Toxicogenomics of the flame retardant tris (2-butoxyethyl) phosphate in HepG2 cells using RNA-seq

Toxicogenomics of the flame retardant tris (2-butoxyethyl) phosphate in HepG2 cells using RNA-seq

Boris V. Krivoshiev[1]*, Gerrit T. S. Beemster[2], Katrien Sprangers[2], Bart Cuypers[3,4], Kris Laukens[3], Ronny Blust[1], Steven J. Husson[1]

[1] Systemic Physiological & Ecotoxicological Research (SPHERE), Department of Biology, University of Antwerp, Antwerp, Belgium

[2] Integrated Molecular Plant Physiology Research (IMPRES), Department of Biology, University of Antwerp, Antwerp, Belgium

[3] Advanced Database Research and Modelling (ADReM), Department of Mathematics and Computer Science, University of Antwerp, Antwerp, Belgium

[4] Unit of Molecular Parasitology, Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium

*Corresponding author:

E-mail: boris.krivochiev@uantwerpen.be

Telephone: +3232653501

Fax: +3232653497

Abstract

Tris (2-butoxyethyl) phosphate (TBOEP) is a compound produced at high volume that is used as both a flame retardant and a plasticizer. It is persistent and bioaccumulative, yet little is known of its toxicological modes of action. Such insight may aid risk assessment in a weight-of-evidence approach supplementing current testing strategies. We used an RNA sequencing approach as an unbiased and sensitive tool to explore potential negative health effects of sub-cytotoxic concentrations of TBOEP on the transcriptome of the human liver hepatocellular carcinoma cell line, HepG2, with the lowest concentration used potentially holding relevance to human physiological levels. Over-representation and gene set enrichment analysis corresponded well and revealed that TBOEP treatments resulted in an upregulation of genes involved in protein and energy metabolism, along with DNA replication. Such increases in cell and macromolecule metabolism could explain the increase in mitochondrial activity at lower TBOEP concentrations. In addition, TBOEP affected a wide variety of biological processes, the most notable one being the general stress response, wound healing. Finally, TBOEP showed effects on steroid hormone biosynthesis and activation, regulation, and potentiation of immune responses, in agreement with other studies. As such, this study is the first study investigating genome-wide changes in gene transcription in response to TBOEP in human cells.

Keywords: Tris (2-butoxyethyl) phosphate, HepG2, RNA-seq, toxicogenomics, mode of action

1. Introduction

Flame retardants (FRs) are compounds produced in high volume and present in nearly all manufactured items and materials with the purpose of averting fire. Their use is expected to increase due to the ever-increasing global population and urbanisation. These compounds have been shown to migrate out of consumer goods and contaminate surrounding environments such as house dust (de Boer et al., 2015; Dodson et al., 2012), biota (Malarvannan et al., 2014; Santín et al., 2016), and food sources (Von Eyken et al., 2016; Zheng et al., 2015). Detection of FRs as far as the Arctic (Salamova et al., 2014) further lends evidence to the pervasiveness and persistence of these chemicals. Bioaccumulation is also indicated by the detection of FRs in human milk (Kim et al., 2014; Ryan and Rawn, 2014), birds' eggs (Bouwman et al., 2014; Braune et al., 2015), and in bird offspring (Greaves and Letcher, 2014). FRs therefore could pose a significant risk to both human and environmental health.

One of the most-abundantly detected organophosphate FRs is tris (2-butoxyethyl) phosphate (TBOEP). It is continuously detected at higher amounts compared to other brominated and organophosphate FRs in house dust (ranging from 2.3-5300 μg/g house dust) (Dodson et al., 2012; Fan et al., 2014; Marklund et al., 2003; Wei et al., 2015), drinking water (19.5-81.7 ng/L) (Li et al., 2014), human milk (0-206 ng/g lipid) (Kim et al., 2014), and human placenta (0-77.8 ng/g lipid) (Ding et al., 2016). Its presence in mothers' milk and placenta along with its high octanol-water coefficient (log K_{ow} = 3.75) indicating lipophilicity demonstrates its potential for bioaccumulation (van der Veen and de Boer, 2012). Regulation of the use and production of such chemicals falls under the Registration, Evaluation, Authorisation, and restriction of Chemicals (REACH) directive tasked with protecting human end environmental health through the better classification and identification of potentially hazardous chemicals. Several classical toxicological endpoints are used to assess their threat to health, including; determination of lethal dose concentrations, investigating effects on skin sensitisation, effects on immune and reproductive systems, as well as genotoxicity and

carcinogenicity potential. However, little is uncovered concerning the mode of action (MOA) that may bring about these adverse health effects when looking at the above-mentioned classical toxicological endpoints. Such insight would allow for better prediction of potential health effects, along with improved characterisation of potentially hazardous chemicals, which would ultimately aid in a more comprehensive risk assessment.

In vivo toxicological data used to assess the toxicity risk of TBOEP have marked it as a chemical of low mammalian toxicity and low irritation potential, while neurotoxic effects in rats were inconsistent (World Health Organisation, 2000). *In vitro* assays also identified TBOEP as harbouring no carcinogenic or clastogenic potential (World Health Organisation, 2000). Subchronic studies have revealed that the liver is a major target organ in rats, with a lowest-observed-adverse-effect level for liver effects being 150 mg/kg body weight per day (World Health Organisation, 2000). Short-term repeated exposure studies in rats have found effects on the liver by alterations to cholinesterase and gamma-glutamyltransferase activity, along with hepacellular hypertrophy (World Health Organisation, 2000). Neurotoxic effects included reduction nerve conductivity, increase in refractory periods, degeneration of myelin sheaths, and swelling and degeneration of nerve fibres (World Health Organisation, 2000). Similarly, neurotoxic effects were observed by decreased free swimming and photomotor responses in both Japanese medaka (Sun et al., 2016a) and zebrafish larvae (Sun et al., 2016b).

While the data described above were key in elucidating the toxicological risk of TBOEP, little is uncovered about the MOA that may give rise to such adverse effects. Some studies have investigated potential molecular MOA by which TBOEP may affect health. For instance, the neurotoxic effects of TBOEP in both the Japanese medaka and zebrafish larvae were brought about by changes in gene transcription of genes involved in the nervous system (Sun et al., 2016a, 2016b). TBOEP was also shown to increase sex hormone production by altering genes involved in sex hormone metabolism in human adrenal cortex cells (Liu et al., 2012), while potentiating estrogen

signalling by altering receptor expression in zebrafish (Ma et al., 2015). Additionally, TBOEP has shown to effect development of zebrafish embryos by inducing malformations, ultimately resulting in death (Ma et al., 2016). These developmental effects were also observed concurrently with alterations to endocrine functions, by potentially reducing thyroid hormone production, altering cortisol homeostasis, and sex-hormone homeostasis (Ma et al., 2016). In line with the disruption of sex-hormone homeostasis, TBOEP was shown to alter serum 17β-estradiol and testosterone levels which led to a decrease in egg production, hatching success, and survival rates while also retarding oocyte maturation and spermiation in zebrafish (Xu et al., 2017). Further developmental effects of TBOEP in zebrafish was shown by a decrease in survival and hatching percentage along with a decrease in heart rate and body length (Han et al., 2014). These effects were accompanied by the inhibition of the use of vitellogenin, and affecting proteins involved in cell proliferation and DNA repair which brought about an increase in cells undergoing apoptosis (Han et al., 2014). In daphnia, TBOEP was shown to alter genes involved in protein and energy metabolism (Giraudo et al., 2015) while having very limited effects in chicken embryos (Egloff et al., 2014). Furthermore, effects on membrane and protein integrity were seen and likely brought about by oxidative damage as found using a bacterial gene profiling assay (Krivoshiev et al., 2015). However, many of these studies are limited to investigating only a subset of genes, and could therefore be biased and fail to identify other possible MOA.

In vitro toxicological assays able to elucidate MOA are seen as the initial strategy in trying to predict adverse health effects. Such insight may be used to prioritise hazardous chemicals for further study in more time-consuming animal experiments (Colnot et al., 2014), while contributing to risk assessment concurrently with *in vivo* toxicological data to aid better risk assessment in a weight-of-evidence approach (Ankley et al., 2010; Rouquie et al., 2015). Global gene expression profiling allows for greater utility in identifying possible toxicological MOA, which in turn can be used to identify biomarkers of exposure and adverse health effects (Beane et al., 2011; Bjerrum et al., 2013; Connor

et al., 2010; Ren et al., 2013, 2012; Yang et al., 2008). It may also generate hypotheses and identify candidate genes of interest that may be further examined in order to understand phenotypic responses (Smith et al., 2013).

Given that the liver is another major target organ for TBOEP toxicity, biotransformation of FRs has shown to potentiate their toxicity compared to parent compounds (Dingemans et al., 2011, 2008; van Boxtel et al., 2008). *In vitro* liver models, such as HepG2 cells, have successfully been used to investigate drug metabolism for improved drug efficacy (Bai J et al., 2010; Stormo et al., 2014), and to investigate hepatotoxicity (Summeren et al., 2011; Valentin-Severin et al., 2003), while also being used in transcriptomics studies that monitor hepatic responses to xenobiotics (Van Delft et al., 2012). Given the lack of insight into the MOA for TBOEP toxicity, particularly in human cells, we adopted RNA-seq to identify molecular changes that may give rise to TBOEP toxicity in HepG2 cells.

2. Materials and methods

2.1 Chemicals and reagents

TBOEP (CAS 78-51-3) (94% purity) and all other chemicals and reagents were purchased from Sigma-Aldrich (USA) unless otherwise stated. TBOEP stock solution was made up in ≥99.9% dimethyl sulfoxide (DMSO) and stored at -20 ^{o}C.

2.2 Cell culture

The human hepatocellular carcinoma cell line, HepG2 (ATCC HB-8065), was maintained as a monolayer in T-25 Nunc culture flasks in Minimum Essential Medium supplemented with Earle's salts (Gibco, USA), 1 mM sodium pyruvate, 4 mM L-glutamine, 1% non-essential amino acids, 50 IU/ml penicillin, 50 mg/ml streptomycin, and 10% heat-inactivated foetal bovine serum (FBS). Cells were cultured in a 37^{o}C incubator under 5% CO_2, and once reaching 70-80% confluency, were passaged using 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA), without reaching a maximum of 25 passages. Cells were routinely assessed for mycoplasma contamination using the LookOut® Mycoplasma PCR Detection Kit (Sigma-Aldrich, USA) following manufacturer's instructions.

2.3 Cytotoxicity

Dose-dependent cytotoxicity was determined by means of a resazurin cell-viability assay. The use of resazurin as a measure of cell viability and therefore also as cytotoxicity assays is well documented (McMillian et al., 2002; Mikus and Steverding, 2000; Page et al., 1993). The assay relies on the reduction of resazurin into resofurin by metabolically active cells. A spectrophotometer can be used to qualify the level of reduction, which is dependent on cell number, and whether cells are metabolically active or not (viable or dead). Cytotoxicity assays were designed to mimic proposed treatments. Briefly, 1 x 10^4 HepG2 cells were seeded in 96-well plates and allowed to attach and acclimatise for 72 hrs. Cells were then treated with a range of TBOEP concentrations made up in

0.1% DMSO as indicated in Supplementary Figure 1. Final exposure DMSO concentration was 0.1%. Following 72 hrs treatment, cells were washed with PBS and 50 mM resazurin sodium salt was added per well. Cells were allowed to incubate at 37 ^{o}C for 45 min and fluorescence at 570 nm was measured. Assays were conducted in triplicate on cells at three different passages on separate days. Statistical differences between exposure conditions and control treatment (0.1% DMSO) were determined using the unpaired t-test.

2.4 Treatments

3 x 10^5 HepG2 cells were seeded per well in 6-well plates and allowed to culture for 72 hrs for acclimatisation after detachment/reattachment. The number of seeding cells was optimised to allow for the necessary amount of RNA to be extracted after treatments while cells were at 70-80% confluency by the end of treatments to prevent over-confluency. 2.5 µM and 125 µM TBOEP in 0.1% DMSO were used for exposures to low and high concentrations respectively (concentrations at which there was an absence of cytotoxicity). Final DMSO concentrations in treatments did not exceed 0.1%, equivalent or even lower than the final concentrations used in other MOA transcriptomic studies of other chemicals in HepG2 cells (Jennen et al., 2011; Magkoufopoulou et al., 2012; Souza et al., 2016; Zhang et al., 2016, 2015a, 2015b). Control treatments included cells treated with 0.1% DMSO only. Cells were exposed for 72 hrs before RNA isolation. Treatments were conducted as triplicates, involving separate seeding of cells (thus forming three separate cell populations), separate treatment of each cell population with compound solutions made up independently between replicates, and separate RNA isolations and sequencing reactions for each population of cells.

2.5 RNA isolation and sequencing

The combined TRIzol (Invitrogen, USA) and RNeasy (Qiagen, GER) approach was adopted for RNA isolation as it yields high RNA quality required for RNA-seq (Hook et al., 2014). Briefly, this involved

extracting RNA from cells with TRIzol as per manufacturer's instructions through to phase separation, at which point the RNA in the aqueous phase was treated as lysate and then subjected to RNA clean up using the RNeasy kit according to manufacturer's instructions. Isolated, pure total RNA was then quantified and qualified (260/280 and 230/280 ratios greater than 2) using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). RNA integrity was determined visually using the QIAxcel System (Qiagen, GER) (Yednock et al., 2015). RNA was stored at -80 ^{O}C prior to library preparation. Sequencing libraries were prepared using the TruSeq® Stranded mRNA Sample Preparation kit (Illumina, USA) following the manufacturer's protocol. Prepared libraries were 2 x 50bp paired-end sequenced using the Illumina HiSeq® 1500 platform (Illumina, USA).

2.6 Data and pathway analysis

The average number of total reads per sample was 18.4 million, ranging from 6.4 – 30.3 million reads. Reads were mapped to the human reference genome (UCSC hg19) and only reads that were uniquely mapped and mapped concordantly in pairs were retained. Alignment, mapping, and annotation steps were performed with CLC Genomics Workbench (CLC Bio, DEN) using default parameters. Samples were normalised by quantile normalisation. Differential expression *p*-values were generated using Baggerly's test statistic (Baggerly et al., 2003). These *p*-values were subsequently corrected with the Benjamini-Hochberg procedure to limit the false discovery rate (FDR) to 5% of the significant genes (Benjamini and Hochberg, 1995). Over-representation analysis of Gene Ontology (GO) terms characterising biological processes for differentially expressed genes (DEGs) was performed using the Cytoscape plugin, BiNGO (Maere et al., 2005). Only GO enrichments that were supported by at least three genes within that specific GO term were considered for further analysis and interpretation. Clustering of similar GO terms was done using the web applet REVIGO (Supek et al., 2011). Pathways annotated within the KEGG database were elucidated using Gene Set Enrichment Analysis (GSEA), normalised expression data for each triplicate (Subramanian et al., 2005). Parameters for GSEA were used as previously published (Weidner et al., 2016). Pathways of

interest were clustered using a combination of the Cytoscape plugin, Enrichment Map (Merico et al., 2010), and MultipleExperiment Viewer (Saeed et al., 2003).

2.7 Quantitative real-time PCR validation

Overall, thirteen DEGs were selected to be validated by quantitative real-time PCR (qRT-PCR). This was done on samples completely independent of the samples used for RNA-seq, using cells from a different frozen stock of cells and a different stock of TBOEP that was used for the samples that underwent RNA-seq. This therefore also validated the robustness and accuracy of our approach, albeit only using a limited number of genes as examples. HepG2 cells were seeded, treated, and underwent RNA isolation as for the samples that were used for RNA-seq. Only 125 µM treatments were conducted and verified by qRT-PCR. Once purified for mRNA, cDNA libraries were generated using the iScript™ Advanced cDNA Synthesis Kit (Bio-Rad, USA) as per manufacturer's instructions. qRT-PCR was conducted using SsoAdvanced™ Universal SYBR Green Supermix and gene primers ordered as PrimePCR™ assays from Bio-rad (USA), and was performed according to manufacturer's instructions. Primer sequences are unavailable given that PrimePCR™ assays are proprietary. Primer efficiencies of all primers used exceeded 95%. Based on the RNA-seq data, *HSPD1* was selected as a reference gene given its high and unvaried expression over the three treatment conditions (0.1% DMSO, 2.5 µM TBOEP, and 125 µM TBOEP). Relative gene expression was analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3. Results

3.1 Effects on cell viability

Cytotoxic effects of TBOEP on cell viability was first investigated over a range of concentrations (0 – 1000 µM). Acute testing for 72 hrs showed toxicity effects only at 1000 µM (Supplementary Figure 1), where a 50% decrease in cell viability was observed, with increases in mitochondrial activity at lower concentrations. Using a toxicogenomics approach, we aimed to identify molecular targets that were affected by exposure to 2.5 µM and 125 µM concentrations of TBOEP (denoted as low- and high-dose from here on out). RNA-seq was carried out using non-cytotoxic concentrations in an effort to limit effects of general stress response genes on the observed transcriptome.

3.2 Differential gene expression by RNA-seq

Extracted RNA from HepG2 cells originating from three exposure scenarios (low-dose TBOEP, high-dose TBOEP, and control) were analysed in triplicate by RNA-seq. Supplementary Table 1 shows the sequencing and mapping statistics. Of the 57,774 genes annotated within the hg19 human reference genome, a total of 18,999, 19,163, and 18,367 genes were detected as expressed with a count of at least one in the TBOEP high-dose, TBOEP low-dose, and control treatments respectively (Supplementary Table 2). To identify DEGs between TBOEP and control treatments, a false-discovery (FDR) threshold of 5% was used. When applied, 4060 and 1055 genes were detected as differentially expressed for high- and low-dose TBOEP treatments respectively. In addition, a further restriction of DEGs with a fold-change (FC) of greater than 2 compared to control resulted in 939 and 80 DEGs for both high-dose and low-dose TBOEP group respectively (Supplementary Table 2).

A majority of DEGs (73%) detected for the low-dose treatment were shared with the DEGs detected for high-dose TBOEP. Of the 1055 DEGs revealed for low-dose TBOEP treatments at FDR<0.05, 772 of those were shared with high-dose treatments, while only 283 DEGs were detected as differentially

expressed for low-dose TBOEP exclusively (Figure 1A). Similarly when employing a further restriction of absolute fold-changes greater than 2 (|FC|>2), of the 80 DEGs detected in response to low-dose TBOEP, 56 were shared with high-dose treatments while only 24 DEGs were detected as differentially expressed in response to low-dose TBOEP treatment only (Figure 1B).

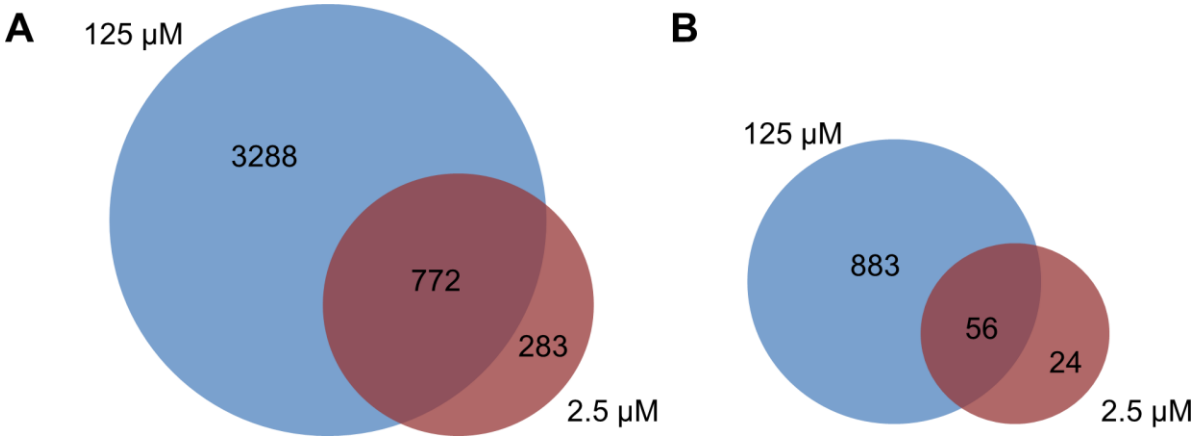


Figure 1. Differentially expressed genes in response to TBOEP. Venn diagrams of number of differentially expressed genes highlighting the number of common DEGs for high- and low-dose TBOEP treatments. DEGs were detected at (A) FDR<0.05, and at (B) FDR<0.05, |FC|≥2. A gene was differentially expressed, irrespective of actual gene fold-changes, when the expression change across the three replicates was significant from control treatments at FDR<0.05 following the Benjamini-Hochberg correction for multiple comparisons.

3.3 Over-representation analysis of biological processes

To identify the biological processes affected by TBOEP exposure, DEGs were subjected to Gene Ontology (GO) term enrichment using over-representation analysis. Initially all DEGs detected at FDR<0.05 were used, and GO terms were enriched using BiNGO (Maere et al., 2005). All significant terms (Benjamini-Hochberg FDR<0.05) were then clustered based on similarity into representative clusters using REVIGO (Supek et al., 2011) (Supplementary Figure 2). 353 and 217 terms were enriched in the high- and low-dose treatments respectively (Supplementary File 1).

For TBOEP high-dose treatments, GO terms typical of translation were effected most significantly, followed by GO term clusters characteristic of oxidative phosphorylation, negative regulation of cellular processes, cellular metabolism, and cellular response to stress, with minor clusters indicative of intracellular transport and organisation, biosynthesis, cell cycle, and cell death (Figure 2A).

Consistently, low-dose TBOEP treatments resulted in enrichment of GO terms representative of translation, oxidative phosphorylation, and cellular metabolism, with minor clusters suggestive of cellular organisation, biosynthesis, cell cycle, and response to chemical stimulus (Supplementary Figure 2B). The 283 genes detected as differentially expressed for TBOEP low-dose treatments only revealed effects primarily on cell cycle and spindle organisation (Supplementary Figure 3).

Given the high number of DEGs induced by both TBOEP treatment doses, DEGs found to be expressed at |FC|>2 were also subjected to GO enrichment in an effort to identify biological processes that were most affected. The 939 DEGs found to be differentially expressed by high-dose treatments at these constraints were characteristic of a number of cellular processes, including; response to stress, response to stimulus and regulation of immune function, cell migrations and proliferation, tissue development and organ regeneration, and metabolism (Figure 2A). These general biological processes were further characterised to identify more specific responses that are affected by TBOEP (Figure 2B). Response to stress was mainly characterised by responses to wound healing, organic substances, and to corticosteroid stimulus. Response to stimulus and regulation of immune function was distinguished by regulation of wound healing, blood coagulation, chemotaxis, and inflammatory responses. Regulation of cell cycle, smooth muscle cell proliferation, and leukocyte migration amongst other processes were found to be typical of the cell migration and proliferation group. Tissue development and organ regeneration was denoted by blood vessel development and organ regeneration, while metabolism was represented by lipid, steroid hormone, and organophosphate metabolism. Specifically for steroid hormone metabolism, TBOEP was shown to significantly alter the expression of genes encoding for all the major enzymes (except for P450scc) in this pathway (Supplementary Figure 4). However, changes in gene expression for all enzymes involved in this pathway were not unidirectional (Supplementary Figure 5). GO enrichment of the 80 DEGs found to be differentially expressed following treatment with low-dose TBOEP failed to enrich for any GO terms (data not shown).

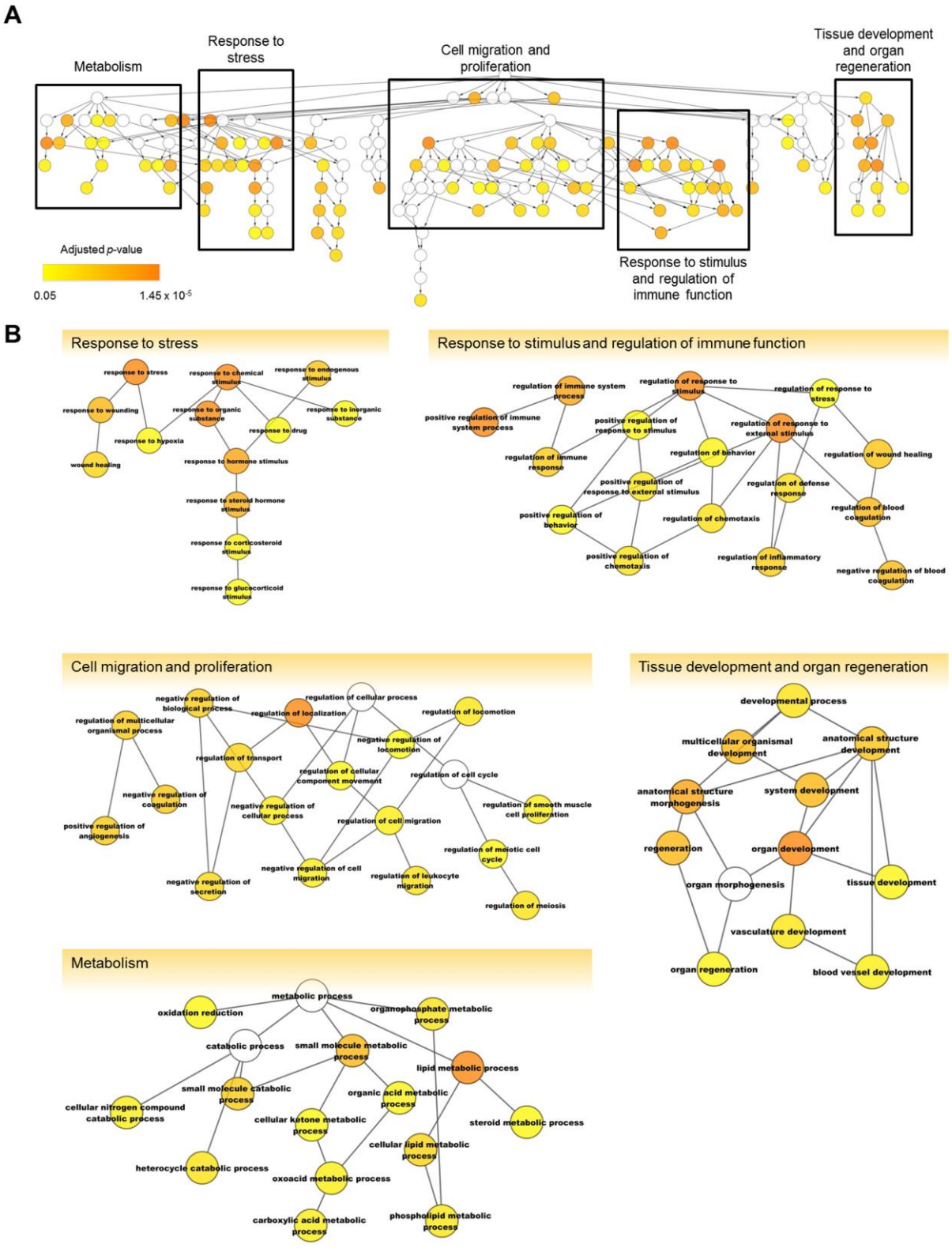


Figure 2. Biological processes over-represented by DEGs detected for high-dose TBOEP treatments (FDR<0.05, |FC|≥2). (A) A GO tree obtained using BiNGO showing the hierarchy of GO terms representative of biological processes over-represented by DEGs. A GO term was considered significantly altered at FDR<0.05 after Benjamini-Hochberg correction for multiple testing corrections. Biologically similar GO terms were further grouped into five groups representing general biological functions associated with each group. (B) The five biological process groups and characteristic responses for each are shown in greater detail. Coloured nodes indicate significant nodes (FDR<0.05), with more orange indicate greater

significance. For ease of reading, GO terms are arranged in a hierarchy, with more general GO terms being present near the top, and more specific GO terms being found at the bottom. Data from GO term enrichments are found in Supplementary File 2.

3.4 Gene set enrichment of pathways

Normalised expression data of all transcripts detected were analysed for enriched pathways using GSEA and the curated KEGG database. Analysis revealed enrichment (FDR<0.25) of ribosome (hsa03010), oxidative phosphorylation (hsa00190), tricarboxylic acid (TCA) cycle (hsa00020), and pyrimidine metabolism (hsa00240) KEGG pathways as over-expressed for TBOEP high-dose treatments (Supplementary Table 3), while no pathways were detected as under-expressed (Supplementary File 3). Similarly for low-dose TBOEP treatments, ribosome (hsa03010), oxidative phosphorylation (hsa00190), and pyrimidine metabolism (hsa00240) KEGG pathways were also enriched as over-expressed (FDR<0.25) along with the DNA replication (hsa03030) KEGG pathway (Supplementary Table 4). Once again, no pathways were detected as under-expressed (Supplementary File 3).

Heatmaps of enriched KEGG pathways were generated using Log_2 converted fold-change values of averaged expression data over the three triplicates per condition (Figure 3), allowing the visualisation of expression data for each gene making up each enriched pathway. Genes making up the separate pathways and treatment conditions were then clustered for each enriched KEGG pathway. Clustering reveals expression levels of a vast majority of genes making up these pathways were affected following treatment with TBOEP, both at high- and low-dose treatments. Most of these altered gene expressions concern increases in gene expression in response to TBOEP treatments.

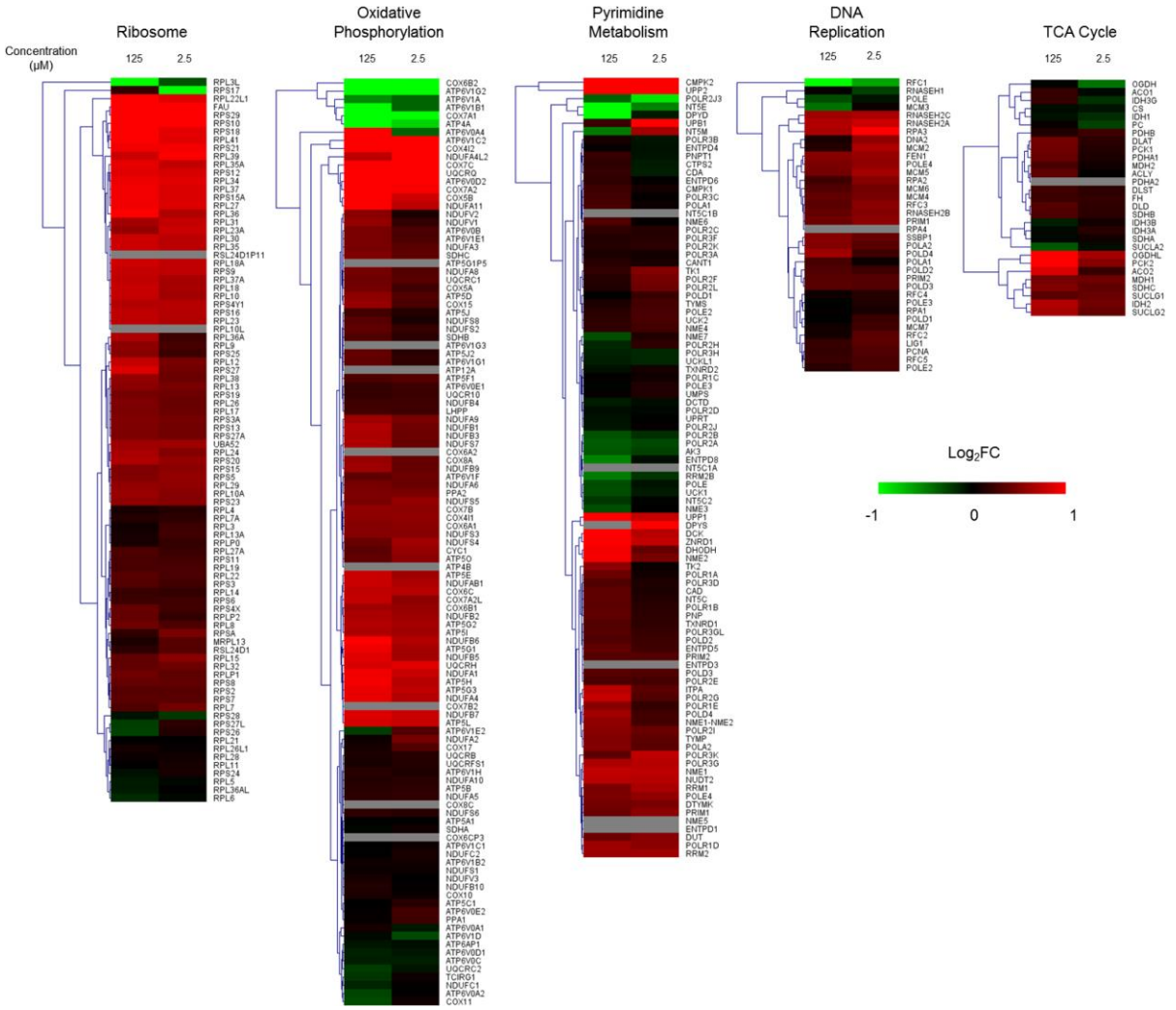


Figure 3. KEGG pathways affected by TBOEP treatment. Heatmap visualisation and cluster analysis of genes characterising significantly enriched (FDR<0.25) KEGG pathways for both TBOEP high- and low-dose treatments. Expression data used was the averaged of triplicates per condition. Data represents Log_2FC expression values compared to control. KEGG pathways were considered significantly altered at FDR<0.25 following adjustments for multiple hypothesis testing as recommended by the original authors (Subramanian et al., 2005). Variation between replicates for significantly affected KEGG pathways can be visualised in Supplementary Figure 6.

3.5 qRT-PCR validation

All thirteen genes selected for qRT-PCR validation showed good correlation to the results generated by RNA-seq for 125 µM treatments (Supplementary Figure 7), highlighting the robustness of our approach. Of the thirteen genes, eight (*CYP19A1, AKR1C1, AKR1C2, HSD17B7, HSD17B8, CYP17A1, CYP21A2,* and *UGT24B4*) that were detected as differentially expressed by RNA-seq and enriched for steroid hormone metabolism were included in the panel of genes selected for qRT-PCR. Five genes

(*AKAP12, CYP24A1, RAB17, CLMN,* and *ASB2*) were selected given their high expression and magnitude of differential-expression in response to TBOEP.

4. Discussion

Given its presence in many different matrices, including; drinking water (Li et al., 2014), human milk (Kim et al., 2014), and placenta (Ding et al., 2016), along with limited insight into its potential MOA, there is a need to expand our understanding on the potential effects of TBOEP on human and environmental health. The present study elucidated toxicological MOAs of TBOEP using a hypothesis-free approach in an attempt to identify potential human health effects, and aid better risk assessment. For this, a toxicogenomic approach was followed, which has already proven its worth in identifying toxicological MOA that may give rise to potential negative health effects (Guyot et al., 2014; Hook et al., 2014; Li et al., 2015; Xu et al., 2015).

Cytotoxicity

Firstly, acute cytotoxicity of TBOEP was measured by means of a cell viability assay. Concentration-dependant cytotoxicity by monitoring mitochondrial activity revealed that TBOEP had limited cytotoxicity in HepG2 cells, which is in line with other similar studies investigating the *in vitro* cytotoxicity of TBOEP in immortalised human cell lines (Krivoshiev et al., 2016; Liu et al., 2012) and in *Daphnia magna* (Giraudo et al., 2015). However, different levels of cytotoxicity are seen between different cell lines and different *in vivo* models (Table 1).

	Model (origin)	Concentration (μM)	Cytotoxicity/ mortality (%)	Exposure time (hrs)	Reference
in vitro	HepG2 (human liver)	1000	80%	72	This study
	MCF-7 (human breast)	1000	0%	24	(Krivoshiev et al., 2016)
	H295R (human adrenal cortex)	250	<80%	48	(Liu et al., 2012)
	Chicken embryonic hepatocytes	60	50%	36	(Porter et al., 2014)
	Herring gull embryonic hepatocytes	95	50%	36	(Porter et al., 2014)
	E.coli	500	20%	1.5	(Krivoshiev et al., 2015)
in vivo	*Daphnia magna*	370	50%	48	(Giraudo et al., 2015)
	Zebrafish embryos	32	50%	48	(Han et al., 2014)
	Zebrafish embryos	8	50%	96	(Du et al., 2015)
	Zebrafish embryos	0.72	50%	96	(Ma et al., 2016)

Table 1. Comparison of TBOEP concentration and level of cytotoxicity/mortality investigated in other studies.

Variations seen within different species, both *in vitro* and *in vivo*, indicates that TBOEP may elicit dissimilar levels of acute toxicity in a species-specific manner. However, it should be noted that different exposure times could cause these dissimilar levels of toxicity. Additionally, dissimilar levels of cytotoxicity between different cell types of the same species origin may be suggestive of differing TBOEP toxicity in an exposure time- and cell type-dependent manner. For instance, longer exposure times generally resulted in higher levels of cytotoxicity, while adrenal cortex cells appear to be more vulnerable to TBOEP toxicity compared to cells of liver and breast origin. Perhaps indicative of this, is the need to establish levels of both acute and chronic toxicity in not only mammals, but also invertebrates, plants, and sediment organisms for registration of a chemical with REACH. Here, we observed cells responding to TBOEP treatment firstly by increasing mitochondrial activity, with levels increasing to 30% above that of controls directly before noting cytotoxic effects at 1000 µM treatments only. This would suggest that TBOEP has limited cytotoxicity to HepG2 cells, however, it should be noted that only acute toxicity was measured here, and that mixture toxicity was not accounted for, given that mixtures of certain FRs have already been shown to potentiate their individual toxicity (Breitholtz et al., 2008).

Alterations to the transcriptome

The changes in mitochondrial activity were accompanied by changes in global gene expression. 4060 and 1055 genes were differentially expressed in the HepG2 cells that were treated with high dose (125 µM) and low dose (2.5 µM) TBOEP respectively. Several data analysis strategies were employed to identify a wide variety of biological processes being altered.

Firstly, over-representation analysis revealed that treatment with TBOEP impacts cell metabolism at many different points, with significant alternations in pathways related to protein metabolism, response to stress and chemical stimuli, tissue regeneration, and lipid metabolic processes. Clustering of the numerous significant GO terms to remove redundancy and thus group similar GO

terms based on similarity revealed that the most significant biological processes affected by TBOEP were typically related to mRNA translation and energy metabolism for both high- and low-dose treatments. The 283 genes detected as differentially expressed in low-dose TBOEP treatments indicated potential non-monotonic responses of some genes and therefore potentially some biological processes in response to TBOEP. Over-representation analysis of these genes revealed effects primarily on cell cycle and spindle formation processes, highlighting that TBOEP may affect some biological functions in a non-monotonic manner, while a vast majority of remaining effects seemed to be monotonic in response given that a majority of genes detected as differentially expressed in low-dose TBOEP were also detected as differentially expressed in high-dose TBOEP treatments.

As a second analysis approach, we performed GSEA, which in contrast to Gene Ontology analysis (GOA) does not rely on cut-offs to identify DEGs (Subramanian et al., 2005), and can therefore be an interesting complementary analysis to GOA (Hedegaard et al., 2009). Gene set enrichment analysis revealed effects on TCA cycle, oxidative phosphorylation, pyrimidine metabolism, DNA replication, and ribosomal subunits. This is in agreement with the over-representation analysis that found alterations to protein and energy metabolism. However, effects of DNA metabolism were detected exclusively with GSEA. Overall, these results suggest that increases in these pathways may result in an increased production of macromolecules such as proteins and DNA, along with an increase in energy metabolism potential. Such an increase in basic cellular function might explain the significant increase in mitochondrial activity we observed at these TBOEP concentrations. Additionally, similar gene alterations to such pathways have already been characterised for some disorders. For example, out of three pathways found to be altered in an obese cohort compared to a lean cohort, ribosome and oxidative phosphorylation pathways were found to be upregulated (Ghosh et al., 2010). These alterations to specific pathways caused by TBOEP may also alter normal aging of the cell, given that modification of pathways involved in protein turnover and energy metabolism have been shown to

play a role in normal cell aging (Linford et al., 2007). Taken together, this indicates that changes in such general cellular pathways may give rise to a wide variety of adverse outcomes.

GOA using DEGs with fold-changes of greater than two-fold was performed since a majority of DEGs exhibited only minor, though significant fold-changes, and are therefore less likely to affect biology compared to DEGs showing greater levels expression changes. Treatment with 125 µM revealed five broad biological processes being affected. A biological process consistently being seen as altered was wound healing. Wound healing is the complex process by which a wound may properly heal, and is comprised by the co-ordination of four biological functions; haemostasis, inflammation, proliferation, and remodelling (Guo and DiPietrio, 2010). Unsurprisingly, TBOEP was shown to alter genes involved in many of these processes as evidenced by enrichment of the GO terms representative of the regulation of angiogenesis, regulation of immune function, cell migration and proliferation, and organ development and regeneration. Additionally, responses to corticosteroid GO terms were also detected as enriched. Given that corticosteroids are released in response to stress and serve as a negative-feedback loop in regulating the wound healing process (Wicke et al., 2000), this therefore further reaffirms that the cellular response to stress induced by TBOEP mimics that of wound healing. Liver fibrosis is the wound healing response which involves the development of scar tissue that may originate from damage to hepatocytes and may ultimately lead to liver cirrhosis (Vinken, 2013). Given the enrichment of wound healing responses here, this would suggest that TBOEP may result in damage to hepatocytes and may hold potential to induce liver fibrosis. This is in agreement with other studies conducted in rats that have already revealed effects of TBOEP on the liver; including a decrease in cholinesterase activity, an increase in gamma-glutamyltransferase activity, and hepatocellular hypertrophy (World Health Organisation, 2000). A decrease and increase in cholinesterase and gamma-glutamyltransferase have been associated with liver cirrhosis (Meng et al., 2013) and liver damage (Alam et al., 2013) respectively, indicating that using these as biomarkers, TBOEP also showed potential to induce liver fibrosis by way of liver damage in rats.

To further explore how TBOEP exposure may alter immune responses, genes comprising the makeup such GO terms were investigated. Subsequently, TBOEP was found to alter the expression of a number genes involved in various aspects of immune function. For instance, genes involved in the formation (*C8A, C8B, C8G*) and regulation of the complement system (*CFI*), regulation of antigen presentation (*TLR3, CD74, CD79A*), pathogen pattern recognition (*DDX58*), modulation of immune responses (*BCL6, DPP4, F2RL1, HMOX1*) and the upregulation of immune response by upregulating interleukin receptors (*IL4R, IL6R*), were found to be altered in response to TBOEP. It should be noted that while HepG2 cells should not be used as a model cell line for investigating effects on immunity, their ability to mount innate immune responses (Israelow et al., 2014) have allowed for them to be used to investigate the pro-inflammatory effects of drugs on liver cells (Yuhas et al., 2011). Nevertheless, the potential hepatic pro-inflammatory effects of TBOEP seen here are agreement with previous studies documenting effects of some brominated FRs in affecting immune responses by either altering antigen presentation-related molecule expression in mouse immune cells (Koike et al., 2013), or by altering cytokine signalling in human immune cells (Mynster Kronborg et al., 2016).

Finally, TBOEP had effects on lipid metabolism, specifically steroid hormone metabolism. Here, TBOEP was shown to alter the expression of genes encoding for all the major enzymes in steroid hormone biosynthesis. Genes encoding for the major enzymes; 17α-hydroxylase, 17,20 lyase, 17β-hydroxysteroid dehydrogenase, 21-hydroxylase, and aromatase were all shown to be altered at high levels ($|FC|>2$) in response to TBOEP treatment, with nearly all other enzymes involved in steroid hormone biosynthesis barring one (P450-scc) exhibiting altered expression levels compared to control, albeit at smaller fold-change magnitudes. However, changes in expression for all enzymes were not unidirectional. Of interest, estrogenic effects of TBOEP have already been documented. Following a five-day treatment of 0.5 µM TBOEP in zebrafish embryos, an increase in estrogen receptor gene expression was observed, thereby potentiating normal estrogen signalling (Ma et al., 2015). TBOEP was also shown to alter steroid hormone metabolism in human cells (Liu et al., 2012).

Our study therefore confirms that the estrogenic effects of TBOEP are likely as a result of alterations to steroid hormone biosynthesis. The non-unidirectional changes of gene expression in the steroid hormone biosynthesis cascade could be a sign of its lack of gene expression coordination of participating genes. The rationale is that genes making up a specific pathway or function should be regulated in a more coordinated manner than a random set of genes. However, this is not the case for all pathways. For instance, pathways with lower levels of expression coordination between its member genes are usually a result of cross-talk between pathways and that some genes have different roles in different pathways, while gene members of biological processes that are highly conserved and vital to basic biological function (such as transcription and translation) were found to be highly coordinated in expression (Huang et al., 2006). The steroid hormone biosynthesis pathway has already shown to hold little expression coordination amongst its gene members in human cancer cells (Huang et al., 2006), which is perhaps why we observed non-unidirectional changes in gene expression for all genes making up this process.

In vitro to in vivo extrapolation

Given that *in vitro* culturing practices are far removed from blood serum dynamics found *in vivo* and that *in vitro* cell cultures are not comparable to *in vivo* target tissue, models are needed to extrapolate *in vitro* findings to an *in vivo* setting. Firstly, cell culturing practices impart alterations to chemical behaviour (e.g. binding to plastic, evaporation, binding to proteins, etc.) that are not found in an *in vivo* setting. To control for such factors affecting free concentrations *in vitro*, biokinetic models can be used to describe accurate dosimetry (Blaauboer, 2010). Effective concentrations rather than nominal concentrations may then be extrapolated to an *in vivo* setting taking into account factors affecting dosimetry in cell culture (Blaauboer, 2010). Furthermore, simplified *in vitro* models lack complex biological functions such as *in vivo* absorption, distribution, metabolism, and excretion while also lacking the tissue complexity found *in vivo*. To overcome this, the physiologically based pharmacokinetic modelling (PBPK) approach has been introduced which involves

mathematical descriptions of routes of exposure, physiological parameters used to described the *in vivo* biological system of interest, and chemical-specific parameters used to describe the interaction between the chemical and the biological system of interest (D'Yvoire et al., 2007). The above-mentioned modelling approaches are key to quantitative in vitro to in vivo extrapolations (Yoon et al., 2015), a process that has already been successfully used to identify margins of safety for the potential estrogenic effects of parabens using *in vitro* and *in silico* data (Campbell et al., 2015).

These modelling approaches rely on chemical-specific data to be correctly executed. Multiple physiochemical, permeability, and clearance parameters are needed in conjunction with partitioning coefficients, all of which must be empirically derived (D'Yvoire et al., 2007; Kuepfer et al., 2016). Perhaps indicative of the need for empirical data before attempting such an undertaking is exemplified by the recent and only publication involving PBPK modelling and a flame retardant that has been studied for decades, polybrominated diphenyl ether (Song et al., 2016). Given that TBOEP is less well-studied, with its *in vitro* metabolism only recently being characterised in human liver microsomes (Van den Eede et al., 2015) and primary human hepatocytes (Van den Eede et al., 2016), such complex modelling approaches were not attempted to extrapolate our findings to *in vivo* settings. However, as a measure of the nominal concentrations used in this study, along with the only existing study investigating the levels of TBOEP in humans as weight per volume blood, a crude approximation relating the concentrations used in this study to physiologically relevant levels can be made. Whole blood samples from 257 adult individuals revealed that TBOEP was detected in 98.1% of subjects, with a median concentration of 0.54 ng/ml whole blood, and a range of not detected to 16 ng/ml whole blood (Zhao et al., 2016). Considering that for small lipophilic molecules blood flow to the tissue becomes the limiting factor (Jones and Rowland-Yeo, 2013), and that TBOEP has a moderate clearance rate and therefore limited potential to bioaccumulate (Van den Eede et al., 2015), 16 ng/ml whole blood corresponds to approximately 0.04 µM effective concentration that liver epithelial cells might be exposed to, roughly 62 times lower than our lowest nominal

concentration used here. However, given that TBOEP is rapidly metabolised (Van den Eede et al., 2015), and that only the parent compound of TBOEP was measured in human blood (Zhao et al., 2016), it is likely that human body-burden to TBOEP may be higher than indicated by measuring the parent TBOEP compound in blood. This, together with the fact that cell culturing with lipophilic compounds may result in reduced effective free concentrations that the cells may be exposed to and therefore lead to underestimation of toxic potency (Gülden et al., 2015), indicates that the lowest concentration used in this study may be even more relevant to physiological exposure levels. This is further compounded by the fact that toddlers are exposed to much higher levels of TBOEP than adults by a factor of nearly 10 times and perhaps more by measuring dust intake (Van den Eede et al., 2011), further making the lowest nominal concentration used in this study more physiologically relevant.

Comparing our findings to those conducted *in vivo*, nominal external concentrations similar to the ones used here were found to elicit adverse effects by altering steroid hormone biosynthesis. For instance, treatment with 1.25 µM TBOEP for 21 days resulted in decreased egg production, hatching success, and survival rates in zebrafish offspring while also being found to affect gonadal development and sex hormone homeostasis (Xu et al., 2017). It should be stressed that the approach used above serves as a crude approximation to correlate the concentrations used here to physiologically relevant levels of TBOEP while also correlating our findings to other *in vivo* studies. For a thorough evaluation that can not only qualitatively but also quantitatively extrapolate our findings *in vitro* to *in vivo* settings, a fully described PBPK model would be needed. Nevertheless, despite this, given that risk assessment involves both hazard identification (which is the identification of potential health effects irrespective of potential exposure levels) and exposure assessments (which identifies potential exposure levels likely to be encountered), this study serves as a hazard identification of TBOEP to elicit changes in the transcriptome that may give rise to health effects independent of concentrations that might be experienced in a practical setting. Such studies

in conjunction with *in vivo* toxicological studies could prove useful in predicting adverse health effects (Rouquie et al., 2015), and can also support risk assessment in a weight-of-evidence approach (Behl et al., 2015; Colnot et al., 2014).

Future perspectives

While HepG2 cells are of liver origin, they appear to maintain lower metabolism of xenobiotics compared to primary hepatocytes as evidenced by lower expression of some phase I and phase II enzymes, both at a gene (Wilkening et al., 2003) and protein level compared to primary hepatocytes (Wiśniewski et al., 2016). However, even primary human hepatocytes had a low level of sensitivity when detecting for known hepatotoxins (Gerets et al., 2012), highlighting that there is currently no perfect *in vitro* model for predicting drug metabolism. As such, further research using other *in vitro* liver models is required to develop a comprehensive understanding of potential hepatotoxic effects of TBOEP and its metabolites.

5. Conclusion

Given the gaps in toxicological MOA data for TBOEP, we adopted RNA-seq to identify molecular changes in human hepatocellular carcinoma cells in response to TBOEP. TBOEP showed limited cytotoxicity, with mitochondrial activity increasing directly before cytotoxic effects occurred at very high concentrations. A transcriptome-wide approach was employed using two non-cytotoxic concentrations, the lowest of which might hold relevance to human physiological levels. Results from over-representation and gene-set enrichment analysis correlated well and revealed upregulation of protein, DNA, and energy metabolism. These changes were accompanied by enrichment of GO terms for a wide variety of biological processes, but most significantly for wound healing, immune function, and steroid hormone biosynthesis, which is in agreement with other studies.

Acknowledgements

This study was performed within the framework of a Marie Curie Initial Training Network (INFLAME, grant agreement 264600), funded by the 7th EU Framework Programme. Research was funded by a Ph.D grant of the Agency for Innovation by Science and Technology (IWT) to BVK. This work was also supported by the Next Generation Sequencing Consortium Antwerp. We specifically thank the staff of the Centre of Medical Genetics for their help with the sequencing runs.

References

Alam, S., Noor-E-Alam, S.M., Chowdhury, Z.R., Alam, M., Kabir, J., 2013. Nonalcoholic steatohepatitis in nonalcoholic fatty liver disease patients of Bangladesh. World J. Hepatol. 5, 281–287. doi:10.4254/wjh.v5.i5.281

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrrano, J. a, Tietge, J.E., Villeneuve, D.L., 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ. Toxicol. Chem. 29, 730–41. doi:10.1002/etc.34

Baggerly, K.A., Deng, L., Morris, J.S., Aldaz, C.M., 2003. Differential expression in SAGE: Accounting for normal between-library variation. Bioinformatics 19, 1477–1483. doi:10.1093/bioinformatics/btg173

Bai J, Sadrolodabaee L, Ching CB, Chowbay B, Chen WN, 2010. A Comparative Proteomic Analysis of HepG2 cells Incubated by S(-) and R(+) Enantiomers of Anti-coagulating Drug Warfarin. Proteomics 10, 1463–1473. doi:10.1002/pmic.200900785

Beane, J., Vick, J., Schembri, F., Anderlind, C., Gower, A., Campbell, J., Luo, L., Zhang, X.H., Xiao, J., Alekseyev, Y.O., Wang, S., Levy, S., Massion, P.P., Lenburg, M., Spira, A., 2011. Characterizing the impact of smoking and lung cancer on the airway transcriptome using RNA-Seq. Cancer Prev. Res. 4, 803–817. doi:10.1158/1940-6207.CAPR-11-0212

Behl, M., Hsieh, J.-H., Shafer, T.J., Mundy, W.R., Rice, J.R., Boyd, W.A., Freedman, J.H., Hunter, E.S., Jarema, K., Padilla, S., Tice, R.R., 2015. Use of alternative assays to identify and prioritize organophosphorus flame retardants for potential developmental and neurotoxicity. Neurotoxicol. Teratol. doi:10.1016/j.ntt.2015.09.003

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. B 57, 289–300.

Bjerrum, J.T., Rantalainen, M., Wang, Y., Olsen, J., Nielsen, O.H., 2013. Integration of transcriptomics and metabonomics: improving diagnostics, biomarker identification and phenotyping in ulcerative colitis. Metabolomics 1–11. doi:10.1007/s11306-013-0580-3

Blaauboer, B.J., 2010. Biokinetic modeling and in vitro-in vivo extrapolations. J. Toxicol. Environ. Health. B. Crit. Rev. 13, 242–52.

Bouwman, H., Booyens, P., Govender, D., Pienaar, D., Polder, A., 2014. Chlorinated, brominated, and fluorinated organic pollutants in Nile crocodile eggs from the Kruger National Park, South Africa. Ecotoxicol. Environ. Saf. 104, 393–402. doi:10.1016/j.ecoenv.2013.12.005

Braune, B.M., Letcher, R.J., Gaston, A.J., Mallory, M.L., 2015. Trends of polybrominated diphenyl ethers and hexabromocyclododecane in eggs of Canadian Arctic seabirds reflect changing use patterns. Environ. Res. 142, 651–661. doi:10.1016/j.envres.2015.08.010

Breitholtz, M., Nyholm, J.R., Karlsson, J., Andersson, P.L., 2008. Are individual NOEC levels safe for mixtures? A study on mixture toxicity of brominated flame-retardants in the copepod Nitocra spinipes. Chemosphere 72, 1242–1249. doi:10.1016/j.chemosphere.2008.05.004

Campbell, J.L., Yoon, M., Clewell, H.J., 2015. A case study on quantitative in vitro to in vivo extrapolation for environmental esters: Methyl-, propyl- and butylparaben. Toxicology 332, 67–

76. doi:10.1016/j.tox.2015.03.010

Colnot, T., Kacew, S., Dekant, W., 2014. Mammalian toxicology and human exposures to the flame retardant 2,2',6,6'-tetrabromo-4,4'-isopropylidenediphenol (TBBPA): implications for risk assessment. Arch. Toxicol. 88, 553–73. doi:10.1007/s00204-013-1180-8

Connor, S.C., Hansen, M.K., Corner, A., Smith, R.F., Ryan, T.E., 2010. Integration of metabolomics and transcriptomics data to aid biomarker discovery in type 2 diabetes. Mol. Biosyst. 6, 909–21. doi:10.1039/b914182k

D'Yvoire, M.B., Prieto, P., Blaauboer, B.J., Bois, F.Y., Boobis, A., Brochot, C., Coecke, S., Freidig, A., Gundert-Remy, U., Hartung, T., Jacobs, M.N., Lavé, T., Leahy, D.E., Lennernäs, H., Loizou, G.D., Meek, B., Pease, C., Rowland, M., Spendiff, M., Yang, J., Zeilmaker, M., 2007. Physiologically-based kinetic modelling (PBK modelling): Meeting the 3Rs agenda. ATLA Altern. to Lab. Anim. 35, 661–671.

de Boer, J., Ballesteros-Gómez, A., Leslie, H.A., Brandsma, S.H., Leonards, P.E.G., 2015. Flame retardants: Dust - And not food - Might be the risk. Chemosphere 150, 2014–2017. doi:10.1016/j.chemosphere.2015.12.124

Ding, J., Xu, Z., Huang, W., Feng, L., Yang, F., 2016. Organophosphate ester flame retardants and plasticizers in human placenta in Eastern China. Sci. Total Environ. 554–555, 211–217. doi:10.1016/j.scitotenv.2016.02.171

Dingemans, M.M.L., de Groot, A., van Kleef, R.G.D.M., Bergman, Å., van den Berg, M., Vijverberg, H.P.M., Westerink, R.H.S., 2008. Hydroxylation increases the neurotoxic potential of BDE-47 to affect exocytosis and calcium homeostasis in PC12 cells. Environ. Health Perspect. 116, 637–643. doi:10.1289/ehp.11059

Dingemans, M.M.L., van den Berg, M., Westerink, R.H.S., 2011. Neurotoxicity of brominated flame retardants: (In)direct effects of parent and hydroxylated polybrominated diphenyl ethers on the (Developing) nervous system. Environ. Health Perspect. 119, 900–907. doi:10.1289/ehp.1003035

Dodson, R.E., Perovich, L.J., Covaci, A., Van den Eede, N., Ionas, A.C., Dirtu, A.C., Brody, J.G., Rudel, R. a, 2012. After the PBDE phase-out: a broad suite of flame retardants in repeat house dust samples from California. Environ. Sci. Technol. 46, 13056–66. doi:10.1021/es303879n

Du, Z., Wang, G., Gao, S., Wang, Z., 2015. Aryl organophosphate flame retardants induced cardiotoxicity during zebrafish embryogenesis: By disturbing expression of the transcriptional regulators. Aquat. Toxicol. 161, 25–32. doi:10.1016/j.aquatox.2015.01.027

Egloff, C., Crump, D., Porter, E., Williams, K.L., Letcher, R.J., Gauthier, L.T., Kennedy, S.W., 2014. Tris (2-butoxyethyl) phosphate and triethyl phosphate alter embryonic development , hepatic mRNA expression , thyroid hormone levels , and circulating bile acid concentrations in chicken embryos. Toxicol. Appl. Pharmacol. 279, 303–310. doi:10.1016/j.taap.2014.06.024

Fan, X., Kubwabo, C., Rasmussen, P.E., Wu, F., 2014. Simultaneous determination of thirteen organophosphate esters in settled indoor house dust and a comparison between two sampling techniques. Sci. Total Environ. 491–492, 80–86. doi:10.1016/j.scitotenv.2013.12.127

Gerets, H.H.J., Tilmant, K., Gerin, B., Chanteux, H., Depelchin, B.O., Dhalluin, S., Atienzar, F.A., 2012. Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human

hepatotoxins. Cell Biol. Toxicol. 28, 69–87. doi:10.1007/s10565-011-9208-4

Ghosh, S., Dent, R., Harper, M.-E., Gorman, S. a, Stuart, J.S., McPherson, R., 2010. Gene expression profiling in whole blood identifies distinct biological pathways associated with obesity. BMC Med. Genomics 3, 56. doi:10.1186/1755-8794-3-56

Giraudo, M., Douville, M., Houde, M., 2015. Chronic toxicity evaluation of the flame retardant tris (2-butoxyethyl) phosphate (TBOEP) using Daphnia magna transcriptomic response. Chemosphere 132, 159–165. doi:10.1016/j.chemosphere.2015.03.028

Greaves, A.K., Letcher, R.J., 2014. Comparative body compartment composition and in ovo transfer of organophosphate flame retardants in north american great lakes herring gulls. Environ. Sci. Technol. 48, 7942–50. doi:10.1021/es501334w

Gülden, M., Schreiner, J., Seibert, H., 2015. In vitro toxicity testing with microplate cell cultures: Impact of cell binding. Toxicology 332, 41–51. doi:10.1016/j.tox.2013.11.006

Guo, S., DiPietrio, L., 2010. Factors Affecting Wound Healing. J. Dent. Res. 89, 219–229. doi:10.1177/0022034509359125

Guyot, R., Chatonnet, F., Gillet, B., Hughes, S., Flamant, F., 2014. Toxicogenomic analysis of the ability of brominated flame retardants TBBPA and BDE-209 to disrupt thyroid hormone signaling in neural cells. Toxicology 325, 125–132. doi:10.1016/j.tox.2014.08.007

Han, Z., Wang, Q., Fu, J., Chen, H., Zhao, Y., Zhou, B., Gong, Z., Wei, S., Li, J., Liu, H., Zhang, X., Liu, C., Yu, H., 2014. Multiple bio-analytical methods to reveal possible molecular mechanisms of developmental toxicity in zebrafish embryos/larvae exposed to tris(2-butoxyethyl) phosphate. Aquat. Toxicol. 150, 175–181. doi:10.1016/j.aquatox.2014.03.013

Hedegaard, J., Arce, C., Bicciato, S., Bonnet, A., Buitenhuis, B., Collado-Romero, M., Conley, L.N., SanCristobal, M., Ferrari, F., Garrido, J.J., Groenen, M.A., Hornshøj, H., Hulsegge, I., Jiang, L., Jiménez-Marín, Á., Kommadath, A., Lagarrigue, S., Leunissen, J.A., Liaubet, L., Neerincx, P.B., Nie, H., Poel, J. van der, Prickett, D., Ramirez-Boo, M., Rebel, J.M., Robert-Granié, C., Skarman, A., Smits, M.A., Sørensen, P., Tosser-Klopp, G., Watson, M., 2009. Methods for interpreting lists of affected genes obtained in a DNA microarray experiment. BMC Proc. 3, S5. doi:10.1186/1753-6561-3-s4-s5

Hook, S.E., Twine, N.A., Simpson, S.L., Spadaro, D.A., Moncuquet, P., Wilkins, M.R., 2014. 454 pyrosequencing-based analysis of gene expression profiles in the amphipod Melita plumulosa: Transcriptome assembly and toxicant induced changes. Aquat. Toxicol. 153, 73–88. doi:10.1016/j.aquatox.2013.11.022

Huang, R., Wallqvist, A., Covell, D.G., 2006. Comprehensive analysis of pathway or functionally related gene expression in the National Cancer Institute's anticancer screen. Genomics 87, 315–328. doi:10.1016/j.ygeno.2005.11.011

Israelow, B., Narbus, C.M., Sourisseau, M., Evans, M.J., 2014. HepG2 cells mount an effective antiviral interferon-lambda based innate immune response to hepatitis C virus infection. Hepatology 60, 1170–1179. doi:10.1002/hep.27227

Jennen, D., Ruiz-Aracama, A., Magkoufopoulou, C., Peijnenburg, A., Lommen, A., van Delft, J., Kleinjans, J., 2011. Integrating transcriptomics and metabonomics to unravel modes-of-action of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in HepG2 cells. BMC Syst. Biol. 5, 139. doi:10.1186/1752-0509-5-139

Jones, H., Rowland-Yeo, K., 2013. Basic Concepts in Physiologically Based Pharmacokinetic Modeling in Drug Discovery and Development. CPT Pharmacometrics Syst. Pharmacol. 2, e63. doi:10.1038/psp.2013.41

Kim, J.-W., Isobe, T., Muto, M., Tue, N.M., Katsura, K., Malarvannan, G., Sudaryanto, A., Chang, K.-H., Prudente, M., Viet, P.H., Takahashi, S., Tanabe, S., 2014. Organophosphorus flame retardants (PFRs) in human breast milk from several Asian countries. Chemosphere 116, 91–97. doi:10.1016/j.chemosphere.2014.02.033

Koike, E., Yanagisawa, R., Takigami, H., Takano, H., 2013. Brominated flame retardants stimulate mouse immune cells in vitro. J. Appl. Toxicol. 33, 1451–9. doi:10.1002/jat.2809

Krivoshiev, B. V, Dardenne, F., Blust, R., Covaci, A., Husson, S.J., 2015. Elucidating toxicological mechanisms of current flame retardants using a bacterial gene profiling assay. Toxicol. Vitr. doi:10.1016/j.tiv.2015.09.001

Krivoshiev, B. V, Dardenne, F., Covaci, A., Blust, R., Husson, S.J., 2016. Assessing in-vitro estrogenic effects of currently-used flame retardants. Toxicol. Vitr. 331. Krivo, 153–162. doi:10.1016/j.tiv.2016.03.006

Kuepfer, L., Niederalt, C., Wendl, T., Schlender, J.F., Willmann, S., Lippert, J., Block, M., Eissing, T., Teutonico, D., 2016. Applied Concepts in PBPK Modeling: How to Build a PBPK/PD Model. CPT Pharmacometrics Syst. Pharmacol. 5, 516–531. doi:10.1002/psp4.12134

Li, J., Giesy, J.P., Yu, L., Li, G., Liu, C., 2015. Effects of Tris (1,3-dichloro-2- propyl) Phosphate (TDCPP) in Tetrahymena Thermophila : Targeting the Ribosome. Nat. Publ. Gr. 1–9. doi:10.1038/srep10562

Li, J., Yu, N., Zhang, B., Jin, L., Li, M., Hu, M., Zhang, X., Wei, S., Yu, H., 2014. Occurrence of organophosphate flame retardants in drinking water from China. Water Res. 54, 53–61. doi:10.1016/j.watres.2014.01.031

Linford, N.J., Beyer, R.P., Gollahon, K., Krajcik, R.A., Malloy, V.L., Demas, V., Burmer, G.C., Rabinovitch, P.S., 2007. Transcriptional response to aging and caloric restriction in heart and adipose tissue. Aging Cell 6, 673–688. doi:10.1111/j.1474-9726.2007.00319.x

Liu, X., Ji, K., Choi, K., 2012. Endocrine disruption potentials of organophosphate flame retardants and related mechanisms in H295R and MVLN cell lines and in zebrafish. Aquat. Toxicol. 114–115, 173–181. doi:10.1016/j.aquatox.2012.02.019

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and. Methods 25, 402–408. doi:10.1006/meth.2001.1262

Ma, Z., Tang, S., Su, G., Miao, Y., Liu, H., Xie, Y., Giesy, J.P., Saunders, D.M.V., Hecker, M., Yu, H., 2016. Effects of tris (2-butoxyethyl) phosphate (TBOEP) on endocrine axes during development of early life stages of zebrafish (Danio rerio). Chemosphere 144, 1920–1927. doi:10.1016/j.chemosphere.2015.10.049

Ma, Z., Yu, Y., Tang, S., Liu, H., Su, G., Xie, Y., Giesy, J.P., Hecker, M., Yu, H., 2015. Differential modulation of expression of nuclear receptor mediated genes by tris (2-butoxyethyl) phosphate (TBOEP) on early life stages of zebrafish (Danio rerio). Aquat. Toxicol. 169, 196–203. doi:10.1016/j.aquatox.2015.10.017

Maere, S., Heymans, K., Kuiper, M., 2005. BiNGO: A Cytoscape plugin to assess overrepresentation of

Gene Ontology categories in Biological Networks. Bioinformatics 21, 3448–3449. doi:10.1093/bioinformatics/bti551

Magkoufopoulou, C., Claessen, S.M.H., Tsamou, M., Jennen, D.G.J., Kleinjans, J.C.S., Van delft, J.H.M., 2012. A transcriptomics-based in vitro assay for predicting chemical genotoxicity in vivo. Carcinogenesis 33, 1421–1429. doi:10.1093/carcin/bgs182

Malarvannan, G., Belpaire, C., Geeraerts, C., Eulaers, I., Neels, H., Covaci, A., 2014. Assessment of persistent brominated and chlorinated organic contaminants in the European eel (Anguilla anguilla) in Flanders, Belgium: Levels, profiles and health risk. Sci. Total Environ. 482–483, 222–33. doi:10.1016/j.scitotenv.2014.02.127

Marklund, A., Andersson, B., Haglund, P., 2003. Screening of organophosphorus compounds and their distribution in various indoor environments. Chemosphere 53, 1137–46. doi:10.1016/S0045-6535(03)00666-0

McMillian, M.K., Li, L., Parker, J.B., Patel, L., Zhong, Z., Gunnett, J.W., Powers, W.J., Johnson, M.D., 2002. An improved resazurin-based cytotoxicity assay for hepatic cells. Cell Biol. Toxicol. 18, 157–173. doi:10.1023/A:1015559603643

Meng, F., Yin, X., Ma, X., Guo, X.-D., Jin, B., Li, H., 2013. Assessment of the value of serum cholinesterase as a liver function test for cirrhotic patients. Biomed. reports 1, 265–268. doi:10.3892/br.2013.60

Merico, D., Isserlin, R., Stueker, O., Emili, A., Bader, G.D., 2010. Enrichment map: A network-based method for gene-set enrichment visualization and interpretation. PLoS One 5. doi:10.1371/journal.pone.0013984

Mikus, J., Steverding, D., 2000. A simple colorimetric method to screen drug cytotoxicity against Leishmania using the dye Alamar Blue. Parasitol. Int. 48, 265–269. doi:10.1016/S1383-5769(99)00020-3

Mynster Kronborg, T., Frohnert Hansen, J., Nielsen, C.H., Ramhøj, L., Frederiksen, M., Vorkamp, K., Feldt-Rasmussen, U., 2016. Effects of the Commercial Flame Retardant Mixture DE-71 on Cytokine Production by Human Immune Cells. PLoS One 11, e0154621. doi:10.1371/journal.pone.0154621

Page, B., Page, M., Noel, C., 1993. A new fluorometric assay for cytotoxicity measurements in-vitro. Int. J. Oncol. 3, 473–476.

Porter, E., Crump, D., Egloff, C., Chiu, S., Kennedy, S.W., 2014. Use of an avian hepatocyte assay and the avian toxchip polymerse chain reaction array for testing prioritization of 16 organic flame retardants. Environ. Toxicol. Chem. 33, 573–82. doi:10.1002/etc.2469

Ren, S., Peng, Z., Mao, J.-H., Yu, Y., Yin, C., Gao, X., Cui, Z., Zhang, J., Yi, K., Xu, W., Chen, C., Wang, F., Guo, X., Lu, J., Yang, J., Wei, M., Tian, Z., Guan, Y., Tang, L., Xu, C., Wang, L., Gao, X., Tian, W., Wang, J., Yang, H., Wang, J., Sun, Y., 2012. RNA-seq analysis of prostate cancer in the Chinese population identifies recurrent gene fusions, cancer-associated long noncoding RNAs and aberrant alternative splicings. Cell Res. 22, 806–821. doi:10.1038/cr.2012.30

Ren, S., Wang, F., Shen, J., Sun, Y., Sun, Y., Xu, W., Lu, J., Wei, M., Xu, C., Wu, C., Zhang, Z., Gao, X., Liu, Z., Hou, J., Huang, J., 2013. Long non-coding RNA metastasis associated in lung adenocarcinoma transcript 1 derived miniRNA as a novel plasma-based biomarker for diagnosing prostate cancer. Eur. J. Cancer 49, 2949–2959. doi:10.1016/j.ejca.2013.04.026

Rouquie, D., Heneweer, M., Botham, J., Ketelslegers, H., Markell, L., Pfister, T., Steiling, W., Strauss, V., Hennes, C., 2015. Contribution of new technologies to characterization and prediction of adverse effects. Crit. Rev. Toxicol. 45, 172–183. doi:10.3109/10408444.2014.986054

Ryan, J.J., Rawn, D.F.K., 2014. The brominated flame retardants, PBDEs and HBCD, in Canadian human milk samples collected from 1992 to 2005; concentrations and trends. Environ. Int. 70, 1–8. doi:10.1016/j.envint.2014.04.020

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V., Quackenbush, J., 2003. TM4: A Free, Open-Source System for Microarray Data Management and Analysis. Microarray Technol. 34, 374–377.

Salamova, A., Hermanson, M.H., Hites, R.A., 2014. Organophosphate and halogenated flame retardants in atmospheric particles from a European arctic site. Environ. Sci. Technol. 48, 6133–40. doi:10.1021/es500911d

Santín, G., Eljarrat, E., Barceló, D., 2016. Simultaneous determination of 16 organophosphorus flame retardants and plasticizers in fish by liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1441, 34–43. doi:10.1016/j.chroma.2016.02.058

Smith, S., Bernatchez, L., Beheregaray, L.B., 2013. RNA-seq analysis reveals extensive transcriptional plasticity to temperature stress in a freshwater fish species. BMC Genomics 14, 375. doi:10.1186/1471-2164-14-375

Song, G., Peeples, C.R., Yoon, M., Wu, H., Verner, M.A., Andersen, M.E., Clewell, H.J., Longnecker, M.P., 2016. Pharmacokinetic bias analysis of the epidemiological associations between serum polybrominated diphenyl ether (BDE-47) and timing of menarche. Environ. Res. 150, 541–548. doi:10.1016/j.envres.2016.07.004

Souza, T., Jennen, D., van Delft, J., van Herwijnen, M., Kyrtoupolos, S., Kleinjans, J., 2016. New insights into BaP-induced toxicity: role of major metabolites in transcriptomics and contribution to hepatocarcinogenesis. Arch. Toxicol. 90, 1449–1458. doi:10.1007/s00204-015-1572-z

Stormo, C., Kringen, M.K., Lyle, R., Olstad, O.K., Sachse, D., Berg, J.P., Piehler, A.P., 2014. RNA-Sequencing Analysis of HepG2 Cells Treated with Atorvastatin. PLoS One 9, e105836. doi:10.1371/journal.pone.0105836

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M. a, Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., Mesirov, J.P., 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U. S. A. 102, 15545–50. doi:10.1073/pnas.0506580102

Summeren, A. Van, Renes, J., Bouwman, F.G., Noben, J., Delft, J.H.M. Van, Kleinjans, J.C.S., Mariman, E.C.M., 2011. Proteomics Investigations of Drug-Induced Hepatotoxicity in HepG2 Cells 120, 109–122. doi:10.1093/toxsci/kfq380

Sun, L., Tan, H., Peng, T., Wang, S., Xu, W., Qian, H., Jin, Y., Fu, Z., 2016a. Developmental neurotoxicity of organophosphate flame retardants in early life stages of Japanese medaka (Oryzias latipes). Environ. Toxicol. Chem. doi:10.1002/etc.3477

Sun, L., Xu, W., Peng, T., Chen, H., Ren, L., Tan, H., Xiao, D., Qian, H., Fu, Z., 2016b. Developmental exposure of zebrafish larvae to organophosphate flame retardants causes neurotoxicity.

Neurotoxicol. Teratol. 55, 16–22. doi:10.1016/j.ntt.2016.03.003

Supek, F., Bosnjak, M., Skunca, N., Smuc, T., 2011. Revigo summarizes and visualizes long lists of gene ontology terms. PLoS One 6. doi:10.1371/journal.pone.0021800

Valentin-Severin, I., Le Hegarat, L., Lhuguenot, J.-C., Le Bon, A.-M., Chagnon, M.-C., 2003. Use of HepG2 cell line for direct or indirect mutagens screening: comparative investigation between comet and micronucleus assays. Mutat. Res. Toxicol. Environ. Mutagen. 536, 79–90. doi:10.1016/S1383-5718(03)00031-7

van Boxtel, A.L., Kamstra, J.H., Cenijn, P.H., Pieterse, B., Wagner, J.M., Antink, M., Krab, K., van der Burg, B., Marsh, G., Brouwer, A., Legler, J., 2008. Microarray analysis reveals a mechanism of phenolic polybrominated diphenylether toxicity in zebrafish. Environ. Sci. Technol. 42, 1773–9.

Van Delft, J., Gaj, S., Lienhard, M., Albrecht, M.W., Kirpiy, A., Brauers, K., Claessen, S., Lizarraga, D., Lehrach, H., Herwig, R., Kleinjans, J., 2012. Rna-seq provides new insights in the transcriptome responses induced by the carcinogen benzo[a]pyrene. Toxicol. Sci. 130, 427–439. doi:10.1093/toxsci/kfs250

Van den Eede, N., de Meester, I., Maho, W., Neels, H., Covaci, A., 2016. Biotransformation of three phosphate flame retardants and plasticizers in primary human hepatocytes: untargeted metabolite screening and quantitative assessment. J. Appl. Toxicol. 36, 1401–1408. doi:10.1002/jat.3293

Van den Eede, N., Dirtu, A.C., Neels, H., Covaci, A., 2011. Analytical developments and preliminary assessment of human exposure to organophosphate flame retardants from indoor dust. Environ. Int. 37, 454–461. doi:10.1016/j.envint.2010.11.010

Van den Eede, N., Erratico, C., Exarchou, V., Maho, W., Neels, H., Covaci, A., 2015. In vitro biotransformation of tris (2-butoxyethyl) phosphate (TBOEP) in human liver and serum. Toxicol. Appl. Pharmacol. 284, 246–253. doi:10.1016/j.taap.2015.01.021

van der Veen, I., de Boer, J., 2012. Phosphorus flame retardants: Properties, production, environmental occurrence, toxicity and analysis. Chemosphere 88, 1119–1153. doi:10.1016/j.chemosphere.2012.03.067

Vinken, M., 2013. The adverse outcome pathway concept: A pragmatic tool in toxicology. Toxicology 312, 158–165. doi:10.1016/j.tox.2013.08.011

Von Eyken, A., Pijuan, L., Marti, R., Blanco, M.J., Diaz-Ferrero, J., 2016. Determination of Dechlorane Plus and related compounds (dechlorane 602, 603 and 604) in fish and vegetable oils. Chemosphere 144, 1256–1263. doi:10.1016/j.chemosphere.2015.10.001

Wei, G., Li, D., Zhuo, M., Liao, Y., Xie, Z., 2015. Organophosphorus flame retardants and plasticizers : Sources , occurrence , toxicity and human exposure. Environ. Pollut. 196, 29–46. doi:10.1016/j.envpol.2014.09.012

Weidner, C., Steinfath, M., Opitz, E., Oelgeschläger, M., Schönfelder, G., 2016. Defining the optimal animal model for translational research using gene set enrichment analysis. EMBO Mol. Med. 8, 831–838. doi:10.15252/emmm.201506025

Wicke, C., Halliday, B., Allen, D., Roche, N.S., Scheuenstuhl, H., Spencer, M.M., Roberts, a B., Hunt, T.K., 2000. Effects of steroids and retinoids on wound healing. Arch. Surg. 135, 1265–1270. doi:10.1001/archsurg.135.11.1265

Wilkening, S., Stahl, F., Bader, A., 2003. Comparison of primary hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation properties. Drug Metab. Dispos. 31, 1035–1042.

Wiśniewski, J.R., Vildhede, A., Norén, A., Artursson, P., 2016. In-depth quantitative analysis and comparison of the human hepatocyte and hepatoma cell line HepG2 proteomes. J. Proteomics 136, 234–247. doi:10.1016/j.jprot.2016.01.016

World Health Organisation, 2000. Flame retardants : tris(2-butoxyethyl) phosphate, tris(2-ethylhexyl) phosphate and tetrakis(hydroxymethyl) phosphonium salts.

Xu, Q., Wu, D., Dang, Y., Yu, L., Liu, C., Wang, J., 2017. Reproduction impairment and endocrine disruption in adult zebrafish (Danio rerio) after waterborne exposure to TBOEP. Aquat. Toxicol. 182, 163–171. doi:10.1016/j.aquatox.2016.11.019

Xu, T., Zhao, J., Yin, D., Zhao, Q., Dong, B., 2015. High-throughput RNA sequencing reveals the effects of 2,2′,4,4′ -tetrabromodiphenyl ether on retina and bone development of zebrafish larvae. BMC Genomics 16, 1–12. doi:10.1186/s12864-014-1194-5

Yang, N., Kaur, S., Volinia, S., Greshock, J., Lassus, H., Hasegawa, K., Liang, S., Leminen, A., Deng, S., Smith, L., Johnstone, C.N., Chen, X.M., Liu, C.G., Huang, Q., Katsaros, D., Calin, G.A., Weber, B.L., B??tzow, R., Croce, C.M., Coukos, G., Zhang, L., 2008. MicroRNA microarray identifies Let-7i as a novel biomarker and therapeutic target in human epithelial ovarian cancer. Cancer Res. 68, 10307–10314. doi:10.1158/0008-5472.CAN-08-1954

Yednock, B.K., Sullivan, T.J., Neigel, J.E., 2015. De novo assembly of a transcriptome from juvenile blue crabs (Callinectes sapidus) following exposure to surrogate Macondo crude oil. BMC Genomics 16, 521. doi:10.1186/s12864-015-1739-2

Yoon, M., Blaauboer, B.J., Clewell, H.J., 2015. Quantitative in vitro to in vivo extrapolation (QIVIVE): An essential element for in vitro-based risk assessment. Toxicology 332, 1–3. doi:10.1016/j.tox.2015.02.002

Yuhas, Y., Berent, E., Ashkenazi, S., 2011. Effect of rifampin on production of inflammatory mediators in HepG2 liver epithelial cells. Antimicrob. Agents Chemother. 55, 5541–5546. doi:10.1128/AAC.05149-11

Zhang, J., Abou-Elwafa Abdallah, M., Williams, T.D., Harrad, S., Chipman, J.K., Viant, M.R., 2016. Gene expression and metabolic responses of HepG2/C3A cells exposed to flame retardants and dust extracts at concentrations relevant to indoor environmental exposures. Chemosphere 144, 1996–2003. doi:10.1016/j.chemosphere.2015.10.014

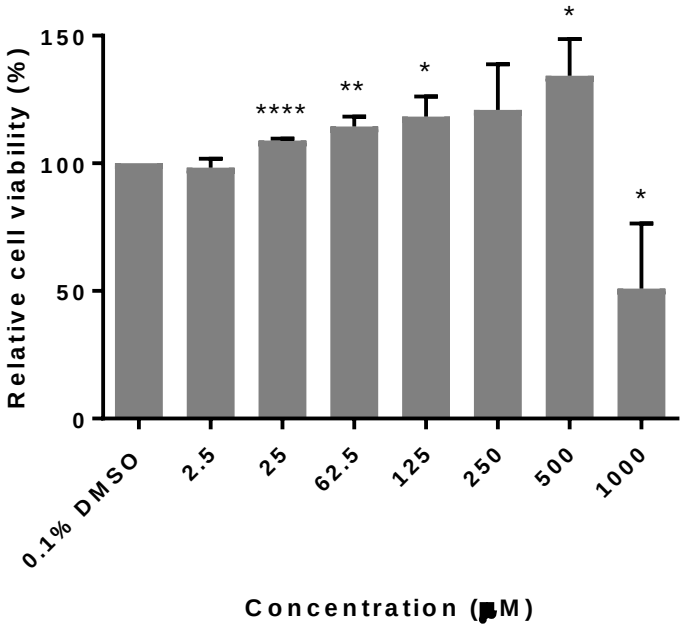
Zhang, J., Williams, T.D., Abdallah, M.A.-E., Harrad, S., Chipman, J.K., Viant, M.R., 2015a. Transcriptomic and metabolomic approaches to investigate the molecular responses of human cell lines exposed to the flame retardant hexabromocyclododecane (HBCD). Toxicol. Vitr. doi:10.1016/j.tiv.2015.08.017

Zhang, J., Williams, T.D., Chipman, J.K., Viant, M.R., 2015b. Defensive and adverse energy-related molecular responses precede tris (1, 3-dichloro-2-propyl) phosphate cytotoxicity. J. Appl. Toxicol. n/a-n/a. doi:10.1002/jat.3194

Zhao, F., Wan, Y., Zhao, H., Hu, W., Mu, D., Webster, T.F., Hu, J., 2016. Levels of Blood Organophosphorus Flame Retardants and Association with Changes in Human Sphingolipid Homeostasis. Environ. Sci. Technol. acs.est.6b02474. doi:10.1021/acs.est.6b02474

Zheng, X., Xu, F., Luo, X., Mai, B., Covaci, A., 2015. Phosphate flame retardants and novel brominated flame retardants in home-produced eggs from an e-waste recycling region in China. Chemosphere 150, 545–550. doi:10.1016/j.chemosphere.2015.09.098

Supporting Information



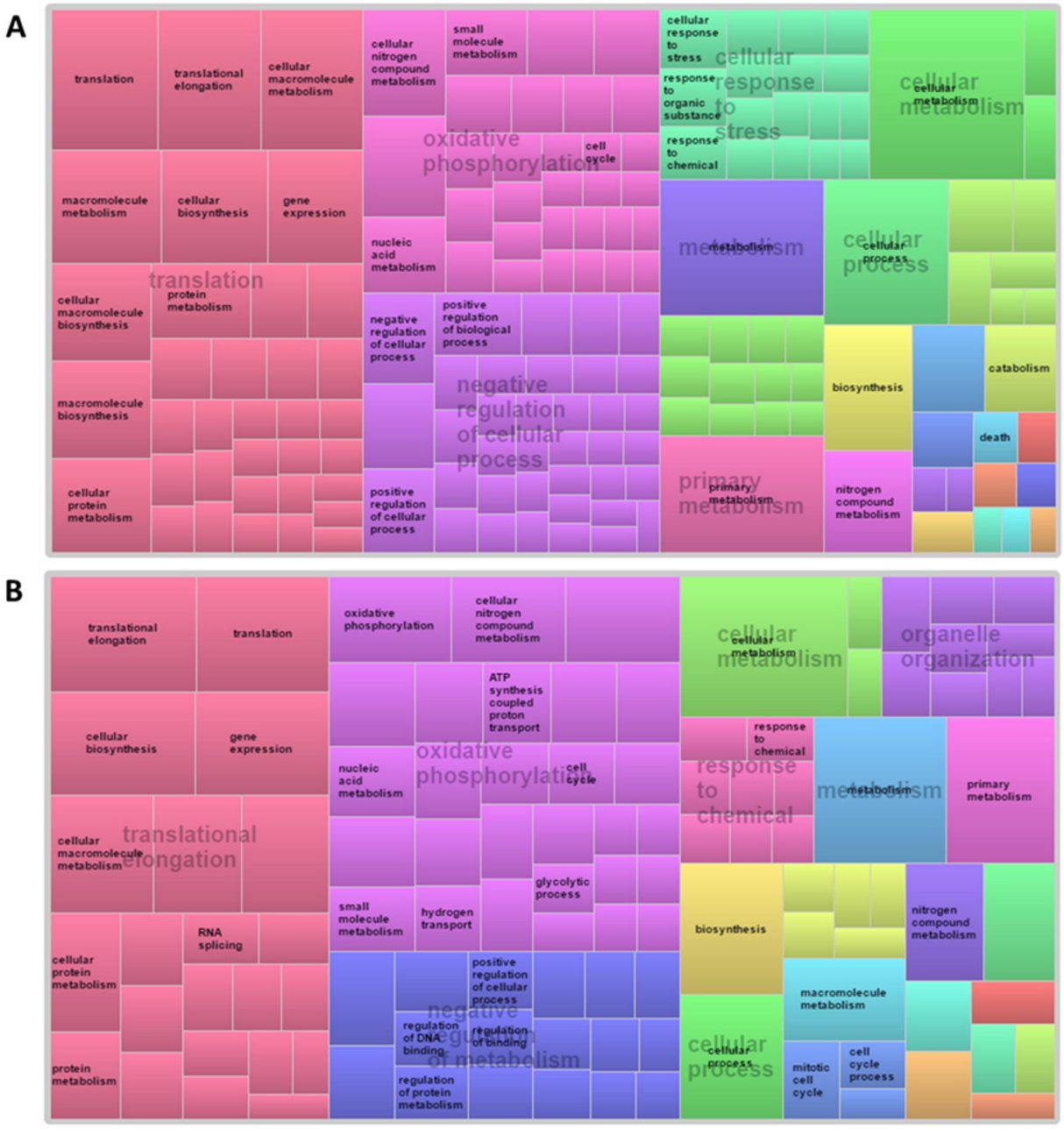
Supplementary Figure 1. Cytotoxic effects of TBOEP on HepG2 cells indicated as a measurement of cell viability. Alterations to mitochondrial activity was considered significant across experiments compared to control following the unpaired t-tests test statistic; *p<0.05 **p<0.01 ***P<0.001 ****p<0.0001.

Treatment	Dose	Triplicate	Number of reads	Reads mapped in pairs (%)	Reads mapped in broken pairs (%)	Reads not mapped (%)	Total fragments	Total unique fragments (%)	Total non-specific fragments (%)	Uncounted fragments (%)	Total fragments mapped to exons (%)
TBOEP	High	1	20,924,414	78.51	5.58	15.90	10,462,207	78.48	0.03	21.49	91.40
	125µM	2	30,297,034	79.46	4.34	16.19	15,148,517	79.43	0.03	20.54	91.53
		3	7,070,344	76.48	7.33	16.18	3,535,172	76.45	0.03	23.52	91.92
	Low	1	25,725,122	80.57	3.75	15.67	12,862,561	80.53	0.04	19.43	89.44
	2.5µM	2	26,667,750	78.68	4.70	16.61	14,333,875	78.65	0.03	21.32	91.63
		3	6,478,332	77.64	5.50	16.86	3,239,166	77.61	0.03	22.36	92.24
DMSO	0.1%	1	25,784,896	79.49	5.14	15.36	12,892,448	79.45	0.04	20.51	89.68
(Control)		2	12,643,388	79.26	5.70	15.03	6,321,694	79.23	0.04	20.74	89.93
		3	10,381,144	78.77	5.50	15.74	5,190,572	78.73	0.03	51.23	90.80
Average			18,441,380	78.76	5.28	15.95	9,331,801	78.73	0.03	24.57	90.95

Supplementary Table 1. Sequencing and mapping statistics of RNA-seq reads to the reference human genome, hg19.

Treatment	Dose	Number of genes detected as expressed	Number of DEGs (FDR<0.05)	Upregulated DEGs	Downregulated DEGs	Number of DEGs (FDR<0.05, |FC≥2|)	Upregulated DEGs	Downregulated DEGs
TBOEP	High	18,999	4,060	2,127	1,933	939	501	438
	Low	19,163	1,055	579	476	80	51	29
DMSO	-	18,367	-	-	-	-	-	-

Supplementary Table 2. Number of DEGs in response to TBOEP. Number of differentially expressed genes (DEGs) detected for 125 µM (high dose) and 2.5 µM (low dose) TBOEP compared to control treatments at FDR<0.05, and at FDR<0.05 |FC|≥2.



Supplementary Figure 2. Treemap clustering and visualisation of GO enrichment analysis representative of biological processes. GO enrichment was done using BiNGO, and all significant GO terms were clustered and visualised using REVIGO for all DEGs (FDR<0.05) detected in both **(A)** high-dose, and **(B)** low-dose TBOEP treatments. Size of rectangles represent significance |log(*p*-value)| of the GO term. The specific GO terms that make up the super clusters are characterised in Supplementary File 4.

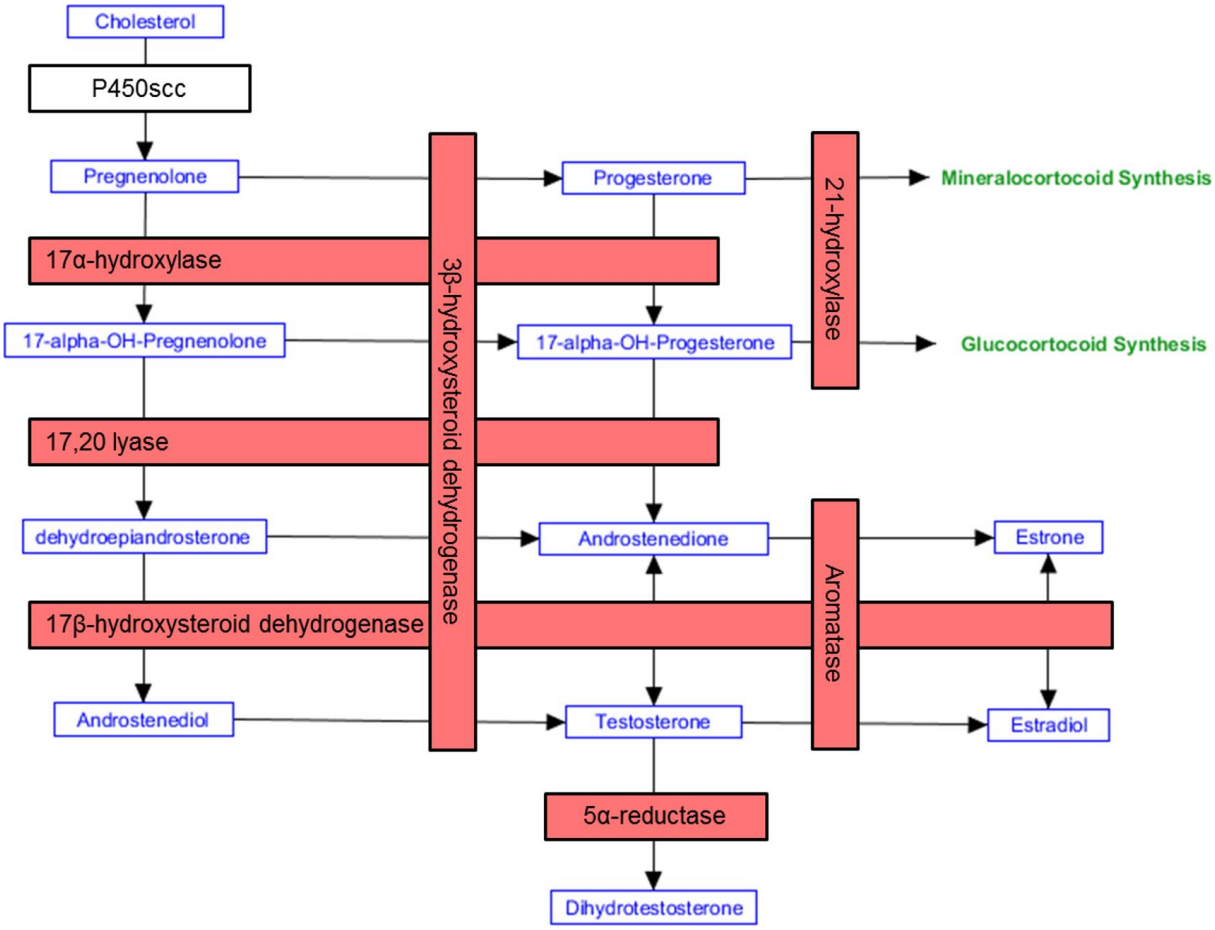
Supplementary Figure 3. Treemap clustering and visualisation of GO enrichment analysis for DEGs (FDR<0.05) detected in TBOEP low-dose treatments only. Size of rectangles represent significance |log(*p*-value)| of the GO term. The specific GO terms that make up the super clusters are characterised in Supplementary File 5.

KEGG pathway	Description	Enrichment score	Normalised enrichment score	*p*-value	FDR *q*-value
hsa03010	Ribosome	0.86	1.82	0	0
hsa00190	Oxidative phosphorylation	0.77	1.71	0.002	0.004
hsa00020	TCA cycle	0.81	1.46	0.02	0.22

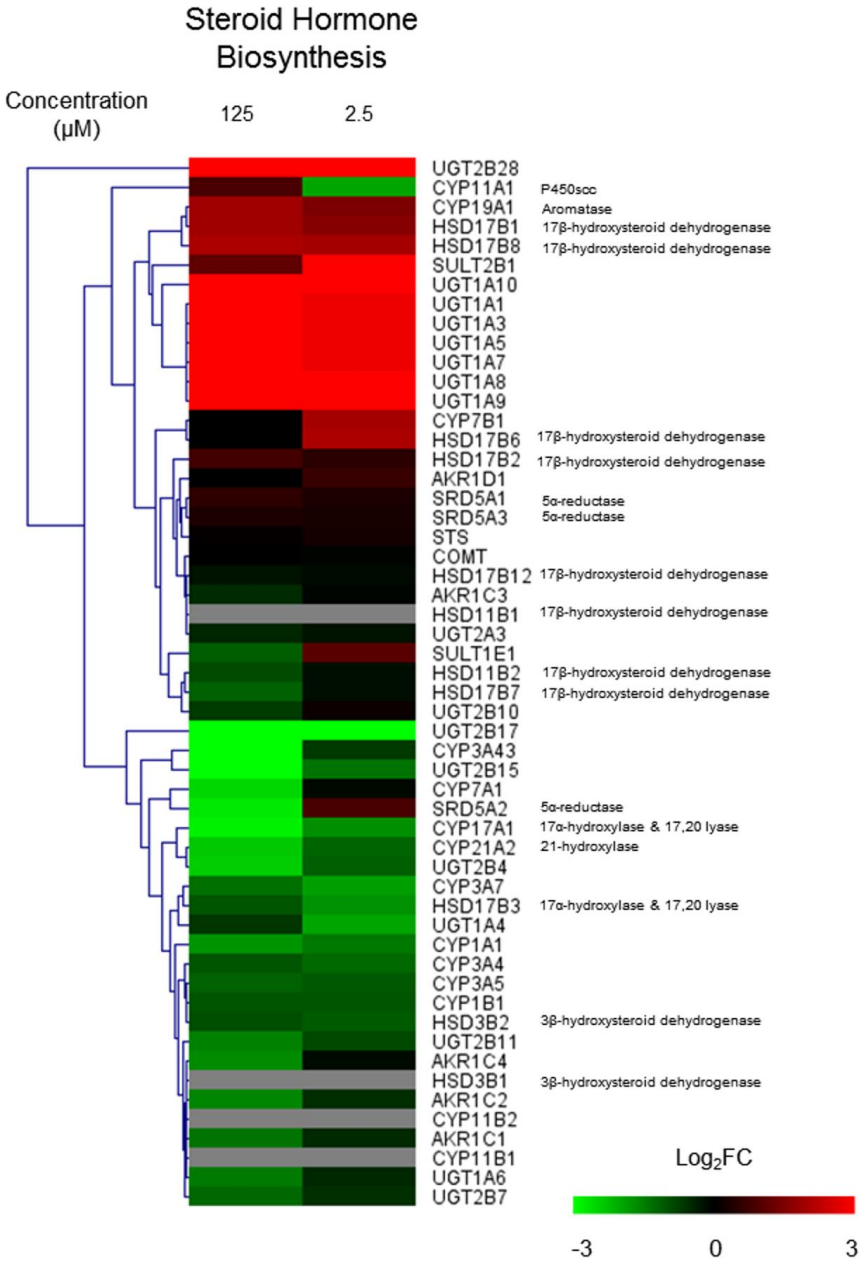
Supplementary Table 3. KEGG pathways enriched at a level of FDR<0.25 for high-dose TBOEP treatments.

KEGG pathway	Description	Enrichment score	Normalised enrichment score	*p*-value	FDR *q*-value
hsa03010	Ribosome	0.866	1.868	0	0
hsa00190	Oxidative phosphorylation	0.780	1.745	0	0.001
hsa00240	Pyrimidine metabolism	0.722	1.589	0	0.051
hsa03030	DNA replication	0.806	1.537	0.007	0.088

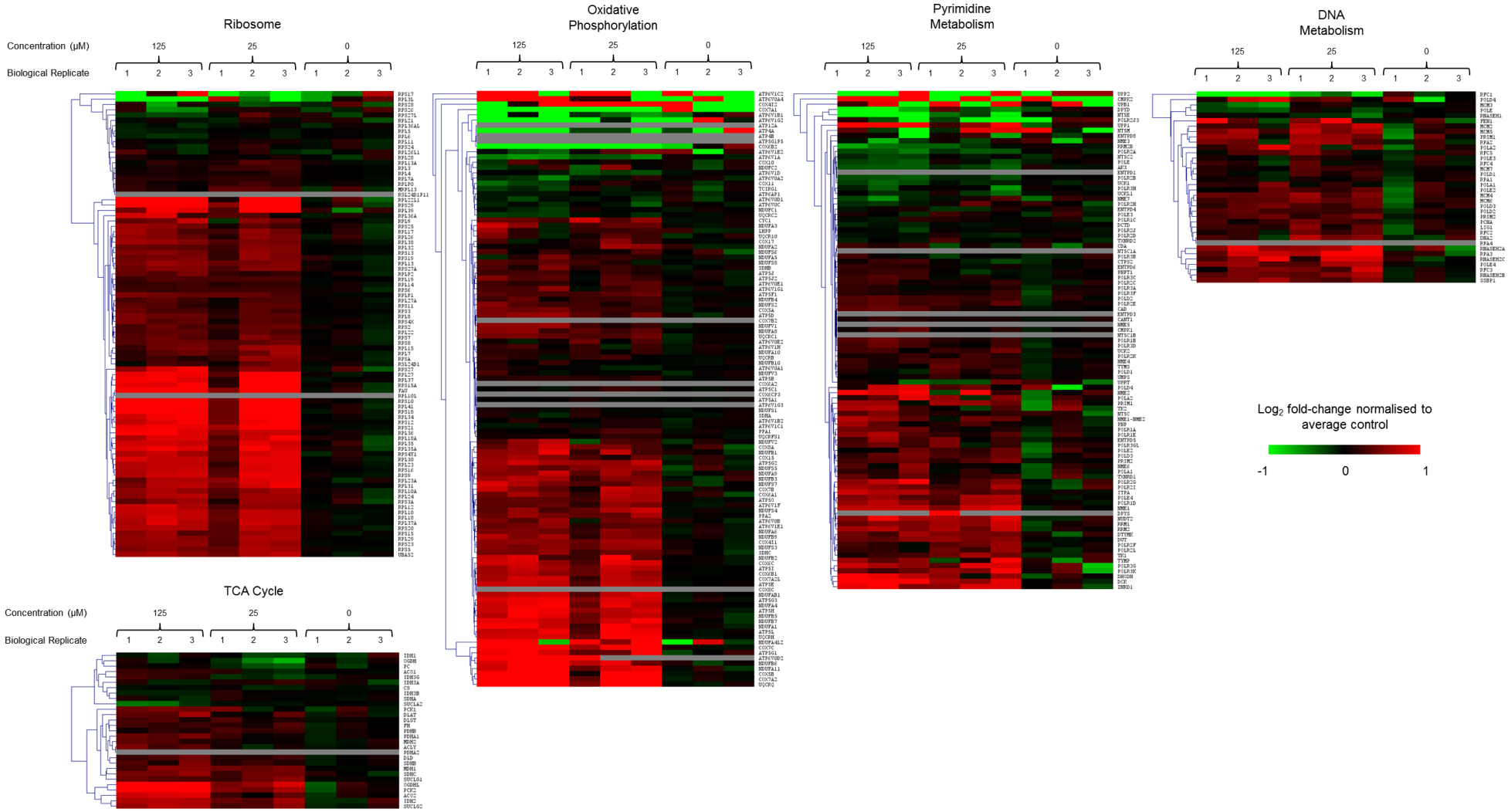
Supplementary Table 4. KEGG pathways enriched at a level of FDR<0.25 for low-dose TBOEP treatments.



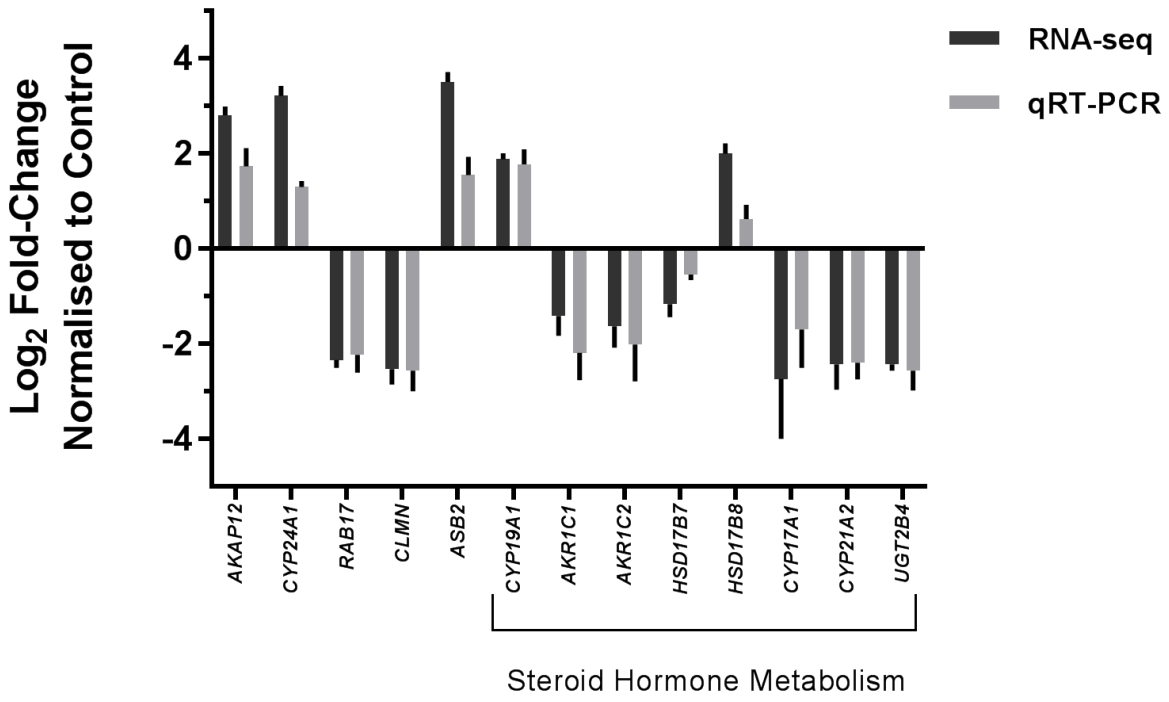
Supplementary Figure 4. Simplified schematic diagram of steroid hormone biosynthesis pathway. Only major metabolites and enzymes are included for ease of reading. Metabolites are blue while enzymes are represented by boxes. Expression of genes encoding for specific enzymes that were significantly ($p<0.05$) altered across triplicates compared to control are coloured red.



Supplementary Figure 5. Heatmap indicating changes of expression of genes involved in the entire steroid hormone biosynthesis KEGG pathway (hsa00140). Genes encoding for the enzymes in Supplementary Figure 4 are also indicated. Data represents Log_2FC expression values compared to control.



Supplementary Figure 6. Visualisation of significantly affected KEGG pathways (FDR<0.25) across all triplicates. KEGG pathways found to be significantly altered by Gene Set Enrichment Analysis were clustered and gene expression of genes involved were visualised across all triplicates. Gene expression is Log_2-converted and normalised to average control.



Supplementary Figure 7. Log_2-converted changes in expression of thirteen genes in response to 125 µM TBOEP by RNA-seq and qRT-PCR. Samples that underwent RNA-seq and qRT-PCR analysis were generated independent of each other. All expression changes for both RNA-seq and qRT-PCR were statistically significant from their respective controls across triplicates as determined by unpaired t-test statistics ($p<0.05$). Genes used for validation are described on the x-axis, while genes involved in steroid hormone metabolism are indicated.