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

Boris V. Krivoshiev[a,*], Gerrit T.S. Beemster[b], Katrien Sprangers[b], Bart Cuypers[c,d], Kris Laukens[c], Ronny Blust[a], Steven J. Husson[a]

[a] Systemic Physiological & Ecotoxicological Research (SPHERE), Department of Biology, University of Antwerp, Antwerp, Belgium
[b] Integrated Molecular Plant Physiology Research (IMPRES), Department of Biology, University of Antwerp, Antwerp, Belgium
[c] Advanced Database Research and Modelling (ADReM), Department of Mathematics and Computer Science, University of Antwerp, Antwerp, Belgium
[d] Unit of Molecular Parasitology, Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium

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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.

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

* Corresponding author.
E-mail address: boris.krivochiev@uantwerpen.be (B.V. Krivoshiev).

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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 by 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; Ren et al., 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; Dingemans et al., 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 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 °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 °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, 1×10^4 HepG2 cells were seeded in 96-well plates and allowed to attach and acclimatise for 72 h. Cells were then treated with a range of TBOEP concentrations made up in 0.1% DMSO as indicated in Supplementary Fig. 1. Final exposure DMSO concentration was 0.1%. Following 72 h treatment, cells were washed with PBS and 50 mM resazurin sodium salt was added per well. Cells were allowed to incubate at 37 °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.