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The flame retardant, 9,10-dihydro-9-oxa-10-phosphaphenanthrene 10-oxide (DOPO), has been receiving great interest given its superior fire protection properties, and its predicted low level of persistence, bioaccumulation, and toxicity. However, empirical toxicological data that is essential for a complete hazard assessment is severly lacking. In this study, we attempted to identify potential toxicological modes of action by transcriptome (RNA-seq) profiling of the human liver hepatocellular carcinoma cell line, HepG2. Such insight may identify compounds of concern and potential toxicological phenotypes. DOPO was found to have little cytotoxic potential, with lower effect concentrations compared to other flame retardants studied in the same cell line. Differentially expressed genes revealed a wide range of molecular effects including changes in protein, energy, DNA, and lipid metabolism, along with changes to cellular stress response pathways. In response to 250 µM DOPO, the most perturbed biological processes were representative fatty acid metabolism, androgen metabolism, glucose transport, and renal function and development, which is in agreement with other studies observing similar effects of other flame retardants in other species. However, treatment with 2.5 µM DOPO resulted in very few differentially expressed genes and failed to indicate any potential effects on biology, despite such concentrations likely being orders of magnitude greater than would be encountered in the environment. This, together with the low levels of cytotoxicity, acknowledges the potential replacement of current flame retardants by DOPO, although further studies are needed to establish the nephrotoxicity and endocrine disruption of DOPO.

Introduction

To meet regulated safety requirements, flame retardants (FRs) are ubiquitously used in consumer products and building materials in an effort to limit the risk of fire, and their demand is expected to increase given our increasingly urbanised lifestyles. These compounds have been shown to leech out of such materials and contaminate immediate and distant environments. Consequently, they have been detected in house dust and air, while also being detected in human milk, placenta, cord blood, and serum, exemplify the daily exposure of humans to these compounds.[1] Their detection as far as the Arctic highlights their pervasiveness and persistence.[2] FRs could therefore be a concern for both human and environmental health.

The phasing-out of some brominated FRs has led to organophosphate FRs surpassing brominated FRs in demand.[3] Nevertheless, some compounds in this broad family of FRs have shown similar levels of toxicity compared to their brominated predecessors. For instance, some organophosphate FRs have exhibited high levels of toxicity in a number of assays investigating cell differentiation, cell proliferation, and *in vivo* developmental effects,[4] while also being linked to decreased cognitive function in children,[5] similarly to brominated FRs.[6] Halogen-free organophosphate FRs are therefore of interest given their limited predicted persistence, bioaccumulation, and toxicity based on QSARs.[7]

In an effort to limit toxicity and environmental concerns while maintaining superior flame extinguishing potential, 9,10-dihydro-9-oxa-10-phosphaphenanthrene 10-oxide (DOPO) has attracted great interest. Predicted to be one of the safest FRs in terms of persistence, bioaccumulation, and toxicity,[7] DOPO has also shown to produce less toxic gases upon combustion while maintaining capable flame retardancy.[8] However, very little empirical toxicological data exists, with a majority of studies focusing on the aquatic toxicology of DOPO.[9–13] Only recently has progress been made in identifying the immunotoxicity, neurotoxicity, and skin sensitisation potential

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of DOPO.[14] As such, more toxicological data is needed to evaluate the potential health effects of this promising FR.

In the European Union, compounds such as FRs undergo several toxicological studies in an effort to estimate their risk to environmental and human health. However, such studies conducted in model organisms monitor classical endpoints such as; lethal dose concentrations, skin sensitisation, carcinogenic potential, immuno-, reproductive effects, and genotoxicity. Conservative estimates are then used to predict their impact to human and environmental health. While useful in identifying toxic substances on a limited number of endpoints, these approaches are often time-consuming and costly to animal welfare. As such, not all chemicals humans are exposed to are tested as rigorously as others, and if tested at all, usually done in organisms of non-human origin. There is therefore a need to develop strategies that are able to discern as many potential health effects as possible in relevant models that may complement current *in vivo* testing strategies to aid risk assessment of chemicals. *In vitro* toxicological assays able to discern modes of action (MOA) are seen as the initial strategy in trying to elucidate health effects. Such insight may be used to prioritise hazardous chemicals for further study,[15] while contributing to risk assessment concurrently with *in vivo* toxicological data to aid better risk assessment in a weight-of-evidence approach.[16] Robust, high-throughput omics techniques able to elucidate molecular effects already have been used to identify potential toxicological MOA which have resulted in the identification of biomarkers of exposure, adverse health effects, and therapeutic targets.[17–22] These strategies have also been applied to generate hypotheses and identify candidate genes of interest that may be further examined in order to understand phenotypic responses.[23]

Given the lack of insight into the potential toxicological effects of DOPO, we have adopted the RNA-seq technology to identify molecular MOA that may give rise to toxic phenotypes in the human hepatocellular carcinoma cell line, HepG2. HepG2 cells have been used as liver models for predicting compound toxicity previously.[24] Overall, this study served as a first-tier unbiased screening approach to investigating potential toxicological effects of DOPO in an effort to identify toxicological MOA in a human-relevant model.

Materials and methods

Chemicals and reagents

Chemicals and reagents were purchased from Sigma-Aldrich unless otherwise stated. DOPO (>97%, CAS 35948-25-5) was purchased from Tokyo Chemical Industry Co. 1 M stock solutions were made up in dimethyl sulfoxide (≥99.9%, DMSO) and stored at -20 ^{o}C.

Cell culture and DOPO treatment

HepG2 cells (ATCC HB-8065), which is of human hepatocellular carcinoma origin, was cultured as a monolayer in Minimum Essential Medium supplemented with Earle's salts, 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). Culturing was done at 37^{o}C, 5% CO_2. Cells were passaged using 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) once 70-80% confluent, without reaching a maximum of 25 passages. Lack of mycoplasma contamination was routinely ensured using the LookOut® Mycoplasma PCR Detection Kit as per manufacturer's instructions. Treatments were done in triplicate involving separate seeding, treatment, RNA isolation, and sequencing independent of each other as described previously.[25,26] Briefly, 300 000 HepG2 cells were seeded per well in 6-well plates and allowed to culture for 72 hrs. 250 µM and 2.5 µM DOPO in DMSO was used for exposures to high and low concentrations respectively. Final DMSO concentrations in treatments did not exceed 0.1%. Cells were exposed for 72 hrs in triplicate before RNA isolation.

Cytotoxicity

Cytotoxicity was determined by means of a resazurin cell-viability assay and were designed to mimic proposed treatments. Briefly, 10 000 HepG2 cells were seeded in 96-well plates and treated with a range of DOPO concentrations (1000 µM to 62.5 µM). Following 72 hrs treatment, cells were exposed to 50 mM resazurin sodium following incubation at 37 ^{o}C for 45 min. Fluorescence at 570 nm was measured and non-cytotoxic concentrations were determined when no statistical significance (unpaired t-test, p<0.05) between treatment concentration and control treatment was found.

RNA isolation and sequencing

RNA was isolated using the hybrid TRIzol and RNeasy extraction and clean up approach.[27] RNA was first extracted from cells with TRIzol as per manufacturer's instructions. The protocol was followed through to phase separation, where the aqueous phase containing RNA was treated as lysate and then subjected to RNA clean up using the RNeasy kit as per manufacturer's instruction. Quantification of pure total RNA was then done along with assessing RNA purity (260/280 and 230/280 ratios >2) using the NanoDrop ND-1000 spectrophotometer. Integrity of pure total RNA was determined using the QIAxcel System. Sequencing libraries were prepared using the TruSeq RNA preparation kit as per manufacturer's instructions. Prepared libraries were paired-end sequenced (2x 50BP) using the Illumina HiSeq 1500 platform.

Data and pathway analysis

Sequenced reads were mapped to the human reference genome (UCSC hg19) using CLC Genomics Workbench version 9.0.1, the parameters of which are found in Supplementary File 1. Only reads that were uniquely mapped and mapped in pairs were used for further analysis. *P*-values were obtained with Baggerly's test,[28] and the Benjamini-Hochberg method was used to limit the false discovery rate to 5%. Over-representation analysis and Gene Ontology Analysis (GOA) of differentially-expressed genes (DEGs) was performed with

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Cytoscape plugins BiNGO[29] and ClueGO.[30] GO terms were only considered enriched if supported with at least three genes, and similar GO terms were clustered into descriptive clusters by finding representative subsets of terms using REVIGO.[31] Gene Set Enrichment Analysis (GSEA) was subsequently used to identify affected cellular pathways using the stand-alone java GSEA program downloaded from the Broad Institute website (www.broadinstitute.org) and the curated Kyoto Encyclopaedia of Genes and Genomes (KEGG) database.[32] Expression data of genes comprising specific KEGG pathways was extracted from the whole sequencing dataset and row-normalised using the Cytoscape plugin, Enrichment Map (version 2.0.1).[33] These values were then hierarchically clustered by Euclidian distance and visualised using MultipleExperiment Viewer (version 4.9.0).[34]

Qualitative real-time PCR validation

Eleven DEGs were selected to be validated by quantitative real-time PCR (qRT-PCR) on an independent set of samples that was treated with 250 µM DOPO. Given that this involved the start-up of new stocks of HepG2 cells and repeating the treatments, this also served to validate the robustness and accuracy of our approach, albeit only using a limited number of genes as examples. Isolated mRNA was converted to cDNA using the iScript™ Advanced cDNA Synthesis Kit 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, and was performed according to manufacturer's instructions. Primer sequences are unavailable given that PrimePCR™ assays are a proprietary product. *RPL17* was selected as a reference gene given its unaltered expression over the three treatment conditions (0.1% DMSO, 2.5 µM DOPO, and 250 µM DOPO) as revealed by the RNA-seq data. Relative gene expression was analysed using the $2^{-\Delta\Delta Ct}$ method.[35] Relative gene expression was normalised to control treatments and Log_2-converted.

Results

Cytotoxicity of DOPO

Dose-dependent cell viability assays revealed limited cytotoxicity of DOPO to HepG2 cells (Supplementary Figure 1), with an IC50 of 2630 µM (95% CI: 1078-6417 µM). 1000 µM and 500 µM treatments were shown to significantly affect cell viability based on the statistical conditions described in materials and methods. Despite the high concentration used, 1000 µM only resulted in a 29.1% reduction in cell viability, further highlighting the limited cytotoxic effects of DOPO in HepG2 cells. No statistically-significant decrease in cell viability was observed for 250 µM treatments; 250 µM and 2.5 µM concentrations were selected as high- and low-dose treatments respectively for exposure experiments.

Sequence mapping statistics

An average of 20.7 million reads were sequenced per sample, of which 79.2% were mapped uniquely and in pairs while the remaining reads were discarded (Supplementary Table 1). 90.6% of those successfully mapped reads were mapped to exons and used for subsequent differential expression analysis (Supplementary Table 1).

Effects of DOPO on global gene expression

18,961 and 19,718 genes were detected as expressed (transcripts with a count of at least 1 in either triplicate) for DOPO high- and low-dose treatments respectively (Supplementary Table 2). 250 µM DOPO resulted in the differential expression of 1374 genes (FDR≤0.05) (Supplementary Table 2). Comparatively, treatment with 2.5 µM DOPO resulted in much fewer differentially expressed genes (DEGs) with only 131 genes being observed as differentially expressed (FDR≤0.05). The majority of these DEGs exhibited only minor alterations in expression compared to the untreated control (less than 2-fold), with only 167 and 12 DEGs being detected for DOPO high- and low-dose treatments respectively.

Venn diagrams revealed to what degree DEGs were shared between the two treatment concentrations (Figure 1A). Of the 131 DEGs detected at FDR≤0.05 for 2.5 µM DOPO, 84 of those were shared with 250 µM DOPO treatments, while the remaining 47 DEGs were exclusive for 2.5 µM treatments. Similarly, of the 12 DEGs with a |FC|≥2 detected as differentially expressed for 2.5 µM treatments, only five of them were shared with high-dose treatments and the remaining seven were considered distinctive of transcriptomic response to low-dose DOPO treatments. Of the DEGs detected as up- and downregulated at FDR<0.05, 61% of the 75 DEGs detected as upregulated were also detected as upregulated

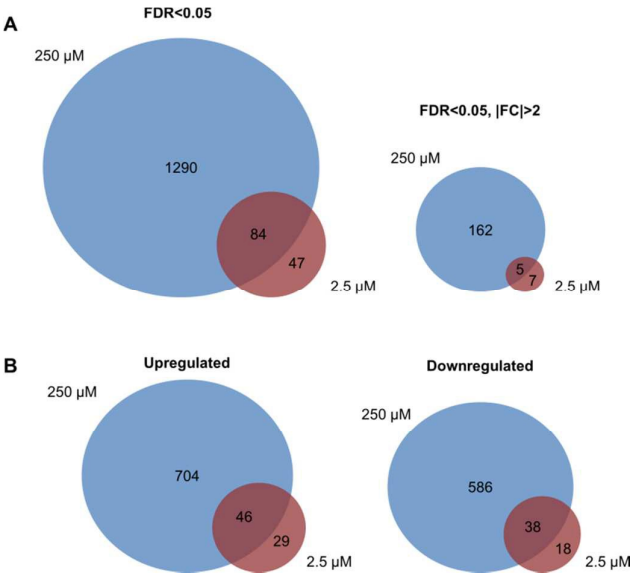

Figure 1: Number of DEGs represented as Venn diagrams highlighting the number of common DEGs for high- and low-dose DOPO treatments. DEGs were detected at **(A)** FDR≤0.05, and at FDR≤0.05, |FC|≥2. **(B)** Number of DEGs shared between low- and high-dose treatments detected as upregulated and downregulated at FDR<0.05.

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following low-dose treatment were also detected as upregulated following high-dose DOPO treatment (Figure 1B). Similarly, for low-dose treatment, 68% of DEGs detected as downregulated were also detected as downregulated for high-dose treatments (Figure 1B).

Biological processes affected by DOPO

Over-representation analysis (ORA) was used to identify which GO terms representative of biological processes were affected by each condition. Initially, all DEGs detected at FDR≤0.05 were used for enrichment of GO terms. 992 and 42 GO terms were enriched for high- and low-dose DOPO treatments respectively (Supplementary File 2). Similar GO terms were then grouped into representative clusters in order to simplify data visualisation (Supplementary Figure 2).

Grouping of similar GO terms revealed that 250 µM treatments mostly affected mRNA metabolism, followed by cell cycle, cellular subunit organisation, response to stress, cellular transport, and metabolism (Supplementary Figure 2A). 2.5 µM similarly affected mRNA metabolism, but effects on amino acid metabolism, transport of untranslated proteins to the endoplasmic reticulum and, muscle cell proliferation were also observed (Supplementary Figure 2B).

To determine which biological processes were influenced most significantly, over-representation analysis was also performed only including DEGs exhibiting a fold-change of |FC|≥2 (Figure 2). The 98 upregulated DEGs detected for 250 µM DOPO treatment were found to play a role in a number of cellular functions including; metabolism, carbohydrate transport, and renal function (Figure 2A, Supplementary File 3). Within the carbohydrate transport category, mostly genes related to glucose transport were altered after treatment with DOPO, while alterations to renal function were denoted by effects on natriuresis and diuresis. In addition, effects on the regulation of coagulation genes were also observed. Interestingly, effects on metabolism was characterised mainly by fatty acid and steroid hormone metabolism, specifically androgen metabolism. However, the androgen metabolism node was described by only three genes (Supplementary File 3), one of them being the gene encoding for aromatase.

For downregulated DEGs, GO terms specific for cell differentiation and organ development were enriched (Figure 2B). Specifically, effects were observed for genes controlling the regulation of cell fate determination, particularly for embryonic morphogenesis, and the formation of primary germ layers. Additionally, organ development was found to be characterised specifically by effects on kidney development. DEGs in response to 2.5 µM DOPO failed to enrich for any GO terms (data not shown).

Pathways affected by DOPO

The complete expression data set for all detected genes was used to identify which cellular pathways were altered in response to DOPO treatment. Using the curated KEGG database, only one pathway was found to be overexpressed in response to 250 µM DOPO treatment, namely the peroxisome pathway (hsa04146) (Supplementary Table 3), while no pathways were detected as underexpressed (Supplementary File 4). For 2.5 µM treatments, several pathways were detected as overexpressed, including; DNA replication (hsa03030), ribosome (hsa03010), pyrimidine metabolism (hsa00240), oxidative phosphorylation (hsa00190), and proteasome pathways (hsa03050) (Supplementary Table 3). As for high-dose treatments, no pathways were detected as underexpressed for low-dose treatments (Supplementary File 4).

Heatmaps allow for the visualisation of expression levels of individual genes relative to each other in large datasets. As

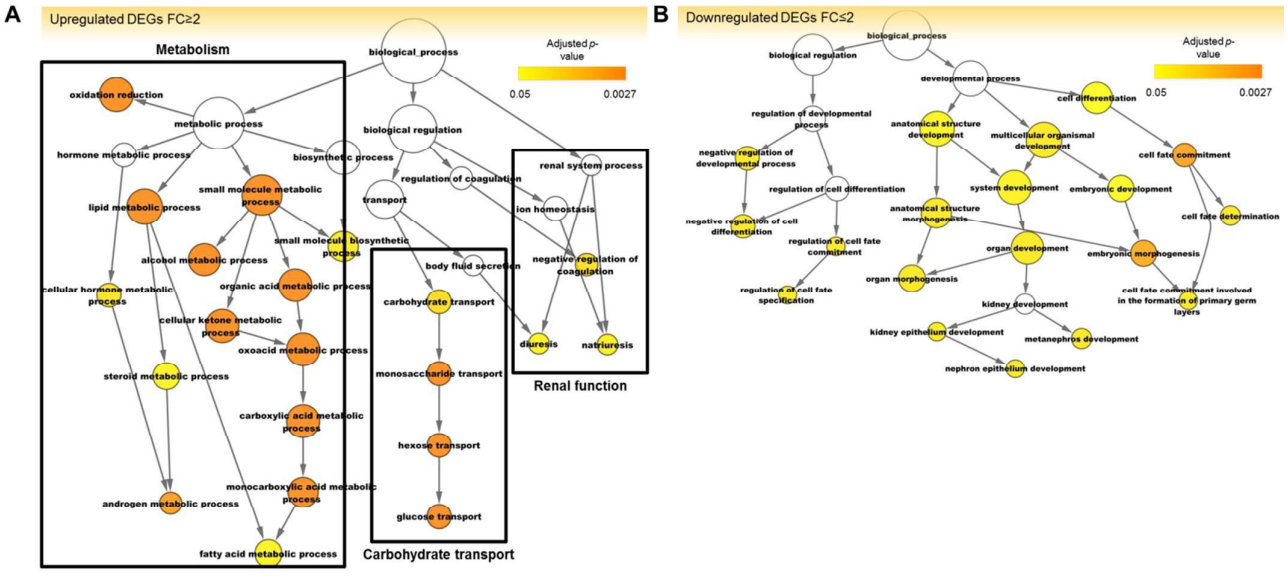

Figure 2: Biological processes over-represented by DEGs detected for high-dose (250µM) DOPO treatments (FDR≤0.05, |FC|≥2). GO trees were obtained using BiNGO and show the hierarchy of effected GO terms for both (A) upregulated, and (B) downregulated DEGs. Coloured nodes indicate significant over-represented terms (FDR≤0.05), with more orange indicate greater significance.

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such, heatmaps for enriched KEGG pathways were generated using row-normalised values of averaged expression data over the three triplicates per condition (Figure 3). Hierarchical clustering revealed that a vast majority of genes making up these pathways were upregulated following treatment with DOPO compared to control treatments, both for high- and low-dose conditions. High- and low-dose treatments were able to cluster together for all enriched KEGG pathways except for peroxisome and proteasome pathways.

qRT-PCR validation of RNA-seq results

The eleven genes selected were based on the results obtained by the RNA-seq approach and subsequent GO term enrichment analysis. The genes *G6PC* and *SLC2A2* were selected to represent effects on glucose transport given that these two genes resulted in the enrichment of GO terms representative of glucose transport (Supplementary File 3). *CYP19A1* and *HSD17B8* were selected to confirm effects on androgen metabolism as identified by RNA-seq data. For effects on cell cycle, the genes *GFI1* and *UHRF1* were chosen, and for effects on natriuresis and diuresis, the genes *END1* and *KNG1* were selected. Finally, additional genes *GPR133*, *ODAM*, and *PTAFR* were selected because of their high fold-changes as detected by RNA-seq. All eleven genes showed good correlation between RNA-seq and qRT-PCR approaches (Supplementary Figure 3), highlighting the accuracy and robustness of our methodology.

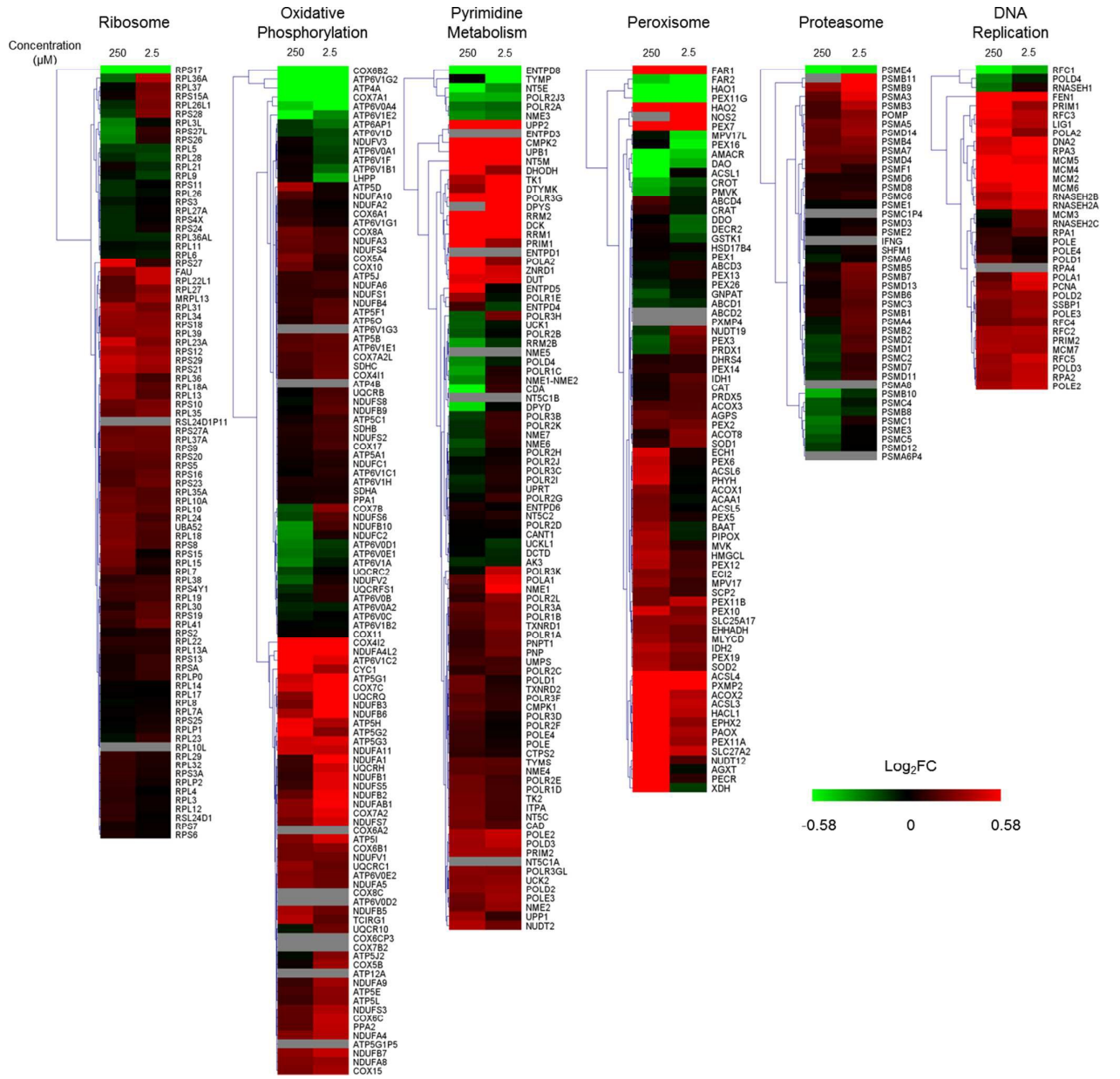

Figure 3: Heatmap visualisation and cluster analysis of genes characterising significantly-enriched (FDR≤0.25) KEGG pathways. Expression data used was the averaged of triplicates per condition. Data represents Log2FC expression values compared to control (Log2FC=0.58 when FC=1.5).

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Discussion

DOPO has received great interest as a halogen-free phosphorus FRs in replacing other halogenated FRs that are omnipresent in both human and natural environments. This is due to its low persistence, bioaccumulation, and toxicity[36] while maintaining capable flame retardancy.[8] In the present study, we performed an investigation of the toxicogenomic effects of DOPO on the human hepatocellular carcinoma cell line, HepG2, in an effort to identify potential MOA that may give rise to toxicological phenotypes in response to human exposure to DOPO.

There is limited empirical evidence on the toxicological potential of DOPO. The few *in vitro* studies that have been conducted have shown limited neurotoxicity,[37] low cytotoxicity, and limited potential to induce oxidative damage and affect calcium-ion homeostasis,[38] while having no estrogen-disruption potential,[39] and very limited effects on bacterial stress genes.[40] *In vivo* studies have demonstrated very limited aquatic toxicity with DOPO exhibiting low acute toxicity in *Daphnia*,[10] while very limited effects on *Daphnia* reproduction and growth rate were observed.[11] Other toxicological data is estimated based on chemical properties and conclude that DOPO has a low potential for acute and chronic toxicit.[7,36] Recently, DOPO was found to have little acute cytotoxicity in a number of human cell lines, while also having little effects on proinflammatory and oxidative stress responses.[14] Within the same study, it was showed that DOPO displayed no epidermal penetration when using a 3D human *in vitro* epidermal model, lacked skin sensitisation potential, and did not harbour developmental toxicity by means of measuring neural crest cell migration.[14] However, it should be noted that while the parent compound DOPO lacked noticeable toxicity in these systems, some of its derivatives showed comparable, and in some cases, increased toxicity to that of the now phased-out FR, PBDE-99, highlighting the importance of using quick and efficient *in vitro* assays to screen chemicals individually.[14]

Hirsh and colleagues have taken great strides in attempting to elucidate the toxicity of DOPO other than studies previously reporting the aquatic toxicity of the compound. While the cytotoxicity potential, release of proinflammatory cytokines, and skin sensitisation potential of DOPO and some its derivatives were investigated, identifying its neurotoxicity in a number of different models remains its greatest conclusion due to its multi-endpoint nature. Neurotoxicity was comprehensively judged by identifying effects on neurodegeneration (by measuring cytotoxicity, mass, and changes in morphology in fully differentiated human neurons), neuronal differentiation (by measuring cytotoxicity and mass in human stem cell-derived neural precursors, and neuronal development (by measuring effects on migration of stem cell-derived human neural crest cells).[14] However, even by their admission, the mechanisms underlying toxic effects were not investigated.[14] Given the importance of mechanism-based data in not only cross-species predictions,[41,42] but also in weight-of-evidence risk assessments[43] and quantitative risk assessment,[44] such data could prove useful in identifying potential toxicity, especially for promising, data-poor compounds such as DOPO.

In this study, DOPO showed very limited cytotoxicity in HepG2 cells. Cell viability was only significantly affected with doses greater than 500 μM, with a high IC_{50} value of 2630 μM. Comparing these cytotoxicity values to those of other FRs investigate in the same cell model, it becomes apparent that DOPO some of the lowest cytotoxicity potential in HepG2 cells. For instance, the organophosphate FRs tributylphosphate, tris (2-butoxyethyl) phosphate, and tris (1-chloro-2-propyl) phosphate showed significant effect on HepG2 cell viability at 200 μM after 24 hrs treatment,[45] while tris (1,3-dichloro-2-propyl) phosphate had an IC_{50} of 84.0 μM after 72 hrs of exposure.[46] Of the brominated FRs tested in HepG2 cells, polybrominated diphenyl ethers exhibit high levels of cytotoxicity with effect concentrations ranging from low μM to nM ranges,[47–49] while hexabromocyclododecane had an IC_{50} of 63.0 μM after treatment for 24 hrs.[50]

To elucidate the concurrent molecular changes upon exposure to DOPO, integrated analysis by several specific statistical and bioinformatics approaches (including over-representation analysis and gene set enrichment analysis) revealed that when taking into account all DEGs, cellular responses to DOPO involve alterations in mRNA and amino acid metabolism, along with potential effects on cell cycle. However, when looking at DEGs that were altered at $|FC| \geq 2$, these affects were largely lost indicating that DEGs with low fold-changes contributed to these biological processes being affected. The biological significance of these low fold-change genes could be argued, especially when considering the number of genes contributing to the enrichment of specific GO terms. For instance, while the GO term cell cycle (GO:0007049) was detected as enriched following DOPO treatment, genes comprising only 10% of the term were found to be differentially expressed, with a majority showing low fold-changes. Additionally, these over-represented GO terms were accompanied by alterations to pathways involved in protein turnover (by overexpression of genes involved in ribosome and proteasome function), and DNA metabolism (by overexpression of genes involved in DNA replication and pyrimidine metabolism), and not cell cycle pathways as determined by GSEA. This highlights the need to analyse omics data sets using different approaches given the differences in each method.[51] Additionally, the enrichment of genes involved in oxidative phosphorylation indicated effects on cellular energy metabolism. Finally, the enrichment of genes involved in peroxisome function suggests effects of DOPO on lipid metabolism.

Effects of FRs on protein turnover are well documented, with *in vivo* studies demonstrating effects of organophospahate and brominated FRs on protein metabolism both in zebrafish[52] and in *Daphnia*.[53] Effects on protein turnover are likely as a result of targeting the ribosomal machinery,[54,55] in agreement with our study. Effects on energy and lipid metabolism are also well documented for other FRs. Triphenyl phosphate was observed to effect energy and lipid

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metabolism in human hepatocellular progenitor cells[56] and in zebrafish liver.[57] Tris (2-butoxyethyl) phosphate revealed effects on energy and lipid metabolism in zebrafish embryos[58] and *Daphnia*,[53] while the same was seen for hexabromocyclododecane in HepG2 cells.[59] In addition, effects of FRs on energy metabolism by targeting oxidative phosphorylation has been previously observed,[46,60–62] in agreement with our results. Finally, the role of the peroxisome in the production and scavenging of reactive oxygen species is well known.[63] Similarly, the link between FR exposure and oxidative stress by the induction of reactive oxygen species is well described.[64–67] This, along with the previous report of DOPO resulting in an *in vitro* increase in reactive oxygen species production[38] reveals that DOPO may induce oxidative stress in HepG2 cells as evidenced by the upregulation of genes involved in peroxisome function.

The majority of DEGs from our transcriptomics profiling had a small fold change compared to the control. We therefore performed a more stringent over-representation analysis that only includes DEGs having a fold-change of |FC|≥2. For 250 µM DOPO, the 98 upregulated DEGs at this level of expression enriched for GO terms representative of metabolism, carbohydrate transport, and renal function, while the 69 downregulated DEGs enriched for terms involved in cell fate determination and specification, organ development, and embryonic development. Effects on metabolism were primarily characterised by expression changes in genes involved in fatty acid and androgen metabolism. Although this was only characterised by three genes, one of them was the gene encoding for aromatase, the key enzyme in converting androgens into estrogens. HepG2 cells have already exhibited the ability to produce estrogens and it is therefore thought that estrogen biosynthesis may play a role in hepatocellular carcinoma.[68] Based on this, it is therefore possible that DOPO may alter normal estrogen homeostasis. In contradiction, DOPO has already been shown to lack any estrogenic effects in the human breast carcinoma cell line, MCF-7.[39] However, MCF-7 cells maintain little xenobiotic metabolism compared to cells of liver origin, and given that FR toxicity has been previously shown to be potentiated by biotransformation,[60,69,70] other *in vitro* assays able to screen DOPO metabolites for estrogenic activity are needed to confirm the findings here.

Additionally, DOPO was found to affect glucose transport by altering the expression of genes encoding for glucose-6-phosphatase (*G6PC*), glucose transporter 2 (*SLC2A2*), and glucose transporter 3 (*SLC2A3*). Considering the role of glucose-6-phosphate in vital process of glycogenesis and gluconeogenesis,[71] DOPO mediated transcriptional alterations of associated genes suggest possible disturbance of glucose homeostasis.

Effects on renal function were most apparent in genes related to diuresis and natriuresis while downregulated DEGs enriched for terms involved in kidney development by affecting nephron development. While HepG2 cells should not be considered a model for monitoring effects on renal function, the role of FRs on kidney toxicity and development is not new. The brominated FR, tetrabromobisphenol A, has been observed to have nephrotoxic effects by the formation of polycystic lesions in new-born rats.[72] Similarly, polybrominated diphenyl ethers have shown to affect kidney function by inducing oxidative stress.[73] The organophosphate FR, tris (2-chloroethyl) phosphate, has also exhibited potential in inducing focal hyperplasia of renal tube epithelium and therefore inducing renal tube adenomas in rats.[74]

Finally, DOPO was found to harbour potential effects on cell fate determination and embryonic development, specifically kidney development and formation. Cells of hepatocellular carcinoma origin have previously been shown to express gene-expression signatures similar to those of embryonic stem cells,[75] and thus HepG2 cells have already been used to develop our understanding of embryonic differentiation.[76] These results therefore confirm other studies reporting developmental toxicity attributed to several FRs in several species by a number of different modes of action,[77–81] However, to conclusively confirm the potential nephrotoxicity of DOPO, suitable experimentation would need to be conducted both *in vitro* and *in vivo*. The few DEGs enriched at fold-change of greater than two fold for 2.5 µM failed to enrich for any GO terms, indicating limited biological effects at this concentration.

Currently, there is no data relating to human exposure to DOPO. Perhaps this is indicative of its status as a promising alternative to other organophosphate FRs and is therefore not widely used yet. To attempt to quantitatively link the concentrations used in this study to human exposure levels would therefore be unfeasible. However, as an estimate using exposure data of other reactive FRs, the concentrations used here are orders of magnitude greater than are likely to be experienced. For instance, the reactive FR, tetrabromobisphenol A, was found in human serum at the highest concentration of 7.49 ng/ml serum.[82] This equates to approximately 0.01 µM when taking the molecular mass of tetrabromobisphenol A into account. Given that reactive FRs are less likely to leach from products than additive FRs, one could expect DOPO to behave similarly to tetrabromobisphenol A when leeching. Therefore, the lowest concentration used in this study (2.5 µM) is likely orders of magnitude greater than one is likely to encounter.

Comparing these results to those generated using other FRs employing the same methodology, some parallels can be drawn between DOPO and tris (2-butoxyethyl) phosphate, the most common halogen-free organophosphate FR in use currently.[25] For instance, RNA-sequencing found that tris (2-butoxyethyl) phosphate also induced changes in pathways involved in protein turnover, energy metabolism, and DNA turnover by upregulating genes involved in the ribosome, oxidative phosphorylation, pyrimidine metabolism and DNA replication pathways in a similar fashion as highlighted here. Additionally, DOPO was found to potentially impact steroid hormone biosynthesis by altering genes involved in estrogen metabolism similarly to tris (2-butoxyethyl) phosphate.[25] However, DOPO was not noted to affect genes involved in immunological and wound healing processes, processes key in liver fibrosis, as discovered for tris (2-butoxyethyl)

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phosphate.[25] DOPO was also found to harbour lower levels of cytotoxicity than that of tris (2-butoxyethyl) phosphate.[25] However, perhaps the biggest distinction between DOPO and tris (2-butoxyethyl) phosphate is not their differences in molecular MOAs or levels of cytotoxicity, but perhaps the difference between concentrations at which these effects occur *in vitro* and the concentrations at which humans would likely be exposed to. For instance, we identified that the lowest concentration used in this study is likely 250 times greater than normal human exposure scenarios using a FR that is similarly incorporated into end-user products as a model. Conversely, tris (2-butoxyethyl) phosphate was seen to affect molecular processes and at concentrations relevant to human exposure.[25] Therefore, while DOPO may affect similar processes as tris (2-butoxyethyl) phosphate, its lower levels of cytotoxicity, likely lower levels of human exposure, and fewer molecular processes being affected compared to tris (2-butoxyethyl) phosphate highlights its candidacy as a potential FR replacement, perhaps even for some other non-halogenated organophosphates currently in use.

Future perspectives

Here, we used an immortalised human hepatocellular carcinoma cell line as a cellular liver model to identify compound toxicity. The HepG2 cell line has already been used for classification of hepatotoxicants,[83] chemical hazard identification,[84] and MOA studies.[85] However, a few caveats need to be mentioned when using HepG2 cells as liver models, chief amongst which is the limited metabolic competence of HepG2 cells compared to primary hepatocytes given their lower expression of some phase I and phase II-metabolising enzymes.[86] However, it should also be noted that even primary hepatocytes fail to detect all known hepatoxins,[87] highlighting the current lack of reliable *in vitro* models for identifying liver toxicants. Therefore, multiple cell lines would be needed to comprehensively identify liver toxicity of DOPO and its metabolites. Nevertheless, even though HepG2 cells maintain little biotransformation capacity compared to other liver models, they still remain useful in identifying a wide variety of effects given their diverse function. For instance, HepG2 cells possess the ability to metabolise sex hormones,[68,88] thereby confirming our results as DOPO holding potential as an endocrine disruptor. Additionally, the ability for HepG2 cells to mount effective innate immune responses[89] and their ability to differentiate[76] also highlight their diverse roles, particularly in immunity and cellular differentiation.

Currently, initial omics studies such as this study should not be considered a replacement of current testing strategies but more as a tool that is able to detect potential health effects on as many biological outcomes as possible to mark emerging chemicals of concern to be further studied at increasingly complex levels of biology. Specifically with transcriptome studies that are able to monitor alterations to gene expression of tens of thousands of genes, interpretation of such data needs to be validated on increasing levels of biological complexity before characterising a chemical of concern, given the issues involved with trying to associate altered mRNA levels to physiological outcomes.[90] Similarly with proteomics studies which are only able to monitor even less targets than RNA-seq approaches, these omics approaches are useful in identifying potential chemicals of concern and potential MOA that may be used to study effects in higher organisms to develop a more comprehensive understanding of a chemical's toxicity and therefore improve risk assessment.[44]

Conclusions

Given the lack of toxicological data for DOPO, particularly mechanistic or molecular data, we adopted a transcriptomics workflow based on RNA-seq to identify potential MOA that may give rise to adverse health effects. DOPO had little cytotoxicity in HepG2 cells, lower than those observed for other FRs in the same system. Subsequent integrated statistical and bioinformatics data analysis revealed effects on protein, DNA, lipid, and energy metabolism, along with effects on androgen metabolism, glucose transport, cell fate determination and specification, organ development, embryonic development, and kidney function. Of note, this unbiased transcriptome-wide study serves to identify potential toxic effects at the molecular level and exposures were performed at relative high concentrations. As such, more targeted studies making use of more suitable cell types in addition to in vivo assays are needed to confirm, for example, effects on sex-hormone metabolism and kidney function. Overall, our results are similar to the molecular effects seen for other FRs. However, the lower levels of cytotoxicity along with the very slight effects seen on the transcriptome for 2.5 μM treatments despite being higher than likely exposure levels might support the potential replacement of current FRs by DOPO.

Conflicts of interest

There are no conflicts to declare.

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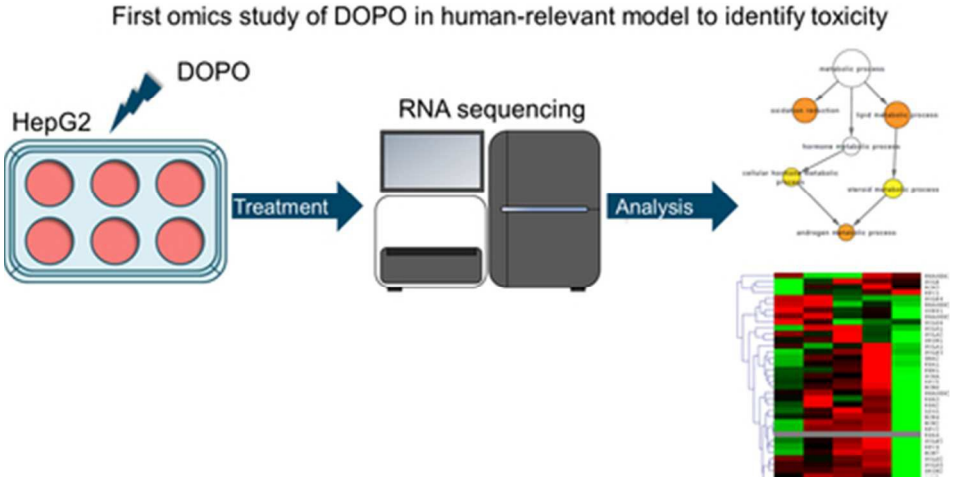

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