Global Transcriptional Analysis of Nontransformed Human Intestinal Epithelial Cells (FHs 74 Int) after Exposure to Selected Drinking Water Disinfection By-Products

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BACKGROUND: Drinking water disinfection inadvertently leads to the formation of numerous disinfection by-products (DBPs), some of which are cytotoxic, mutagenic, genotoxic, teratogenic, and potential carcinogens both in vitro and in vivo.

OBJECTIVES: We investigated alterations to global gene expression (GE) in nontransformed human small intestine epithelial cells (FHs 74 Int) after exposure to six brominated and two chlorinated DBPs: bromoacetic acid (BAA), bromoacetonitrile (BAN), 2,6-dibromo-p-benzoquinone (DBBQ), bromoacetamide (BAM), tribromoacetaldehyde (TBAL), bromate (BrO₃⁻), trichloroacetic acid (TCAA), and trichloroacetaldehyde (TCAL).

METHODS: Using whole-genome cDNA microarray technology (Illumina), we examined GE in nontransformed human cells after 4 h exposure to DBPs at predetermined equipotent concentrations, identified significant changes in gene expression (p ≤ 0.01), and investigated the relevance of these genes to specific toxicity pathways via gene and pathway enrichment analysis.

RESULTS: Genes related to activation of oxidative stress–responsive pathways exhibited fewer alterations than expected based on prior work, whereas all DBPs induced notable effects on transcription of genes related to immunity and inflammation.

DISCUSSION: Our results suggest that alterations to genes associated with immune and inflammatory pathways play an important role in the potential adverse health effects of exposure to DBPs. The interrelationship between these pathways and the production of reactive oxygen species (ROS) may explain the common occurrence of oxidative stress in other studies exploring DBP toxicity. Finally, transcriptional changes and shared induction of toxicity pathways observed for all DBPs caution of additive effects of mixtures and suggest further assessment of adverse health effects of mixtures is warranted. https://doi.org/10.1289/EHP4945

Introduction

Disinfection of drinking water is vital for the protection of public health since it greatly reduces pathogen risks and associated incidences of waterborne diseases (Cutler and Miller 2005) and is considered one of the major public health achievements of the 20th century (Calderon 2000). However, the powerful oxidants used during disinfection (e.g., chlorine or ozone) can react with natural and synthetic organic matter to inadvertently produce a multitude of potentially harmful chemicals, collectively known as disinfection by-products (DBPs) (Richardson and Postigo 2015). Evidence suggests that many DBPs exhibit cytotoxic, genotoxic, mutagenic, teratogenic, neurotoxic, and potentially carcinogenic properties, and may consequently elicit various adverse health effects (Du et al. 2013; Koviusalo et al. 1995; Muellner et al. 2010; Plewa and Wagner 2015; Rahman et al. 2010; Rivera-Núñez and Wright 2013; Villanueva et al. 2004; Wagner and Plewa, 2017; Wright et al. 2017). The presence of toxic DBPs is a concern for legislators and suppliers of drinking water, and identifying the forcing agents for toxicity is a research priority for ensuring responsible water management that protects public health and the environment (Plewa et al. 2017; Li and Mitch 2018).

To date, a limited number of DBPs have been characterized, and only a small fraction of these have been evaluated toxicologically (Wagner and Plewa 2017; Stalter et al. 2016). Brominated DBPs (Br-DBPs) tend to display higher toxicity than their chlorinated analogs (Cl-DBPs) (Escobar-Hoyos et al. 2013; Plewa and Wagner 2015; Yang et al. 2014) and are readily produced through chlorination of bromide-containing source waters (Postigo et al. 2018). This is common in coastal areas suffering from seawater intrusion (Wang et al. 2010), where bromide anions undergo rapid oxidation reactions with hypochlorous acid to produce hypobromous acid (Bougeard et al. 2010; Manas et al. 2016; Wang et al. 2010). Br-DBPs are also a dominant by-product in swimming pools using chlorinated seawater and may thus represent a concern for exposure routes other than drinking water (Manas et al. 2016). Notwithstanding the apparent differential risks, few studies have comprehensively evaluated or compared mechanistic molecular toxicity of different DBPs. There is consequently a pressing need for research aimed at identifying those by-products posing the greatest threat to humans and the environment and at understanding the molecular mechanisms leading to adverse health effects such as cancer (Hanigan et al. 2017; Plewa and Wagner 2015).

The potential association of DBPs with urinary bladder cancer (Villanueva et al. 2004, 2007) and colorectal cancer (Rahman et al. 2010; Villanueva et al. 2015) is an area of high interest. Both are among the most common types of cancer globally and display increased incidences in developed countries that benefit from higher levels of water disinfection (Siegel et al. 2016; Ploeg et al. 2009). The exact mechanism(s) leading to genotoxic and carcinogenic outcomes are still unclear but are believed to relate in some capacity to the production of reactive oxygen species (ROS) and the subsequent activation of oxidative stress pathways (Pals et al. 2013). Interestingly, other effects, such as alterations to immune function and inflammation, have also been associated
with exposure to DBPs (Vlaanderen et al. 2017; Munson et al. 1982). However, despite a rather well-established relationship between inflammation responses and the development and progression of cancer (Coussens and Werb 2002; Westbrook et al. 2009; Ioannidou et al. 2016), there have been limited mechanistic studies in this area in relation to DBPs. This clearly warrants further investigation, since chronic inflammation is known to generate ROS through a variety of mechanisms (Ioannidou et al. 2016; Reuter et al. 2010).

Transcriptomics is a molecular technique that can help elucidate underlying mechanisms of toxicity by quantifying expression changes of various genes with known biological functions (Cui and Paules 2010). Microarrays are particularly useful as an untargeted, or global, approach to gene expression profiling, yielding information for the entire set of genes expressed in a biological sample at a given time (Joseph 2017). Based on our limited knowledge of how DBP exposure elicits adverse health effects and ultimately cancer, there are significant benefits to be gained from using untargeted transcriptomics to explore chemical–gene interactions caused by DBPs.

The objective of the present study was to build on prior research to address the identified knowledge gaps. We characterized the effects of low concentrations of selected DBPs on global gene expression (GE) in normal nontransformed human enterocytes (FHs 74 Int) and used the generated GE profiles to identify affected toxicity signaling pathways through pathway enrichment analysis.

Methods

Disinfection By-Product Selection

We selected six brominated and two chlorinated DBPs: bromoacetamide (BAM), tribromoacetalddehyde (TBAL), bromate (BrO3−), tribromochloroacetamide (TBAA), tribromoacetone (TBAAO), and tribromoacetaldehyde (TBAL) (IC50s) for each of the DBPs were then calculated using Equation 1

\[ \text{IC}_{50} = \frac{\text{mean absorbance of the negative control}, A_{0}}{A_{v}} \times \frac{A_{c}}{A_{v}} \times \frac{100}{100} \]

where \( A_{0} \) is the mean absorbance of the negative control, \( A_{v} \) is the mean background absorbance from the absolute absorbance of each test well, \( A_{c} \) is the mean absorbance of the control, and \( 100 \) is the value of each test well, then dividing the resulting value by the mean background absorbance from the absolute absorbance of each test well. Normalization in GraphPad Prism for Windows (version 6.05; GraphPad) using the program X

Cytotoxicity Assay

The cytotoxicity assays were performed using neutral red dye uptake (NRU) as an indicator of cell viability. NRU is one of the most widely applied in vitro cytotoxicity assays with numerous biomedical (Cavanaugh et al. 1990) and environmental applications (Llorente et al. 2012; Sawyer 1995). The assays were carried out using a previously described method for Caco-2 cells (Leusch et al. 2014) with minor modifications for FHs 74 Int cells. Briefly, each of the tested DBPs was prepared as a concentrated stock in methanol (MeOH) up to a concentration of 1 M or, in the case of DBBQ, to the limit of solubility (0.25 M). On day 1 of the assay, plates were seeded at a density of 1 × 10^5 per well (100 μL) in clear, sterile, flat-bottom 96-well microtiter plates (Greiner Bio-One CELLSTAR™; catalog no. 655-180), using PBS (pH 7.4; Invitrogen), 0.25% (wt/vol) trypsin/EDTA (Invitrogen) solution and growth medium (Hybri-Care medium (Hybri-Care medium) supplemented with 30 ng/mL EGF (90%, Thermo Fisher Scientific) and 10% FBS (Gibco). Eighteen hours later, the growth medium was removed by use of a vacuum aspirator, and wells were washed twice with 150 μL of warm (37°C) PBS (pH 7.4). The assay medium, spiked with serially diluted DBPs, was added into the test wells to a total volume of 100 μL per well. After 4 h of incubation at 37°C and 5% CO2, cells were again washed with PBS (2 ×), 100 μL of NR solution (0.33%, 3.3 g/L in DPBS) was added and the plate incubated for 1 h. Finally, the NR solution was aspirated from the wells, cells were gently washed with warm PBS (150 μL per well), 150 μL of NR desorbing fixative (50% EtOH/H2O, 1% acetic acid) was added, and the plate was incubated for 5 min at room temperature. Absorbance was measured at 540 nm using a FLUOstar Omega® (BMG LABTECH) plate reader.

A cytotoxicity concentration–effect curve was generated using FHs 74 Int cells for each of the DBPs combining the data from all the individual runs (n = 12; a minimum of two individual runs on two separate days) (Figure 1). Absolute absorbance values were converted to percent mean absorbance of untreated cell control wells (i.e., percent negative control) by first subtracting the mean background absorbance from the absolute absorbance value of each test well, then dividing the resulting value by the mean absorbance of the negative control, and finally multiplying by 100. Data were normalized in GraphPad Prism for Windows (version 6.05; GraphPad) using the program’s Normalize function to standardize slight fluctuations between each run, and the percentages of negative control values were plotted against the log concentration (M). The median inhibition concentrations (IC50s) for each of the DBPs were then calculated using Equation 1
The IC_{10} value was calculated from those parameters by use of Equation 2:

\[
\log (\text{IC}_{10}) = \log (\text{IC}_{50}) - \frac{\log (\text{top} - \text{bottom})}{10 - \text{bottom}} - 1
\] 

(2)

The IC_{10} values for all eight DBPs obtained with FHs 74 Int cells in this study were compared to previously published IC_{50} values in CHO cells (Table 5 in Wagner and Plewa 2017) by correlation analysis [Pearson Product Moment Correlation in SPSS Statistics for Windows (version 22; IBM Corporation)].

**Exposure and RNA Preparation**

FHs 74 Int cells (passages 3–7) were seeded in 6-well plates at a density of 1 × 10^6 per well 24 h prior to treatment with the DBPs. Each well received an IC_{10} concentration (Table 1) of test DBP or vehicle control, in duplicate, and was incubated at 37°C for 4 h. The 4-h treatment time was selected empirically based on experiments on the induction of genomic DNA damage by DBPs in CHO cells. That data demonstrated that a 4-h period allowed for the induction of DNA damage before the effect of DNA repair was observed (Komaki et al. 2009). In addition, the 4-h exposure time was established in other studies on the toxic mode of action by DBPs by use of CHO (Dad et al. 2013; Komaki et al. 2014) and FHs 74 Int cells (Pals et al. 2013). Finally, using CHO cells, the 4-h treatment period was established as a standard procedure to determine genomic DNA damage across a wide range of DBP chemical classes (Wagner and Plewa 2017).

The wells were then washed with warm PBS and cells were lysed with QIAzol (Qiagen) (1 mL per well), collected in 1.5-mL microcentrifuge tubes, and frozen (−20°C) overnight.
aqueous layer was then used for RNA extraction using an RNaseq Mini Kit (Qiagen) following the manufacturer’s protocol with minor modification. In brief, the cell lysates in 1.5-mL tubes were brought to room temperature (15–25°C), homogenized by vortexing for 1 min, and placed on the benchtop for 5 min. Chloroform [200 μL, molecular biology (MB) grade, Sigma-Aldrich] was added to each tube, which was then shaken vigorously for 15 s and placed on the benchtop for 2–3 min. The lysates were then centrifuged for 15 min at 12,000 × g at 4°C to ensure efficient phase separation. A 500-μL aliquot of the top aqueous layer of each sample was then carefully transferred to a fresh, nuclease-free, 1.5-mL microcentrifuge tube, to which 750 μL of 100% ethanol (MB grade, Sigma-Aldrich) was added and mixed thoroughly by pipetting up and down several times. Each sample (700 μL) was then immediately loaded onto the RNaseq Mini spin column and centrifuged at ≥8,000 × g for 15 s at room temperature. The flow-through was discarded and the process repeated until all of the sample was processed. Next, 500 μL of Buffer RPE (from the RNaseq Mini Kit) was added to each spin column and centrifuged at ≥8,000 × g for 15 s to wash the column, and the flow-through was discarded. Another 500 μL of Buffer RPE was added to each spin column and centrifuged at ≥8,000 × g for 2 min to dry the spin column membrane. The spin column was placed into a fresh 2-mL collection tube and centrifuged at full speed for 1 min. Finally, each spin column was transferred into a fresh 1.5-mL microcentrifuge tube, 20 μL of nuclease-free water was pipetted directly onto the spin column membrane, and the column was centrifuged at ≥8,000 × g for 1 min to elute the RNA. This final process was repeated with an additional 20 μL of nuclease-free water, for a total of 40 μL of total RNA extract.

The yield and purity of extracted RNA was measured spectrophotometrically using a BioSpectrometer (Eppendorf South Pacific) equipped with a Traycell microliter measurement cell (Hellma GmbH & Co. KG), and RNA integrity was determined at the Ramaciotti Centre for Genomics using a 2100 Bioanalyzer (Agilent Technologies) prior to hybridization and microarray sample analysis (Table 2).

Microarray Transcriptomics and Statistical Data Analysis

The RNA extracts were analyzed using HumanHT-12 v4 Expression BeadChip arrays (Illumina). Hybridization and scanning were performed at the Ramaciotti Centre for Genomics with the supplied total RNA extracts. The raw fluorescence data was then transformed using the preprocessing variance stabilization algorithm (Lin et al. 2008), base-2 log transformation, and quantile normalization using the lumi package in the Bioconductor application suite (version 3.2) for R statistical programming language (version 3.5, R Development Core Team) (Du et al. 2008; see Supplemental Material for the transformed microarray expression data). Ultimately, sets of statistically significant differentially expressed genes (DEGs) for each sample–control pair (n = 2) were identified with the Multiple Experiment Viewer (MeV) suite (version 4.90; The Institute for Genomic Research) for Windows (Saeed et al. 2003) using rank product algorithm (Breitling et al. 2004) set to 1,500 random permutations. DEGs with p ≤ 0.01 and fold change (FC) ≥ 1.2 were considered statistically significant and were further used in biological context analysis using pathway enrichment.

Confirmatory quantitative real-time polymerase chain reaction. For comparison, the expression of the gene heme oxygenase 1 (HMOX1) was analyzed by quantitative real-time polymerase chain reaction (qPCR) in a parallel set of experiments with the same concentrations of DBPs and exposure durations. We selected to perform the confirmatory real-time qPCR on HMOX1, which: a) was detected consistently in a quantitative manner in our microarray experiments for all tested DBPs; b) has an established role in the response to oxidative stress (Poss and Tonegawa 1997); and c) was previously shown to be dysregulated in response to inflammation in mice (Takagi et al. 2018).

B Briefly, we exposed the FHs 74 Int cells to the selected DBPs and extracted total RNA using the methodology described earlier in this study; we then reverse transcribed 500 ng of the total RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad) and amplified it on a CFX96 Touch™ Real-Time PCR System (Bio-Rad) using iTaq™ Universal SYBR® Green Supermix (Bio-Rad) per the manufacturer’s instructions under the following conditions: initialization 95°C/60 s, followed by 44 cycles of denaturation 95°C/15 s, annealing 59°C/20 s, and extension 72°C/20 s. Next, we transformed the resulting raw data of triplicate cycle threshold values into relative expression quantities considering the primer amplification efficiencies (E, 90% < E < 110%) and normalized them using expression values for the ribosomal protein L27 (RPL27) using the method described in Pfaffl (2004), yielding normalized relative quantities (NRQs). These are shown as the mean values (n = 3) of a minimum of two repeat experiments (Figure 2 and Table 3). Finally, we determined the statistical significance (p ≤ 0.05) of the resulting NRQ values by ordinary one-way analysis of variance with Dunnett’s multiple comparison correction method in GraphPad Prism (version 7.05, GraphPad). For comparison, the qPCR NRQ values are equivalent to the FC values obtained from the microarray analysis (Figure 2).

The primer set sequences for HMOX1 were designed using the Primer-BLAST (NCBI) tool (Ye et al. 2012) (forward primer: 5′-ATCTCCGTGAGATGACTCCC-3′; reverse primer: 5′-GGGG-GCGGAGATCTGACTT-3′), and for RPL27, adopted from Ersahin et al. (2014) (forward primer: 5′-ATCGCCAAAGAGA-TCAAGATATA-3′; reverse primer: 5′-TCTGAAACATCTTGATTGAGC-3′). Both primer sets were synthesized commercially (GeneWorks) and evaluated for amplification efficiency (E) (95.8 and 90.5% for HMOX1 and RPL27, respectively).

Hierarchical Clustering and Biological Context Analysis

Agglomerative hierarchical clustering analysis of the DEG data was performed using unweighted pair-group averages and Pearson’s correlation coefficient (XLSTAT version 2016 for Windows; Addinsoft).

Table 2. Summary of RNA yield (ng/μL), purity (absorbance ratios, A260/A280 and A260/A230), and RNA integrity number (RIN).

| RNA extract ID | RNA concentration (ng/μL) | A260/A280 | A260/A230 | RIN |
|----------------|---------------------------|-----------|-----------|-----|
| BAA 1          | 69.9                      | 2.03      | 2.01      | 9.5 |
| BAA 2          | 47.5                      | 1.97      | 2.11      | 9.9 |
| BAN 1          | 66.9                      | 1.99      | 2.08      | 9.1 |
| BAN 2          | 64.8                      | 1.88      | 1.95      | 10.0|
| DBBQ 1         | 54.3                      | 2.04      | 2.24      | 8.4 |
| DDBQ 2         | 62.7                      | 2.00      | 2.10      | 9.6 |
| BAM 1          | 72.7                      | 2.00      | 2.20      | 8.5 |
| BAM 2          | 57.4                      | 2.06      | 1.96      | 9.9 |
| BrO₂ 1         | 66.9                      | 2.00      | 2.25      | 9.6 |
| BrO₂ 2         | 79.2                      | 1.95      | 2.07      | 9.7 |
| TBAL 1         | 54.8                      | 2.08      | 2.12      | 9.5 |
| TBAL 2         | 58.8                      | 1.98      | 1.90      | 9.6 |
| TCAA 1         | 67.6                      | 1.95      | 2.04      | 9.2 |
| TCAA 2         | 63.7                      | 2.10      | 2.01      | 9.4 |
| TCAL 1         | 62.1                      | 1.97      | 2.05      | 9.4 |
| TCAL 2         | 58.9                      | 2.07      | 2.03      | 9.4 |
| Negative control 1 | 47.8                  | 2.06      | 2.12      | 9.7 |
| Negative control 2 | 63.4                  | 1.92      | 2.11      | 9.5 |

Note: BAA, bromoacetic acid; BAM, bromoacetamide; BAN, bromoacetimide; BrO₂, bromate; DDBQ, dibromobenzquinone; TBAL, tribromoacetaldehyde; TCAA, trichloroacetic acid; TCAL, trichloroacetaldehyde.

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To investigate biological significance of the identified deregulated genes, we enriched the resulting gene sets, based on the Illumina gene identifiers (p ≤ 0.01; FC ≥ 1.2), using tools available in GeneGo’s MetaCore bioinformatics suite (version 6.33 build 69110, Clarivariate Analytics; see Supplementary Material for MetaCore output file). From the obtained data, we focused on statistically significant toxicity networks (p ≤ 0.05). This allowed us to identify a small number of altered biological processes indicative of the mechanisms of toxicity of the selected DBPs.

### Quality Assurance and Quality Control

We performed all the exposure tests with careful consideration of in vitro quality assurance and quality control measures, which included replicate wells for each of the tested concentrations, at least one independent replicate run on a separate day, multiple cell-free wells to correct for baseline variability and to serve as a negative control for DEG determination, multiple wells containing cell culture media only, and solvent control wells. In addition, we employed nontransformed human cells in order to prevent altered gene expression associated with neoplastic cell lines.

### Results

#### Cytotoxicity Assay

The 10% inhibition concentration (IC10) values of the 4-h cytotoxicity assay are summarized in Table 1 (see Figure 1 for concentration–effect curves). The IC10 values for each of the tested DBPs were subsequently used in exposure treatments (4 h) of the same cell culture for microarray analysis. As IC10 values ranged over a factor of 10,000, dosing equimolar concentrations would not have yielded comparable gene expression levels. These DBP concentrations and their resulting cytotoxicity in FHs 74 Int cells were highly correlative with the published median lethal concentration (LC50) values using CHO cell cytotoxicity analyses (r = 0.94; p ≤ 0.001; n = 8) (Wagner and Plewa 2017).

#### Global Gene Expression Microarray Analysis

GE analysis using cDNA microarray revealed that only a small subset of genes was affected by treatment with the selected DBPs at IC10 concentrations. From the total of 47,231 gene probes corresponding to 23,775 genes annotated to Illumina tags (ILMN_ID) by GeneGo’s MetaCore, less than 10% were identified as differentially expressed (Table 1). A slight dissymmetry toward down-regulation was observed, ranging between 1.1 (DBBQ) and 1.6 (TCAL).

Treatment with BAN resulted in the highest number of DEGs (p ≤ 0.01; FC ≥ 1.2), followed by BAA, TBAL, BrO3−, BAM, DBBQ, TCAA, and, finally, TCAL (Table 1). Treatment with Br-DBPs resulted in up to 2-fold higher numbers of DEGs (p ≤ 0.01) than the chlorine-substituted Cl-DBPs (Table 1). All DEGs with p ≤ 0.01 and FC ≥ 1.2 were analyzed by hierarchical clustering, and subsequent biological context analysis (i.e., gene and pathway enrichment analysis) was performed with DEGs relevant to each individual cluster group, separately.

The change in HMOX1 expression upon exposure to the different DBPs in this study was confirmed by qPCR, and both the qPCR and microarray data were in good agreement for this gene (paired t-test, p = 0.87; Figure 2).

#### Hierarchical Clustering and Biological Context Analysis

Similarity-based hierarchical clustering identified three main groups (Figure 3): Cluster 1 consisted solely of the two Cl-DBPs (TCAA and TCAL), while Cluster 2 and Cluster 3 incorporated the remaining six Br-DBPs. Cluster 2 contained BAM and DBBQ, and Cluster 3 contained BAA, BAN, BrO3−, and TBAL (Figure 3). The results of querying GeneGo’s toxicity network libraries, using previously identified DEG signatures, were used to assign the biological context of genes and pathways significantly altered by exposure to the studied DBPs. These were subsequently grouped according to the results of hierarchical clustering analysis (Table 4). This analysis revealed similarities, but also differences, in altered pathways between the three clusters. While there was evidence of effects on oxidative stress pathways in DBPs from all three clusters, the number of altered genes associated with oxidative stress was much lower than other pathways (Figure 4). Most notably, all three groupings exhibited a comparatively large number of altered genes related to inflammation and immune responses (Table 4).

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Each RNA extract was tested for purity and integrity using the Agilent 2100 electrophoresis bioanalyzer by the Ramaciotti Centre for Genomics, and only those with RNA integrity numbers >8 were subsequently used in the microarray hybridization (Schroeder et al. 2006) (see Table 2 for details).
cases, the number of DEGs and pathways associated with in the Int, the induction of several genes associated with oxidative stress DBP-exposed cells. 

forms a significant aspect of the documented cellular injury in DBP-exposed cells.

Discussion

The most recent working hypothesis is that DBPs primarily cause adverse effects through mechanisms related to the production of ROS, which subsequently result in the induction of oxidative stress pathways (Stalter et al. 2016). Our observation of comparatively few altered genes associated with oxidative stress (Figure 4) is therefore an interesting outcome and potentially very important for understanding DBP toxicity. It was recently proposed that ROS formation is not the sole mechanism of DBP toxicity per se (Procházka et al. 2015), since ROS can arise as a physiological result from other forms of cellular dysfunction caused by chemical insult (Reuter et al. 2010). Indeed, while it is generally agreed that cells respond to DBPs through pathways sharing ROS-mediated mechanisms (Stalter et al. 2016; Procházka et al. 2015; Luo et al. 2017), it is becoming clearer that the specific genes associated with oxidative stress involve additional layers of complexity (Pals et al. 2013). One key piece of the puzzle therefore involves identifying the functional basis of ROS production elicited by DBP exposure (Pals et al. 2013). Untargeted transcriptomic analysis offers a powerful means to broadly identify DBP-responsive genes and thus reveal other deregulations that might be associated with ROS production and the manifestation of oxidative stress responses. Results of the present study offer compelling evidence suggesting a role of profound anti-inflammatory response pathways, which we hypothesize forms a significant aspect of the documented cellular injury in DBP-exposed cells.

We observed, in a nontransformed human cell line, FHs 74 Int, the induction of several genes associated with oxidative stress pathways in DBPs from all three clusters, which is consistent with the findings of preceding literature using the same cell line (Pals et al. 2013; Attene-Ramos et al. 2010). However, in all cases, the number of DEGs and pathways associated with inflammation and immune responses were by far the most prominent (Figure 4). Clear differences were observed between the less toxic Cl-DBPs (Cluster 1) and more toxic Br-DBPs (Clusters 2 and 3). This may suggest a more complex and mechanistically distinct set of early cellular responses associated with Br-DBPs and supports existing evidence of a lower toxicity risk for their chlorinated analogs (Procházka et al. 2015; Plewa and Wagner 2015; Wagner and Plewa 2017). For Br-DBPs in particular, we hypothesize an increased risk of genotoxicity associated with considerable activation of inflammatory responses, for example, characterized by the production of pro-inflammatory (e.g., interleukin 1; IL-1) and anti-inflammatory (e.g., IL-6) cytokines. It is plausible that such responses represent a key mechanism initiating various downstream pathways (van der Veen et al. 2016), including the subsequent generation of ROS (Pals et al. 2013, 2017). Based on concurrent up-regulation of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, we speculate that this could involve downstream activation of Toll-like receptors (TLRs) (van der Veen et al. 2016; Gilbert et al. 2004). While it is unlikely that Br-DBPs interact directly with such cellular surface receptors, TLR signaling pathways can be activated by production of pro-inflammatory cytokines (Ceribelli 2016), potentially resulting in chronic inflammation and increased production of ROS (Lucas and Maes 2013). The process whereby activation of TLR pathways contributes to tumorigenesis has been relatively well characterized, albeit not in relation to DBP exposure (Rakoff-Nahoum and Medzhitov 2009; Wang et al. 2014). As indicated, this is merely speculation based on the observed results in relation to existing literature surrounding TLR signaling pathways, and thus, further research is needed to explore this hypothesis and further reveal the mechanistic basis of genotoxicity and potential carcinogenicity related to DBP exposure.

There is a growing realization that inflammatory response pathways are important contributors and regulators of a diverse range of adverse toxicity outcomes (Angrish et al. 2016; Villeneuve et al. 2018). Earlier research has established a strong association between inflammatory networks and ROS production and revealed a high level of interconnectivity that could perpetuate the oxidative damage associated with inflammation responses (Reuter et al. 2010). Observed activation of anti-inflammatory response pathways alongside the pro-inflammatory response is most likely a result of a feedback mechanism, which, in the absence of additional toxic insult or injury, may ultimately lead to homeostasis and recovery (Medzhitov 2010). However, chronic inflammation and oxidative stress may pose an enhanced risk of activating various associated downstream pathways, due to what has been termed a “vicious cycle” of adaptive responsiveness (Fediero et al. 2007). To clarify, inflammatory responses induce the production of ROS, and the resulting ROS lead to further production of intermediaries that, in turn, induce additional inflammation (Reuter et al. 2010). Oxidative stress–related xenobiotic-induced ROS production may therefore help explain the various other affected pathways, for example, cell proliferation and regulation of apoptosis (Burdon 1995).

By increasing cellular level of oxidants, many xenobiotics alter gene expression via activation of cellular signaling pathways, including adenylyl cyclase pathway, calcium-dependent signaling pathways, and transcription factors (TFs) such as nuclear factor (erythroid-derived 2)-like (Nrf2), activator protein 1 (AP-1) and NF-κB. Other pathways reportedly influenced by ROS-mediated oxidative stress include altered expression of MAPKs, for example, extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 kinases (Amstad et al. 1992; Angel and Karin 1991; Brown et al. 1998; Gius et al. 1999). Indeed, these various pathways are highly consistent with the DBP-responsive genes (most notably for Br-DBPs in Clusters 2 and 3) identified using microarray analysis of FHs 74 Int cells in the present study. Importantly, many of these pathways could be activated either indirectly or directly as a consequence of alterations to genes associated with inflammation and immune responses.

The use of nontransformed human epithelial cells and untargeted transcriptomics highlighted this connection where many other studies have not, probably due to the greater scope for cellular transcriptional responses compared with more targeted
transformed cell lines (Zhang et al. 1997; Hoheisel 2006; Chang et al. 2013). A recent study using a transformed human uroepithelial cell line (SV-HUC-1) observed increased expression of several Nrf-2 TF-mediated oxidative stress–response genes, including PTGS2 and HMOX1 (Li et al. 2018), as did our previous work with Caco-2 cells (Pals et al. 2013; Procházka et al. 2015).

The present study evaluated individual DBPs to explore differences in gene expression and subsequently compare the mechanistic basis of toxicity. One limitation of this study is the lack of PCR confirmation of genes of interest other than HMOX1, which we were not able to include due to budgetary constraints. While we do delve into individual DEGs in the discussion, our conclusions are based on analyses of whole pathways, integrative of multiple DEGs, thus providing a degree of resilience against potential occasional inaccuracies in microarray gene expression data. Still, confirmation by qPCR of individual genes affected by exposure to the DBPs highlighted in this study would be warranted in the future.

Future research is now needed to investigate the potential augmented risk associated with the presence of DBPs as complex mixtures (Teuschler and Simmons 2003; Massalha et al. 2018; Massalha et al. 2017).

### Table 4. Cellular processes and associated toxicity networks (p ≤ 0.05) with up- and down-regulated genes (p ≤ 0.01), fold change (FC) ≥ 1.2 identified using GeneGo’s MetaCore toxicity enrichment tool.

| Cluster | Affected cellular process | Dominant toxicity network(s) | Upregulated genes | Downregulated genes |
|---------|---------------------------|-----------------------------|-------------------|---------------------|
| Cluster 1 | Chemotaxis | MAPK cascades | HMOX1, HSP70 (HSPA1A, HSPA1B), CRK, GRP78 | IRF1 |
| | Inflammation/immune response | Antigen presentation/MHC class 1 signaling/MAPK signaling | HMOX1, HSP70, HLA-A, GRP78 | IRF1, BDNF |
| | Protein folding | Unfolded protein response (UPR) via heat shock protein (HSP) 70, HSP90, and p53 | HSP70 (HSPA1A, HSPA1B, HSPA6), HSP40 (DNAJB1), Aha1 (AHSA1), GRP78 | HSP10 (mitochondrial) |
| | Apoptosis | TNF receptor (TNFR) signaling | A5A, TNFR1, GSTM3 | — |
| | Oxidative stress response | NF-κB regulation | — | BIRC2, BIRC3 |
| Cluster 2 | Inflammation | IL-1 pro-inflammatory signalling | IL-6, IRAK2, CXCL2, CXCL5, GNA13, NF-kB, HRH1, JAK1, I-kB (NFKBIA, NFKBIIE), CXCL1 | AP-1, endothelin 1 (EDN1) |
| | Chemotaxis | MAPK cascades/GRO signalling | HSP70 (HSPA6, HSPA1L, HSPA1A, HSPA1B), HMOX1, IRAK2, IL-1a, IL-1b, IL-1R, NF-AT2, JAK1, CRK | AP-1 |
| | Cell cycle dysregulation | Signalling to E2F | c-Abl (ABL1) | H2AX, GADD45α, GADD45β, GADD45γ, cyclin D, cyclin E, cyclin A, CDC45L, MCM6, MAP3K, AP-1, FEN1, CDK1 (p34) |
| | Protein folding | UPR via HSP90 | CRYAB, HSP40 (DNAJB1), HSP105 (HSPA1H), HSP90AB1, DNAJ1, AHSJ1, HSPA8B, HSP70 (HSPA1A) | GADD45α, GADD45β, AP-1, endothelin 1 (EDN1), CDK1 (p34), APC, GADD45α, GADD45β |
| | Apoptosis | MAPK cascades (MAPK4 & MAPK9) | c-Abl (ABL1), IL-1b, IL-1α, I-kB (NFKBIA, NFKBIIE), HMOX1 | GADD45α, GADD45β, AP-1, endothelin 1 (EDN1), CDK1 (p34), APC, GADD45α, GADD45β |
| | DNA damage response | Inhibition of apoptosis, dysregulation of cell cycle, up-regulation of double-stranded DNA repair | — | CDK1 (p34), APC, GADD45α, GADD45β |
| Cluster 3 | Inflammation/immune response | IL-1 pro-inflammatory signalling/IL-6 signalling | COX-2, HMOX1, IL-1α, IL-1β, NF-kB, IRAK2, C/EBPβ, IRF1, IL-6, I-kB, IL4R, IL-13RA2, JAK1, CXCL2 | ERK1, AP-1, HMOX1, IL13RA1, PI3K reg class IA |
| | Chemotaxis | HGF signalling, Cell communication | COX-2, CXCL1, EGFR, IL-8, CXCL5 | AP-1, PI3K reg class IA, calmodulin |
| | Cell cycle dysregulation | Signalling to E2F via cyclin D and cyclin E | BCAR1, MEKK4 (MAP3K4) | GADD45β, GADD45α, AP-1, MCM3, cyclin D, cyclin E, CDC45L, PCNA, TCF |
| | Signal transduction | Signalling via IL-1b and IRF1 | COX-2, IL-1a, IL-1b, HMOX1, NF-kB, IRAK2, IRF1, IFN-α/β receptor, EG15, CCL5 | HMOX1, AP-1, calmodulin |
| | Proliferation induction | PDGF signalling | COX-2, NF-kB p50/RelB, PA24A, PDGF-C | AP-1, ERK1, calmodulin |
| | Apoptosis | MAPK cascades | IL-1a, IL-1b, IRAK2, MEKK4 (MAP3K4), HMOX1 | GADD45α, GADD45β, AP-1, HMOX1 |
| | Oxidative stress response | HNF4 regulation | COX-2, SOD2, HMOX1, TXNRD1, SMAD3 | AP-1, PRDX5, HMOX1 |

Note: Clusters (outlined in Figure 3) are defined as follows: Cluster 1: TCAA, trichloroacetic acid; TCAL, trichloroacetaldehyde; Cluster 2: BAM, bromoacetamide; DBBB, dibromo-benzoquinone; Cluster 3: BAA, bromoacetic acid; BAN, bromoacetoniitrile; BrG-, bromate; TBAL, tribromoacetaldehyde. —, no data; IL, interleukin; MAPK, mitogen-activated protein kinase, Nrf2, nuclear factor (erythroid-derived 2)-like.
Dong et al. 2017; Plewa et al. 2017). Information regarding chemical mode of action, such as that provided by the present study, is critical for determining whether there is a likelihood of enhanced toxicity due to mixture effects (Qin et al. 2011). Despite the identification of three distinct clusters and notable differences between the toxicity and gene expression profiles of Br- and Cl-DBPs, there were similarities in the transcriptional responses of FHs 74 Int cells to all compounds (Figure 4). It is therefore likely that enhanced toxicity might occur from exposure to a mixture compared to individual DBPs (Groten 2000; Yeatts et al. 2010). The observed differences between Cl-DBPs and Br-DBPs may suggest unique mechanisms of action for these compounds. However, it is also possible that the lower toxicity of Cl-DBPs allows cells to compensate through protein repair mechanisms (e.g., heat shock proteins), whereas the greater toxicity of Br-DBPs overloaded such compensatory mechanisms, resulting in subsequent activation of other more damaging signaling pathways, including inflammatory responses.

Conclusions
Gene expression–based toxicogenomic analysis can be a sensitive and robust tool for comparative assessment of biological activity of chemical compounds. However, several crucial factors must be considered to obtain meaningful data. For example, the provision of IC₁₀ concentrations from cytotoxicity data employing specific and constant exposure times to DBPs at predetermined equipotent concentrations was an important step aimed at reducing the possibility of transcriptome alterations associated with...
dead or dying cells. Additionally, where many in vitro bioassays use tumor cell lines because of their rapid growth and ease of maintenance, nontransformed human cell lines have the added advantage of further avoiding erroneous gene expression profiles associated with neoplastic cell lines. Finally, the FHs 74 Int cell line offers the additional advantage of being very well suited for evaluating effects on immunomodulatory and inflammatory response pathways. With these strengths, our results offer considerable evidence that ROS-mediated oxidative stress pathways may be associated with inflammatory response pathways, which could contribute to a cycle of toxic insult. Considering the well-documented relationship between inflammation and cancer progression, further research exploring this relationship is warranted. Effects of individual DBPs are needed to unravel mechanistic information, but there is clear potential for complex mixtures to occur, and the toxicity of relevant DBP mixtures must be investigated in the future.

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