due to lacking standardization in experimental setup and data processing, and also due to issues with the definition of adversity. In order to exemplarily determine whether consensus transcript biomarker signatures for a certain toxicological endpoint can be derived from published microarray datasets, we here compared transcriptome data from human HepaRG hepatocarcinoma cells treated with different genotoxins, based on re-analyzed datasets extracted from the literature. Comparison of the resulting data show that even with similarly-acting compounds in the same cell line, considerable variation was observed with respect to the numbers and identities of differentially expressed genes. Greater concordance was observed when considering the whole data sets and biological functions associated with the genes affected. The present results highlight difficulties and possibilities in inter-experiment comparisons of omics data and underpin the need for future efforts towards improved standardization to facilitate the use of omics data in risk assessment. Existing omics datasets may nonetheless prove valuable in establishing biological context information essential for the development of adverse outcome pathways.

KEYWORDS

aflatoxin, benzo[a]pyrene, biomarker, gene expression, HepaRG cells, hepatotoxicity, microarray, toxicogenomics

Abbreviations: 2NF, 2-nitrofluorene; AB1, aflatoxin B1; AHR, aryl hydrocarbon receptor; BaP, benzo[a]pyrene; CYC, cyclophosphamide; DAT, 2,4-diaminotoluene; DEG, differentially expressed gene; DMN, dimethylnitrosamine; FC, fold change; HQO, hydroquinone; IQ, 2-amino-3-methylimidazo(4,5-f)quinolone; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline; NMP, N-nitrosomorpholine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PCA, principle component analysis; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; TAF, 2-acetylaminofluorene.

1 | INTRODUCTION

Mutations in key genes encoding proteins involved in the regulation of cell proliferation, survival, and apoptosis constitute important hallmarks in the development of cancer (Hanahan & Weinberg, 2000, 2011). Gene mutations may, for example, be caused by genotoxic compounds which are able to form covalent DNA adducts, often following metabolic activation (Lutz, 1990; Phillips & Arlt, 2009). Carcinogenicity of compounds is generally assessed in long-term rodent bioassays in vivo, while genotoxicity is routinely analyzed by one or more established in vitro tests using either bacterial or mammalian cell systems, followed by appropriate in vivo tests (Corvi & Madia, 2017). On the one hand, rodent carcinogenicity studies have been criticized due to ethical issues, time and effort, as well as possible inter-species differences impeding extrapolation to humans (Doe et al., 2019; Gottmann, Kramer, Pfahringer, & Helma, 2001; Maronpot, Flake, & Huff, 2004), and there is a need to reduce animal testing in the context of the 3R principle. On the other hand, predictivity of established in vitro genotoxicity tests has also been questioned (Cox, Fellows, Hashizume, & White, 2016; Fowler et al., 2012; Nesslany, 2017).

Omics analyses have been extensively used in the past years to characterize the toxicological profile of various chemical entities using both, samples from in vivo and in vitro studies. This holds especially true for transcriptomics, whereas proteomic or metabolomics studies are less frequently conducted. While not considered core components of regulatory test batteries, such omics analyses may deliver substantial information about the mechanisms of toxicological action of a given test compound. Thereby omics data can substantially contribute to the development of so-called adverse outcome pathways (AOPs), which causally connect molecular events with resulting effects at the cellular and organismal level; e.g. see Vinken (2015). Harmonization and standardization of omics analysis platforms and data processing techniques have been identified as major hurdles for regulatory acceptance of omics approaches, together with the issue of a missing adversity definition when interpreting the results from omics analyses (Marx-Stoelting et al., 2015). Numerous publications have successfully demonstrated that transcriptomics are suitable to distinguish genotoxic from non-genotoxic carcinogens (Lee et al., 2013; Magkoufopoulou, Claessen, Jennen, Kleinjans, & van Delft, 2011; Mathijs et al., 2009). Additional effort has been undertaken to use omics data to define specific biological functions or pathways which are connected to cellular responses to genotoxins, often using liver cells as a model system due to the need of metabolic activation of many genotoxic compounds: for example, deregulation of genes related to cell cycle and proliferation (Luckert, Hessel, Lenze, & Lampen, 2015; Rieswijk et al., 2016), apoptosis and cell death (Luckert et al., 2015; van Kesteren et al., 2011), and/or cellular and oxidative stress (Deferme, Wolters, Claessen, Briede, & Kleinjans, 2015; Smit, Souza, Jennen, Kleinjans, & van den Beucken, 2017) have been reported in vivo and in vitro.

Using data from transcriptomic analyses, it might be possible to establish biomarker sets and/or transcriptional networks for genotoxicity. These could be, most likely, sets of specific transcripts or computationally determined underlying biochemical functions, rather than individual transcripts, due to the fact that a single gene or transcript will most likely not provide substantial specificity for a certain toxicological mechanism (Braeuning & Lampen, 2017).

A plethora of data on omics analyses from biological systems exposed to genotoxic chemicals has been published. Based on the assumption that marker transcript patterns typical for genotoxic stress can be deduced from such data, we now systematically re-analyzed data from published studies with different model genotoxins, focused on published studies with the well-established and frequently-used state-of-the-art human hepatocyte model HepaRG. Results show considerable differences between individual experiments and compounds, thus demonstrating the necessity of further standardization of omics analyses in order to gain comparable results potentially suitable for regulatory use. Greater concordance at the level of bioinformatically deduced functional changes in genotoxin-treated cells nonetheless point towards the value of published datasets for the identification of underlying biochemical changes and molecular events, thus facilitating the understanding of molecular toxicity mechanisms and subsequent AOP development.

2 | METHODS

2.1 | Literature search

Criteria for literature research were set up in order to identify relevant publications dealing with transcriptomic responses of liver cells upon genotoxic insult. The human hepatoma cell line HepaRG constitutes a well-established in vitro model for the liver and expresses, in contrast to most other hepatoma cell lines available, high levels of xenobiotic-metabolizing phase I and II enzymes important for the metabolic activation of pro-carcinogenic compounds. As a permanent cell line, HepaRG furthermore provide increased reproducibility and robustness, as compared to primary cells. Therefore, literature search was focused on work with HepaRG cells. The databases of the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) were screened using the search term "HepaRG" in combination with the following search terms: "gene expression" AND "genotoxic", "gene expression" AND "carcinogenic", "gene expression" AND "carcinogen", "gene expression profile", "gene expression signatures", "gene expression pattern", "gene expression marker", "gene expression fingerprint", "genotoxic carcinogens", "microarray", or "RNA-sequencing". The identified studies were checked for the availability of microarray raw data. In case no raw data were available, the published lists of differentially expressed genes (DEGs) were used.

2.2 | Data processing

Depending on availability, the raw data of the transcriptome analyses were downloaded from repositories where the respective datasets are archived. In case raw data were not accessible, lists of differentially

expressed genes provided in the respective publications were used instead. Additional information was retrieved about the conditions of incubation of HepaRG cells with respect to growth and differentiation medium, additives, presence of solvents, and duration of exposure to genotoxic agents.

Available raw data was used to compile differentially expressed gene lists. For this purpose, the web-based application GEO2R (www.ncbi.nlm.nih.gov/geo/geo2r/) was used. A common data analysis procedure was applied with the following cutoff values for statistical significance and fold change (FC): adjusted p-value <0.05, and $|\log_2 FC| \geq 0.5$. For one publication (Jennen et al., 2010) no GEO2R access is available, but raw data have been deposited in EBI's ArrayExpress. Here, the raw data were analyzed in R with the same criteria as for GEO2R. For two publications (Dumont et al., 2010; Josse, Dumont, Fautrel, Robin, & Guillouzo, 2012) no raw data was available. Here, the lists of differentially expressed genes from the papers with cutoff values of $p < 0.01$ and $|FC \geq 1.5|$ were used. Hierarchical clustering and heat-map visualization was performed using Morpheus software (www.software.broadinstitute.org/morpheus). Principal component analysis was performed using ClustVis (Metsalu & Vilo, 2015).

3 | RESULTS

3.1 | Literature search for HepaRG transcriptomics with genotoxic compounds

Using the search strategy as outlined in the Methods section, 9 different studies published between 2010 and 2018 were identified. Of these, 7 studies provided raw data or DEG lists and thus appeared suited for further analysis (Ates et al., 2018; Corton, Williams, & Yauk, 2018; Doktorova et al., 2013; Doktorova et al., 2014; Dumont et al., 2010; Jennen et al., 2010; Jetten, Kleinjans, Claessen, Chesné, & van Delft, 2013; Josse et al., 2012; Tryndyak et al., 2018). A list of these publications and important basic information about the studies and approaches is presented in Table 1. Affymetrix microarrays had been used in 6 out of 9 studies, while the other studies were based on Agilent arrays. Incubation times of HepaRG cells with the genotoxic compounds mostly ranged between 24 h and 72 h, with one remarkable exception where treatment had been performed for 28 days. The two genotoxins studied most were aflatoxin B1 (AB1; present in 7 out of 9 studies) and benzo[a]pyrene (BaP; present in 6 out of 9 studies). Other more rarely studied genotoxic compounds were 2-nitrofluorene (2NF), 2,4-diaminotoluene (DAT), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), dimethylnitrosamine (DMN), cyclophosphamide (CYC), 2-acetylaminofluorene (TAF), N-nitrosomorpholine (NMP), 2-amino-3-methylimidazo(4,5-f)quinolone (IQ), hydroquinone (HQO), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx). All those compounds were investigated in only 1 to 3 out of the 9 identified studies. For 2 out of the 9 studies, no microarray data were available. Raw data could be retrieved for 4 studies, DEG lists were extracted from 2 papers, and one publication had utilized the data from another study (Table 1). A more detailed overview of the different datasets available is presented in Table 2, where also individual compound concentrations and incubation times are listed together with a one- or two-letter identifier code assigned to the individual datasets for this work. Please note that some publications contained more than one dataset per compound, e.g. if different concentrations or incubation times had been used. The applied concentrations were mostly in the low micromolar range, with only a few exceptions such as NNK (7500 μM) or CYC (700 μM). Thus, in summary, a collection of datasets was compiled which were all derived from the same cell line

TABLE 1 Published literature for gene expression analysis in HepaRG cells treated with genotoxic compounds

1st author	Year	Array data	Microarray	Time	AB1 [μM]	BaP [μM]	Further toxins
Ates	2018	Not available	Affymetrix U133 Plus 2.0	72 h	2.5	5	2NF, CYC, DMN, NNK, DAT
Corton	2018	See Doktorova et al. (2013)	Affymetrix U133 Plus 2.0	72 h	2.5	5	CYC, 2NF, NNK
Doktorova	2014	GEO acc. no. GSE48990	Affymetrix U133 Plus 2.0	24/72 h	-	-	NMP, HQO, TAF, IQ
Doktorova	2013	GEO acc. no. GSE40117	Affymetrix U133 Plus 2.0	72 h	2.5	5	CYC, 2NF, NNK
Dumont	2010	DEG list available from paper	Agilent 4 × 44 K	24 h/28 d	-	-	MeIQx, PhIP
Jennen	2010	ArrayExpress acc. no. E-MEXP-2458	Affymetrix U133 Plus 2.0	12/48 h	3	10	-
Jetten	2013	Not available	Affymetrix U133A Plus 2.0	24 h	2.5	5	-
Josse	2012	DEG list available in paper	Agilent 4 × 44 K	24 h	0.05/0.25	-	-
Tryndyak	2018	GEO acc. No. GSE87028/ GSE117476	Agilent 8 × 60 K	72 h	5	2	-

TABLE 2 Published HepaRG treatments with genotoxic compounds, incubation details and given identifiers for individual datasets

Authors	Source	Compound	Incubation time	Concentration [μM]	Identifier
Doktorova et al. (2013)	GEO GSE40117	AB1	72 h	2.5	a
		BaP	72 h	5	b
		CYC	72 h	700	c
		NNK	72 h	7500	d
		2NF	72 h	18	e
Doktorova et al. (2014)	GEO GSE48990	IQ	24 h	3	f
		IQ	72 h	3	g
		HQO	24 h	150	h
		HQO	72 h	150	i
		NMP	24 h	216	j
		NMP	72 h	216	k
		TAF	24 h	40.8	l
		TAF	72 h	40.8	m
		BaP	24 h	9.51	aa
		BaP	72 h	9.51	ab
		BaP	24 h	17.44	ac
		BaP	72 h	17.44	ad
		BaP	24 h	5.15	ae
		BaP	72 h	5.15	af
Dumont et al. (2010)	published list in paper	PhiP	24 h	10	n
		MeIQx	24 h	10	o
		PhiP	28 d	10	p
		MeIQx	28 d	10	q
Jennen et al. (2010)	E-MEXP-2458	AB1	12 h	3	r
		BaP	12 h	10	s
		AB1	48 h	3	t
		BaP	48 h	10	u
Josse et al. (2012)	published list in paper	AB1	24 h	0.05	v
		AB1	24 h	0.25	w
Tryndyak et al. (2018)	GEO GSE87028	AB1	72 h	5	x
		AB1	72 h	25	y
	GEO GSE117476	BaP	72 h	2	z

exposed to various genotoxic carcinogens, mostly for similar incubation times and at comparable concentrations, and (to the degree possible) analyzed using the same cutoff criteria.

3.2 | Variations in cell culture conditions

Table 3 gives an overview of medium additives and differentiation conditions used in the 9 identified studies. Most authors either used differentiated cells as commercially provided by Biopredic, or followed the protocol by Gripon et al. (2002). Available details on culture conditions, however, differ considerably between individual publications, and therefore the available information is not suited to systematically connect the experimental outcome to specific conditions of cell differentiation or treatment.

3.3 | High variability of the yield of differentially expressed genes

Based on the frequent use of AB1 or BaP as model genotoxic compound in HepaRG cells, these two compounds were chosen for further in-depth analysis of transcriptomic effects and their comparability between different experiments. For AB1, 7 datasets from 4 individual publications were available, while for BaP 10 datasets from 4 individual publications were included in the analysis (Table 2). When inspecting the number of DEGs derived from the individual datasets using identical data processing, pronounced differences became obvious: the maximum number of 8764 DEGs was yielded with a dataset from Tryndyak et al. (2018) with 25 μM AB1 and 72 h of incubation, whereas 0 DEGs were obtained using data from Jennen et al. (2010) with 3 μM AB1 and 12 h of incubation

TABLE 3 Medium and additives used in HepaRG maintenance

Author	Used medium and additives	Differentiation DMSO/time	Treatment medium
Ates 2018	Differentiated cells from Biopredic		no details
Corton 2018	See Doktorova et al. (2013)		no details
Doktorova 2014	Acc. to Gripon et al. (2002)	2% /7 days	no details
Doktorova 2013	Acc. to Gripon et al. (2002)	2% /7 days	no details
Dumont 2010	Acc. to Gripon et al. (2002) + 2 mM glutamine	2% /14 days	10% FCS and 2% DMSO
Jennen 2010	Differentiated cells from Biopredic		DMSO-containing medium +0.5% DMSO
Jetten 2013	Differentiated cells from Biopredic		DMSO-containing medium +0.5% DMSO
Josse 2012	Acc. to Gripon et al. (2002) + 2 mM glutamine	2% /14 days	FCS-free, final DMSO concentration 0.1%
Tryndyak 2018	William's E with growth additives and differentiation supplement from Biopredic		final DMSO concentration 0.5%

(Figure 1A). No clear-cut correlation of the numbers of DEGs and incubation times or applied concentrations was visible, even though a general trend for more DEGs after longer exposure to higher concentrations may exist (Figure 1A). A similar picture was obtained for BaP, with the highest number of DEGs obtained with a dataset from Doktorova et al. (2014) with 17.44 μM BaP and 72 h of incubation (Figure 1B). By contrast, 2 datasets from Jennen et al. (2010) and Tryndyak et al. (2018), with 10 μM BaP and 48 h of incubation, or with 2 μM BaP and 72 h of incubation, respectively, yielded 0 DEGs. Here, the tendency for higher numbers of DEGs following longer exposure to higher concentrations of BaP was clearer as for AB1. A considerable number of datasets in the BaP analysis (6 out of 10) were derived from one individual paper (Doktorova et al., 2014). This could mean that inter-experiment variability might not be fully reflected in this analysis; however, the latter data were derived from an inter-laboratory reproducibility study. DEG numbers for the other genotoxins are presented in Supplementary Figure S1. In summary, the analysis revealed considerable variability of the yield of DEGs obtained with the same compounds in the same cell line.

3.4 | Limited concordance of individual transcripts regulated by different genotoxins

In order to check whether it is possible to identify relevant consensus genes regulated by the different genotoxins with high reliability, the top genes showing responses to AB1 and/or BaP in most of the available datasets were identified. These are listed in Table 4 (AB1) and Table 5 (BaP), respectively. Please note that datasets not yielding any genes with statistically significant regulation were not included in the tables. Only 5 genes were commonly regulated by AB1 in 6 datasets (*ABCA12*, *ENC1*, *GPR87*, *NECTIN4*, *PRKAG2*; Table 4), while another 25 genes were regulated in 5 of the AB1 datasets (Table 4). The direction of regulation was always identical for all datasets, indicating robustness of the top DEGs. A single gene (*CYP1B1*; Table 5) was identified as regulated in 8 datasets obtained after BaP treatment of HepaRG cells, while another 37 genes were identified as regulated in 6 or 7 BaP datasets (Table 5). Again, the direction of regulation was again was always identical for all datasets. The overlap between the 30 (AB1) and 38 (BaP) genes amounted to only 3 genes (*ABCA12*, *LACC1*, *E2F7*). Thus, while indeed similarities exist for the different datasets obtained with a certain compound, comparison of the data at the DEG level resulted only in very limited similarities between the responses to the two genotoxins.

3.5 | Common cellular functions affected by genotoxins

More similarity was observed when gene functions of the top-regulated transcripts were considered: both, the AB1 and the BaP top gene lists, contained several genotoxin-regulated transcripts linked to proliferation and cell cycle (e.g., AB1: *SERTAD1*, *PCNA*, *CCNE2*, *E2F7*; BaP: *GAREM1*, *E2F7*, *VWC7*, *GPC6*), cell adhesion and extracellular matrix interaction (e.g., AB1: *NECTIN4*, *VSIG1*, *COL17A1*, *PLXNC1*, *ADGRB2*; BaP: *COL5A2*, *COL8A1*, *CD44*, *VCAN*, *NRXN3*), as well as inflammation and oxidative stress response (e.g., AB1: *ENC1*, *LACC1*, *TREM2*; BaP: *CXCL10*, *CXCL13*, *LACC1*, *TNFRSF11B*). Calcium signaling

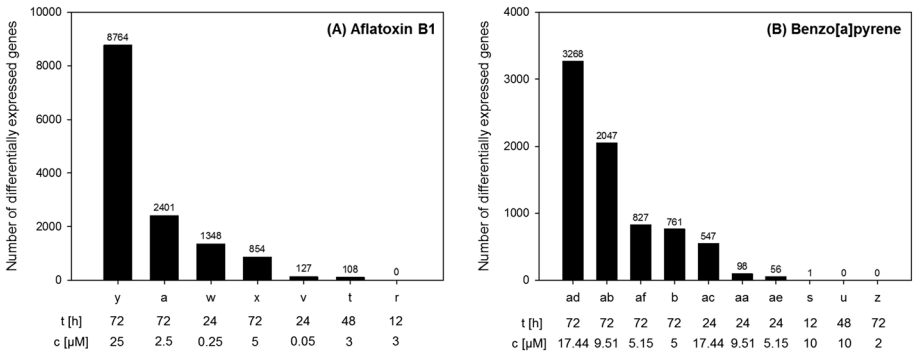

FIGURE 1 Numbers of differentially expressed genes obtained from re-analysis of published microarray data sets from human HepaRG hepatocarcinoma cells exposed to either aflatoxin B1 (A) or benzo[a]pyrene (B). Duration and concentration of treatment are indicated. For assignment of the individual data set identifiers please refer to Table 2

TABLE 4 Most often and strongest differentially expressed genes affected by aflatoxin B1

No	Gene name	Log_2 FC						Regulation
		a	t	v	w	x	y	
1	*ABCA12*	3.30	2.68	1.67	2.31	1.89	1.07	↑
2	*ENC1*	1.79	2.12	1.81	2.56	1.10	2.77	↑
3	*GPR87*	2.01	3.23	1.57	2.16	18.47	27.80	↑
4	*NECTIN4*	3.64	1.68	1.92	3.72	2.51	4.91	↑
5	*PRKAG2*	−1.16	−0.93	−1.62	−2.84	−1.45	−2.69	↓
6	*VSIG1*	2.28	1.32	2.19	3.75	-	2.15	↑
7	*SERTAD1*	1.79	0.92	1.53	-	0.67	1.98	↑
8	*PCNA*	1.38	1.44	1.54	-	4.58	8.78	↑
9	*GLIPR2*	1.73	0.57	-	1.94	2.68	6.43	↑
10	*CCNE2*	1.57	1.48	-	2.19	4.36	8.89	↑
11	*LACC1*	1.24	2.28	-	1.96	0.78	2.31	↑
12	*PFKP*	1.17	0.95	-	1.79	4.88	9.00	↑
13	*CCDC34*	0.89	0.88	-	1.59	1.59	6.07	↑
14	*SLC38A4*	−2.62	−0.92	-	−2.55	−0.97	−1.34	↓
15	*DNAJB2*	1.59	0.70	-	1.94	1.74	3.21	↑
16	*FOXF1*	1.86	1.12	-	2.01	1.92	21.78	↑
17	*GRIN2C*	0.85	-	1.69	2.69	1.80	3.10	↑
18	*TMEM40*	2.02	-	2.33	2.11	1.71	4.66	↑
19	*COL17A1*	1.66	-	4.32	6.07	3.89	6.49	↑
20	*DSE*	1.62	-	1.56	1.89	1.29	1.89	↑
21	*PLXNC1*	−0.61	-	−1.70	−2.67	−1.58	−3.23	↓
22	*E2F7*	1.67	-	2.40	4.12	3.27	9.02	↑
23	*TREM2*	4.10	-	2.47	4.50	4.80	6.96	↑
24	*ADGRB2*	0.67	-	1.57	2.23	3.13	6.62	↑
25	*DRD5*	2.21	-	2.82	7.30	8.05	12.77	↑
26	*DRAXIN*	4.05	-	3.48	6.33	1.85	3.69	↑
27	*ITPR1*	−0.53	-	−1.70	−2.12	−0.84	−4.71	↓
28	*LAMP3*	3.88	-	3.78	3.90	3.00	4.89	↑
29	*KANK3*	2.47	-	1.82	2.93	1.98	4.23	↑
30	*FXYD3*	0.94	-	2.95	3.30	6.61	12.02	↑

and calcium-dependent processes appeared affected especially by BaP but probably less by AB1 (e.g., BaP: *MCTP1*, *GPM6A*, *CADPS2*, *GPC6*; AB1: *ITPR1*). In addition, several genes from both lists contained various genes related to developmental processes, tumor growth, and cell differentiation (Tables 4, 5). A pronounced aryl hydrocarbon receptor (AHR)-dependent response was visible from the BaP top-regulated genes, where several target genes of that receptor were listed (*CYP1B1*, *CYP1A2*, *CYP1A2*, *ALDH3A1*; Table 5). This was not surprising since BaP is a known activator of AHR (Nebert, Dalton, Okey, & Gonzalez, 2004). As expected, the strong AHR-dependent response was not visible in the top genes regulated by AB1 (Table 4). In summary, consideration of affected biological functions provided evidence for considerable similarity of responses to different genotoxins irrespective of marked differences between the identities of individual genes regulated by these compounds.

3.6 | Holistic view of data reveals pronounced similarities of datasets

We were also interested in studying the suspected similarities of transcriptomic responses induced by the different genotoxins on a broader base, to allow for the bioinformatic identification of transcript networks related to genotoxic treatment. A heat-map figure of hierarchical clustering of the datasets was prepared, using all available data to visualize similarities and differences between the individual datasets (Figure 2). While some datasets showed, if at all, only very slight gene regulation, it nonetheless becomes obvious that similar patterns of transcriptomic up- or down-regulation were present in the individual data sets. It should also be noted that most, but not all, AFB1 and BaP datasets clustered together (Figure 2). Thus, despite the very pronounced differences in the numbers of resulting DEGs, a holistic view of the data revealed remarkable similarities. In addition,

TABLE 5 Most often and strongest differentially expressed genes affected by benzo[a]pyrene

No	Gene name	Log_2 FC								Regulation
		b	s	aa	ab	ac	ad	ae	af	
1	*CYP1B1*	1.58	17.78	4.71	3.36	4.44	2.83	4.43	2.48	↑
2	*LRMDA*	−2.26	-	−1.85	−2.26	−2.02	−2.46	−1.43	−1.57	↓
3	*SMYD3*	−0.80	-	−1.50	−1.36	−1.71	−1.80	−0.85	−0.58	↓
4	*CYP1A1*	5.68	-	9.31	8.53	9.54	8.97	9.25	7.56	↑
5	*COL5A2*	−0.53	-	−0.92	−1.04	−1.26	−1.23	−0.75	−0.76	↓
6	*FHIT*	−2.42	-	−1.21	−1.21	−0.82	−1.15	−1.14	−1.20	↓
7	*COL8A1*	−1.80	-	−0.87	−1.02	−1.50	−1.49	−1.25	−1.72	↓
8	*PDZK1IP1*	−0.86	-	−1.40	−1.53	−1.32	−2.10	−0.72	−0.95	↓
9	*GAREM1*	0.68	-	0.94	0.71	0.59	1.02	0.90	0.63	↑
10	*CXCL10*	−1.58	-	−1.54	−2.14	−1.99	−1.98	−1.29	−1.17	↓
11	*GFRA1*	−1.11	-	−1.15	−1.32	−1.13	−1.34	−0.90	−0.85	↓
12	*MCTP1*	−1.06	-	−1.17	−0.72	−1.28	−1.08	−0.91	−0.61	↓
13	*CYP1A2*	1.18	-	6.88	2.98	7.13	3.72	6.09	2.34	↑
14	*ALDH3A1*	2.97	-	3.82	4.07	5.77	5.51	3.43	3.14	↑
15	*FABP4*	−2.74	-	−2.15	−2.74	−1.42	−1.84	−1.94	−2.04	↓
16	*GPM6A*	−0.68	-	−0.79	−0.75	−0.81	−1.05	−0.66	-	↓
17	*CD44*	0.87	-	0.78	1.73	0.88	1.56	-	0.70	↑
18	*CXCL13*	−1.41	-	−1.15	−2.51	−1.82	−4.04	-	−1.52	↓
19	*STX8*	−0.89	-	−1.24	−2.09	−1.32	−2.24	-	−0.61	↓
20	*NUAK1*	−0.63	-	−0.80	−0.93	−0.75	−0.89	-	−0.73	↓
21	*CADPS2*	−1.30	-	−2.17	−2.53	−2.53	−2.26	-	−1.16	↓
22	*ABCA12*	3.30	-	0.87	3.72	1.58	3.57	-	2.22	↑
23	*E2F7*	2.00	-	1.23	1.99	1.50	2.61	-	1.80	↑
24	*LACC1*	0.67	-	0.73	1.58	0.87	2.03	-	0.88	↑
25	*VCAN*	2.43	-	1.01	2.86	0.81	2.52	-	1.51	↑
26	*TNFRSF11B*	−2.02	-	−0.55	−2.11	−0.84	−3.06	-	−1.42	↓
27	*G0S2*	−1.45	-	−1.16	−1.96	−1.15	−3.77	-	−1.22	↓
28	*CPED1*	−1.52	-	−0.98	−1.71	−1.20	−1.76	-	−1.01	↓
29	*VDR*	1.09	-	1.33	1.36	0.82	1.76	-	0.59	↑
30	*VWCE*	1.75	-	-	2.27	1.41	1.95	0.90	1.43	↑
31	*DIAPH2*	−1.07	-	-	−1.34	−1.15	−1.93	−0.64	−0.76	↓
32	*PLXDC2*	−1.09	-	-	−1.88	−1.25	−2.43	−0.88	−0.99	↓
33	*NLGN1*	−1.01	-	-	−1.28	−1.60	−1.21	−0.89	−0.57	↓
34	*GPC6*	−1.94	-	-	−2.15	−2.44	−2.85	−1.27	−1.28	↓
35	*TMEM156*	-	-	1.81	1.35	1.02	0.99	0.77	0.56	↑
36	*SLC7A11*	-	-	2.33	2.07	2.12	1.87	1.29	1.27	↑
37	*NRXN3*	-	-	−1.23	−0.90	−0.81	−0.82	−0.92	−0.90	↓
38	*KIAA0040*	-	-	−1.15	−1.33	−1.15	−1.53	−0.96	−0.78	↓

PCA analysis showed that most datasets clustered together closely in the PCA plot, with only few outliers (Figure 3). Especially the AB1 datasets with the identifiers x and y from Tryndyak et al. (2018) (cp. Table 2) and the NNK data from Doktorova et al. (2013) were different from the other datasets in the PCA analysis. Results were similar when only the DEGs were considered for the PCA instead of the entire datasets (not shown). BaP data visualization in the PCA plot appeared to follow a straight line from lower left to upper right (Figure 3). Data were thereby, at least in parts, reflecting the treatment conditions: datasets derived from longer incubation times and higher BaP concentrations tended to the lower left, whereas in the upper right part of the plot mostly datasets obtained with shorter incubation times and/or lower concentrations of the test compound were present.

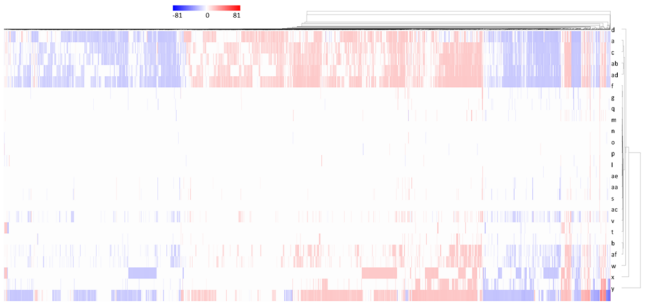

FIGURE 2 Similarity of datasets obtained from aflatoxin B1- or benzo[a]pyrene-treated HepaRG cells using hierarchical clustering and heat-map visualization of the full datasets. Colors indicate up- or downregulation as indicated in the figure. For assignment of the individual data set identifiers please refer to Table 2

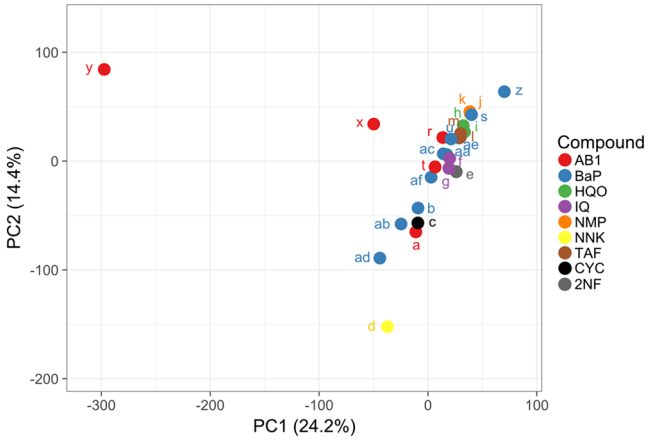

FIGURE 3 Similarity of datasets obtained from aflatoxin B1- or benzo[a]pyrene-treated HepaRG cells using principal component analysis of the full datasets. Individual compounds are indicated by colors as indicated in the figure. For assignment of the individual data set identifiers please refer to Table 2

4 | DISCUSSION

Transcriptome data, often gained using microarray-based technology, have proven their value in the characterization of, for example, disease- and/or toxin-induced cellular changes at the molecular level, especially in basic research-related work. In a regulatory context, however, data obtained by omics techniques are still not routinely considered. This is especially related to a lack of method harmonization, standardization and validation of experimental design and data evaluation; in addition, the current definition of adversity of compound-induced effects impedes the use of omics data in toxicology. Results of a recent workshop on the use of omics technologies in regulatory science have shown that most experts favor an increasing application of omics techniques in toxicological testing in the future, but still feel that additional steps are necessary prior to routine implementation of omics technologies in regulatory test batteries (Marx-Stoelting et al., 2015).

Numerous studies have been published describing gene expression patterns of genotoxic compounds, often with the aim to distinguish genotoxic carcinogens from non-genotoxic carcinogens, for example see Lee et al. (2013), Magkoufopoulou et al. (2011), and Mathijs et al. (2009). These studies often focus on liver tissue from in vivo studies or on hepatic in vitro models like cultivated primary hepatocytes or permanent cell lines, because the liver is required for metabolic activation of numerous carcinogens and also a main target organ of carcinogenicity of many different chemical entities (Davila, Rodriguez, Melchert, & Acosta, 1998). Genotoxic compounds share the ability to damage DNA and are therefore expected to exert, at least partially, similar responses in the cells, for example gene regulation related to DNA damage responses, cell cycle control, or cellular stress. Some divergence is, of course, expected, as the compounds might trigger additional cellular responses, for example via the binding to different nuclear xeno-sensing receptors (see also below). Here, we compared published datasets obtained from treatment of HepaRG cells, a state-of-the-art in vitro model for human hepatocytes (Aninat et al., 2005; Antherieu, Chesne, Li, Guguen-Guillouzo, & Guillouzo, 2012; Josse et al., 2008), with genotoxic compounds. Raw data were re-evaluated using a common procedure in order to gain results yielded under as similar as possible conditions. Nonetheless, the obtained results varied considerably with respect to the number of DEGs detected, suggesting substantial differences between the datasets. When comparing the top-regulated genes identified from datasets of AB1 or BaP treatment, the two most often used model genotoxins, also only a very limited degree of overlap was observed. Nonetheless, top-regulated genes from AB1 and BaP datasets contained several genes linked, for example, to cell cycle and proliferation or to cellular stress responses. This is in line with previous findings, where similar biological functions have been shown to be affected by genotoxic compounds in vitro; e.g. see Lee et al. (2013), Magkoufopoulou et al. (2011), Mathijs et al. (2009), and Smit et al. (2017).

The observed differences between the datasets can be, in parts, attributed to the different molecular targets of the compounds, which exist despite of the common genotoxic properties. For example, the activation of the AHR as a known main molecular target of BaP (Abel & Haarmann-Stemmann, 2010) was clearly visible from the data, as evidenced by the induction of a number of classic AHR target genes by BaP. By contrast, these genes were not induced by AFB1, for which only some indirect evidence exists for a weak activation of AHR in rat hepatocytes (Ayed-Boussema, Pascussi, Maurel, Bacha, & Hassen, 2012), while other nuclear receptors and pathways, for example the pregnane-X-receptor, appear to play a bigger role in mediating transcriptional effects in response to AFB1 activation (Ayed-Boussema et al., 2012; Marchese et al., 2018). In addition, variations in cell culture conditions, concentration and duration of treatment, as well as the choice of the microarray and data analysis approach contribute to data variability. Individual contributions to variability by these factors are difficult to judge, especially with respect to the fact that often not much detail is provided within the materials and methods section of published papers.

It appears that for the inter-experimental comparison of the results, inspection of the overlap of lists of individual DEGs is of rather limited value for the identification of potential common mechanisms

of toxic action. Instead, analysis of datasets as a whole, as well as bioinformatic analyses of biological programs that potentially underlie the observed transcriptomic patterns, should be regarded as valuable tools for network identification and subsequent AOP development based on transcript patterns commonly affected by different compounds sharing an adverse outcome. For example, an AOP for liver genotoxicity and tumorigenesis could be established from such data. Of note, within the 9 selected publications bioinformatic data mining was already routinely used to obtain information about possibly underlying biochemical and/or toxicological mechanisms. For this purpose, however, very different software packages and/or online tools have been used, for example Ingenuity Pathway Analysis, Metacore, Gene Ontology, the KEGG pathway database, ToxProfiler, and others. This high variability of bioinformatic data mining tools makes it difficult to directly compare the predictions obtained with different data sets in different studies. In our analyses, the DEG numbers showed great variance, while the top consensus genes of AB1 and BaP datasets showed only very limited mutual overlap. By contrast, hierarchical clustering and PCA revealed greater similarity than might have been expected based on the above analyses of DEG lists. Nonetheless, care should be given to bioinformatic interpretation of omics results, since those predictions may also contain a substantial fraction of accidental findings. It has recently been shown that even from a random nonsense data set statistically significant predictions of involved biological pathways and transcriptional regulators can be deduced by such approaches (Braeuning, Frenzel, & Lampen, 2018).

In summary, the present work demonstrates substantial variability of published microarray-based transcriptome data in response to genotoxic insults in human HepaRG hepatocarcinoma cells. Analyses which consider affected biological functions and/or make use of the full data set appear more suited to carve out similarities than approaches only investigating short lists of top-deregulated transcripts. More standardization at the levels of experimental setup, bioanalytical procedures and especially omics data analysis are required as a prerequisite for regulatory acceptance of conclusions drawn from omics analyses.

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