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Benchmark Dose Modeling Approaches for Volatile Organic Chemicals using a Novel Air-Liquid Interface In Vitro Exposure System

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Abstract

Inhalation is the most relevant route of volatile organic chemical (VOC) exposure; however, due to unique challenges posed by their chemical properties and poor solubility in aqueous solutions, *in vitro* chemical safety testing is predominantly performed using direct application dosing/submerged exposures. To address the difficulties in screening toxic effects of VOCs, our cell culture exposure system permits cells to be exposed to multiple concentrations at air-liquid interface (ALI) in a 24-well format. ALI exposure methods permit direct chemical-to-cell interaction with the test article at physiological conditions. In the present study, BEAS-2B and primary normal human bronchial epithelial cells (pHBEC) are used to assess gene expression, cytotoxicity, and cell viability responses to a variety of volatile chemicals including acrolein, formaldehyde, 1,3-butadiene, acetaldehyde, 1-bromopropane, carbon tetrachloride, dichloromethane, and trichloroethylene. BEAS-2B cells were exposed to all the test agents, while pHBECs were only exposed to the latter four listed above. The VOC concentrations tested elicited only slight cell viability changes in both cell types. Gene expression changes were analyzed using benchmark dose (BMD) modeling. The BMD for the most sensitive gene set was within one order of magnitude of the threshold-limit value reported by the American Conference of Governmental Industrial Hygienists, and the most sensitive gene sets impacted by exposure correlate to known

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Conflict of Interest/Disclosure

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adverse health effects recorded in epidemiologic and *in vivo* exposure studies. Overall, our study outlines a novel *in vitro* approach for evaluating molecular-based points-of-departure in human airway epithelial cell exposure to volatile chemicals.

Keywords

inhalation; benchmark dose; *in vitro*; cell culture exposure system; VOC; transcriptomics

Introduction

Well-designed *in vitro* exposure models require less time and fewer resources compared to traditional *in vivo* toxicity testing, and in some cases better mimic human biological relevance. Still, air pollutants and inhaled toxicants remain one of the most difficult human exposures to replicate in an *in vitro* laboratory setting. Inhaled volatile organic chemicals (VOC) are particularly challenging to test reliably *in vitro*, given difficulties in generating the appropriate test agent atmospheres, relevance and transferability of the cell or tissue models used, and compatibility with high-throughput screening approaches. VOC inhalation exposure is prevalent in both occupational and environmental settings, representing a significant risk to human health (Alabdulhadi et al. 2019; Cheng et al. 2019; Chiang et al. 2010). Currently, approximately one fourth of chemicals nominated for study in the U.S. Environmental Protection Agency's (EPA) chemical substance inventory are volatile/semi-volatile or are not soluble in DMSO or water and cannot be tested using traditional *in vitro* methods for evaluating chemical safety (USEPA 2020b). *In vivo* rodent inhalation toxicity testing for VOCs has been the gold standard for regulatory agencies but may provide limited relevance to human health effects due to differences in human physiology, such as rodents primarily being obligate nasal breathers (Bates and Irvin 2003; Harkema 1991; Harkema et al.). Additionally, biological effects of VOCs cannot be confidently evaluated in high-throughput test systems that use submerged cultures due to solubility problems and evaporation from the culture wells. Poorly soluble test agents have minimal contact with the cells in a direct-dosing application, further complicating the process of determining delivered dose to the cells. These issues have led to the exclusion of VOCs from testing within the Toxicity Forecaster (e.g. ToxCast) screening program and prevented generation of data from that collection of assays for use in risk assessment (Judson et al. 2010). Thus, new approach methods (NAMs) that include *in vitro* exposure technology for VOCs and associated assays for evaluating biological activity of VOCs in human cells, such as high-throughput transcriptomics, are necessary to provide comprehensive information regarding these data poor chemical species (Harrill et al. 2021).

To circumvent the difficulties in toxicity screening of volatile test agents, we developed a cell culture exposure system (CCES) that permits cells to be exposed to multiple concentrations of test agent at air-liquid interface (ALI) in a 24-well format. ALI exposure methods permit direct chemical-to-cell interaction at physiological conditions, providing a more environmentally realistic airway exposure condition, as compared to submerged cultures (Doyle et al. 2004; Dvorak et al. 2011; Ebersviller et al. 2012). Our previous studies utilizing the CCES established a consistent capacity to deliver high concentrations

of volatiles to ALI cell culture inserts (Zavala et al. 2017; Zavala et al. 2018). A variety of *in vitro* biological effects data can be analyzed in ALI cultures exposed to VOCs using the CCES, including measures of cell viability, release of soluble factors, and gene expression. In comparative studies, ALI exposures have been shown to be a good representation of the *in vivo* airway epithelial transcriptome (Baldrige et al. 2015; Dvorak et al. 2011). Therefore, high-throughput transcriptome profiling using RNA-Seq or targeted RNA-Seq may provide insight into the molecular mechanisms of toxicity for VOCs. Further, the ability of the CCES to conduct concurrent exposure to six concentrations of a test agent through serial dilution allows for a concentration-response assessment to be conducted in a single experiment. By implementing a novel multiple concentration exposure system, we can apply EPA-developed NAMs for dose-response modeling of *in vitro* data.

Using the CCES exposure design, our data is amenable to transcriptomic benchmark dose (BMD) analysis as outlined in previously published methods for integrating BMD estimates with genomic data and the National Toxicology Program approach to genomics dose-response modeling (NTP 2018; Thomas et al. 2007; Thomas et al. 2011; Thomas et al. 2013). Research utilizing TempO-Seq high-throughput transcriptomics (HTTr) analysis has produced technically and biologically reproducible concentration-response modeling data (Gwinn et al. 2020; Harrill et al. 2019; Harrill et al. 2021). These previous studies show that transcriptome-based BMDs accurately predict points of departure (PoD) and mode of action (MoA) for both VOCs and non-VOCs (Jackson et al. 2014; Johnson et al. 2020; Thomas et al. 2007). Optimized analytical protocols utilizing BMDExpress2 consider transcriptomics data at the level of individual genes, as well as at the level of aggregated gene set collections to identify the most sensitive cellular responses to chemical exposure (Filipsson et al. 2003; Phillips et al. 2019; Thomas et al. 2007; Yang et al. 2007). For the first time, our experimental design implements HTTr BMD analysis techniques with *in vitro* ALI VOC exposures.

Over the course of the study, we tested eight widespread VOCs in BEAS-2B cells with four of those test agents (1-bromopropane, carbon tetrachloride, dichloromethane, and trichloroethylene) repeated in primary human bronchial epithelial cells (pHBECs). To stay within relevant concentrations and below overt cytotoxicity, the highest concentration tested was targeted as 10-fold higher than the occupational threshold limit value (TLV®). TLVs are protective occupational exposure limits as determined by the American Conference of Governmental Industrial Hygienists (ACGIH) committee review of existing published and peer-reviewed literature. (ACGIH 2018). In addition to evaluating changes to cell viability, the primary endpoint focused on using TempO-Seq to identify changes in gene expression at both the gene and aggregate gene set level to determine the biological pathway altering concentration (BPAC) for each test agent. Finally, we utilized ACGIH TLVs and *in vivo* toxicity data from EPA Integrated Risk Information System (IRIS) assessments as benchmarks for comparison to *in vitro* transcriptional PoDs. The study reported in this manuscript characterizes the impact VOCs have on human airway epithelial cells and establishes HTTr and BMD analysis as a primary diagnostic tool for ALI gas and vapor exposures. The novel approach also supports a recent research focus on *in vitro* methodologies to support traditional *in vivo* toxicity testing (USEPA 2014).

Materials and Methods:

BEAS-2B Cell Culture

The BEAS-2B cells used are a non-diseased, viral transformed human bronchial epithelial cell line acquired from ATCC (Manassas, VA, CRL-9609). Prior to cell culture, T75 flasks (Corning, One Riverfront Plaza, NY, 430641) and 6.5 mm Transwell® Inserts (Costar, Corning, NY, 3470) were coated with 100 µg/mL bovine collagen (PureCol Collagen [Advanced Biomatrix, San Diego, CA, 5005] diluted in Ultrapure Molecular (nuclease free) Water [Invitrogen, ThermoFisher Scientific, Rockford, IL, 10977023]) for 2 h at room temperature, followed by a water rinse. To prepare the cells for exposure at ALI, cells were thawed from pre-passaged frozen vials, grown on a T75 flask at 37°C 5% CO₂ incubator conditions. The cells proliferated while submerged in Keratinocyte Growth Medium (KGM) (Keratinocyte Basal Medium (KBM) [Lonza, Walkersville, MD] plus KGM Gold Bullet Kit [Lonza, Walkersville, MD, 00192151]) with 25 mM HEPES Buffer (Sigma-Aldrich, St. Louis, MO) or Bronchial Epithelial Growth Medium (BEGM) (Bronchial Epithelial Basal Medium (BEBM) [Lonza, Walkersville, MD, CC-3171] plus BEGM Bullet Kit [Lonza, Walkersville, MD, CC-3170]). The utilized media type was switched to the latter (BEGM) for the experiments with dichloromethane, trichloroethylene, carbon tetrachloride, and 1-bromopropane due to vendor formulation changes and availability. Cells were grown for a minimum of two subsequent passages after thawing. Cells were passaged when approximately 75% confluent and new flasks seeded at 1×10^6 cells. The 6.5 mm inserts were seeded at $2\text{--}5 \times 10^5$ cells/insert and were maintained for 48–72 h before being used for exposure as a fully confluent monolayer. All apical media was removed at least 5 days before using BEAS-2B cells for exposure to the test agent. No FBS was used in the culture of BEAS-2B cells which were observed to have normal basal respiratory cell morphology at ALI.

Primary Human Bronchial Epithelial Cell Culture

Primary human bronchial epithelial cells (pHBEC) were obtained via bronchial brushing from healthy, non-smoking donors aged 21–40 years using a previously published method (Dailey and McCullough 2021a; 2021b; McCullough et al. 2014). Donors gave their consent after being informed of procedures and associated risks. This study utilized cells from 12 healthy non-smoking donors, 10 male and 2 female donors between age 26–41. The consent and collection protocol were approved by the University of North Carolina School of Medicine Committee on the Protection of the Rights of Human Subjects and by the US Environmental Protection Agency. After collection, pHBECs were seeded at approximately 1×10^6 cells and cultured in BEGM (BEGM Bullet Kit, Lonza, Walkersville, MD CC-3170) on plastic T75 tissue culture flasks and expanded until the third passage. For all exposures, cells were lifted using trypsin-EDTA (Gibco 25300062) and plated on uncoated 6.5 mm diameter (24-well format) Transwell™ inserts (Costar, Corning, NY, 3470) at a density of 1×10^5 cells/insert depending on the donor. Each insert of cells was initially grown submerged with 1:1 DMEM-H (Gibco, 11995–065) and BEGM with complete bullet kit of growth supplements, 50 µg/mL of bovine pituitary extract (Lonza) and 1.5 µg/mL Bovine Serum Albumin (Sigma, A9418) on both the basolateral and apical compartments. The pHBECs were then expanded and grown for 2–4 days until they reached confluency as

previously described (Dailey and McCullough 2021a; Ross et al. 2007). The cells were then placed at ALI by removing all apical medium and replacing the basolateral medium with the base media described above, supplemented with 100 nM retinol. The pHBECs continue to differentiate for at least 21 days before being used in an exposure. Previous research categorizing the pHBECs we use show a columnar pseudostratified cell layer with both cilia and mucin producing cells present after day 21 of ALI culture (Ross et al. 2007). All initial pHBEC tissue culture procedures took place at EPA's Human Studies Facility. Cells were transported to the EPA Research Triangle Park campus located approximately 15 miles away and allowed to recover for at least 20 h prior to exposure.

Cell Culture Exposure System

Beginning 2 h prior to test agent exposure, designated cells were prepped for exposure by performing an apical wash with Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco, Grand Island, NY) and replacing the basolateral compartment with 0.5 mL of fresh media with 25 mM HEPES Buffer. Cells remained in the incubator (37°C, 5% CO₂) to recover for 2 h before being placed in the Cell Culture Exposure System (CCES) to undergo test agent exposures. The CCES was developed at EPA's Inhalation Toxicology Facility and is comprised of a base module that accommodates a 24-well tissue culture plate with 6.5 mm diameter Transwell® inserts (patent pending). The CCES was developed to simulate an inhalation exposure chamber design. It supplies air under positive pressure and exhausts air under negative pressure to provide a known concentration of test article over a specific time. The design allows for air flow control (slightly positive pressure) at each set of four wells on a 24-well cell culture plate while also allowing for six concentrations with replicates (four replicate wells per concentration) for each exposure regimen. The inlet nozzles are positioned 2 mm above each of the inserts. The previous study characterizing the CCES showed a uniform distribution of gasses across inserts receiving a single concentration (Zavala et al. 2018). Two CCES modules are used simultaneously. One CCES module receives the test agent at six different concentrations while the other CCES module received filtered humidified dilution air and evaluated as a sham exposure control. The CCES modules (base and top plates) were machined out of anodized aluminum, while the distribution manifolds and inlet nozzles were machined out of stainless steel and Swagelok fittings. Figure 1A shows the dynamic headspace generator, while Figure 1B provides a basic schematic of the entire CCES including the dilution manifold and exposure module chambers used for these experiments. A uniform air flow distribution was maintained through each nozzle so the cells in each Transwell® insert was exposed to either humidified air or diluted test agent at a flow rate of 12.5 mL/min/well. Optimal exposure temperature (37°C), relative humidity (85%) and air flow rates were tested and validated for the CCES in previous studies designed to optimize low variability in delivered material to the replicate wells. (Zavala et al. 2017; Zavala et al. 2018). Cells were exposed to the test agent at ALI for 2 h before being transported back to the incubator (37°C, 5% CO₂) for 4 h prior to sample collection and analysis. A 2 h exposure to the test agent was optimized to avoid stressing cell cultures in initial tests using the CCES for this pilot study. All sample collection and assays were run 4 h post-exposure to complete a 6 h timepoint to reflect both standard *in vivo* inhalation exposure regimes of 4 to 6 h of exposure and high-throughput screening

studies. A sham exposure control cell culture plate which received only humidified air and an incubator control plate were tested in parallel with the plate which received the test agent.

Test Agent Selection and Generation

As part of this pilot study, we selected eight test agents (Table 1) that are relatively common industrial chemicals and have a substantial body of research, including epidemiologic and *in vivo* inhalation exposure studies. Additionally, all the test agents herein have been assessed and assigned occupational exposure limits by professional associations such as the American Conference of Governmental Industrial Hygienists (ACGIH). We utilized the ACGIH TLV to determine the concentration range tested, starting at 10-fold higher than the recommended TLV. A preliminary range finding experiment was conducted in BEAS-2B cells in order to confirm the highest dose did not exceed 50% loss of cell viability. Each chemical was generated based on laboratory specifications as the state which it was procured from the manufacturer. Acrolein, acetaldehyde, and 1,3-butadiene were obtained at a certified gas concentration with the balance being air or nitrogen. 1-Bromopropane, dichloromethane, trichloroethylene and carbon tetrachloride were obtained from the vendors as liquids with certification of analysis for purity. Formaldehyde was generated from solid paraformaldehyde. All vendor information, purity, and specifics to generation methods are listed in Table 1.

The gaseous test agents were used as purchased and the liquid and solid test agents were generated using our in-house dynamic headspace generator, consisting of a sealed vessel (unjacketed pressure filtering reactor, P/N-6384–235; Ace Glass, Vineland, NJ) contained in a warming oven (Heratherm general protocol oven P/N-51028873; ThermoFisher Scientific, Waltham, MA) held at a chemical specific temperature. The sealed vessel contained a reservoir of liquid or solid chemical and had both a carrier gas inlet and outlet that allowed the fixed carrier flow to transport the volatilized chemical vapors. The test agent was then diluted by our in-house compressed air system which provides breathing-quality air, according to the ASTM standards. This pressure-regulated air was delivered to three mass flow controllers, allowing the dilution of test agent (gas or vapor) to be accurately metered and delivered to the first dilution tee on the CCES dilution manifold.

The CCES dilution manifold added the desired quantity of humidified air (80–85% RH at 37°C), to the generated test agent at six points to facilitate a half-log dilution concentration curve (Fig 1b). During exposure, real-time concentrations were measured at each dilution nozzle pair prior to exposing the cells directly (Fig 1a). Concentrations were determined by injecting a 1 mL sample from each chamber inlet nozzle on a Hewlett Packard 5890II or Agilent 6890 gas chromatograph (GC) with a Supelco SBP-624 capillary GC column (30 m × 0.53 mm) coupled to flame ionization detector (FID) or electron capture detector (ECD). Both GCs used hydrogen as the carrier gas at a flowrate of 10 mL/min. The inlets, detectors and oven temperatures were test agent specific and were chosen to give a retention time (RT) of six minutes or less to allow two samples per inlet nozzle and quality control samples. A graphical representation of the actual GC-measured concentrations and the dynamic headspace generator nominal concentration based on dilution are recorded in Supplemental Figure 1.

CellTiter-Glo Luminescent Cell Viability Assay

Cell viability was measured by determining the amount of adenosine triphosphate (ATP) synthesized inside the cells using the Promega CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI). Total cell material was lysed, and ATP was collected on an opaque Corning 96-well plate (Corning, NY) using the reagents from the kit (CellTiter-Glo® Substrate (lyophilized), and CellTiter-Glo® Buffer) and the cell growth media associated with the designated cell type being analyzed. Luminescence was then detected using a SpectraMax® i3 Multi-Mode Microplate Detection Platform plate reader (Molecular Devices, San Jose, CA). An ATP standard curve was used as a positive control and as a quality control parameter to quantify the amount of ATP present based on the luminescence values. This control was made by combining stock ATP standard (Sigma-Aldrich, St. Louis, MO) with the working reagent and performing a serial dilution at μM concentrations of 50, 25, 12.5, 6.25, 3.125, and 0. Data shown are mean \pm SEM from 3 separate biological replicates. A one-way ANOVA and Fisher's LSD post-hoc test was used to determine statistically meaningful differences compared to the sham control exposed cells (p 0.05). Statistical analysis was conducted using GraphPad Prism software (San Diego, CA).

LDH Measurement of Cytotoxicity

Lactate dehydrogenase (LDH) as a measurement of cytotoxicity was determined by sampling the basolateral media from each exposed cell sample 4 h post-exposure. Fifty μL of the basolateral medium from each well were transferred in duplicate to a 96-well clear polystyrene culture plate (Corning, NY). Additionally, three sets of controls were added to the 96-well plate. First, an LDH solution provided as part of the Pierce™ LDH kit (Rockford, IL) was utilized as a positive control. Second, blanks were measured using the corresponding cell culture media. Finally, a completely lysed insert that represented a measure of total (100%) LDH was created by adding lysis buffer to a single incubator control replicate. The LDH reaction was performed using the LDH measurement kit, and the plate was read in a Spectramax i3 (San Jose, CA) with the absorbance set to read at 490 and 680 nm wavelengths. After reading the plate, the raw data was exported into an excel spreadsheet for analysis. Each sample was blank corrected and calculated as percent change from the sham control in relation to the mean of the 100% LDH lysed control inserts. Data shown are mean \pm SEM from three separate biological replicates. A one-way ANOVA and Fisher's LSD post-hoc test was used to determine statistically meaningful differences compared to the sham control exposed cells (p 0.05). Statistical analysis was conducted using GraphPad Prism software (San Diego, CA).

Whole Cell Lysate Collection

An optimized whole cell lysis procedure was used to preserve the RNA of both BEAS-2B and pHBEC samples. After a 4 h recovery post 2 h test agent exposure, the two technical replicate inserts from each exposure condition were placed on a new 24-well plate (Corning, NY). A single replicate insert of the incubator control cells was also preserved for whole cell lysis. Prior to adding the lysis buffer, the cells were thoroughly washed twice with DPBS to remove any remaining apical mucus or surfactant which may interfere with complete lysis. When transferring the BEAS-2B cells or pHBECs, the BioSpyder lysis buffer (Carlsbad,

CA) was added to each of the samples and allowed to incubate for 15 minutes at room temperature. Each sample was mixed and pipetted into an individual well in the 384-well optical imaging plate (Corning, NY). After placing each of the samples into the 384-well plate, it was sealed with ThermalSeal RT 2mil Film (Sigma-Aldrich, MO) and stored in a -80°C freezer until shipment to BioSpyder for analysis.

BioSpyder TempO-Seq Methodology

Plates were shipped to BioSpyder, Inc. frozen (on dry ice) using overnight priority shipping. BEAS-2B and pHBEC lysates were then analyzed by BioSpyder using the TempO-Seq human whole transcriptome version 2 (hWTV2) assay (Yeakley et al. 2017), which includes 22,537 probes covering 19,703 genes. Lysates were processed as described previously (House et al. 2017). In brief, 2 μL of each lysate was hybridized with 2 μL of detector oligos from the hWTV2 assay using the following thermal cycler protocol: 10 min at 70°C , followed by gradual decrease to 45°C over 49 min, terminating with 45°C incubation for 16–24 h. Excess oligos were then removed via nuclease digestion (90 min at 37°C), and hybridized detector oligos were ligated (1 h at 37°C) following respective additions of 24 μL TempO-Seq nuclease and ligation mixes. RNA/DNA duplexes were then heat-denatured, and 10 μL of each ligation product were transferred to an amplification microplate containing 10 μL of PCR master mix per well. Ligation products were then uniquely labeled during product amplification with well coordinate-specific “barcoded” primer pairs containing universal adaptors for sequencing. Samples were pooled into sequencing libraries, distributed across multiple lanes of a HiSeq dual flow cell and analyzed on a HiSeq 2500 Ultra-High-Throughput Sequencing System (Illumina, San Diego, CA). The target depth for each test sample was 6×10^6 total sequenced reads.

HTTr data processing

Raw TempO-Seq data were provided by the vendor as individual FASTQ files for each sample well and were subsequently processed through a custom bioinformatics pipeline previously described (Harrill et al. 2021). Each FASTQ file was aligned to the probe sequences in the hWTV2 assay using HISAT2 v2.1.0 (Kim et al. 2015; Kim et al. 2019) with spliced alignment disabled. Aligned reads in SAM format were processed with SAMtools v1.9 (Li et al. 2009) to compute the number of uniquely aligned reads for each probe. Probe counts and associated metadata for each well were stored for analysis using MongoDB v3.6.14. Source code can be found at https://github.com/USEPA/htrpl_pilot.

HTTr Quality Control

Sample level quality control (QC) criteria were based on the cell viability results for each concentration of the test agents, multiple well-level metrics computed from read mapping rate, and count distribution across probes, as previously described (Harrill et al. 2021). To be considered for BMD analysis, the thresholds for fraction of viable cells (FrVC) and fraction of reads uniquely mapped to probes (FMR) are simple majority cutoffs (50% of cells must be viable; 50% of reads must uniquely map to probe sequences). The threshold for number of uniquely mapped reads (NMR) was set at 10% of target per-sample read depth (600,000 genes). Additional QC metrics outlined in our previous HTTr studies, including Ncov5, Nsig80, and GiC were optimized specifically for MCF7 cells in true high-throughput

screenings and could not be applied as global threshold levels for this limited pilot study design.

BMD Gene Expression Analysis

Transcriptional benchmark dose (BMD) values at the probe level were computed and aggregation of BMD values to the gene set level was performed with BMDExpress2 (Phillips et al. 2019) based on the NTP approach to genomic dose-response modeling (NTP 2018) and previously published manuscripts (Harrill et al. 2021). First, probe-level concentration response analysis was performed as follows: Probe counts for each test agent and corresponding sham exposure controls (subset to samples without QC flags, and probes with mean count > 5) were normalized to log₂ counts per million (CPM) values using the sum of filtered probe counts as the sample depth and addition of a pseudo-count of 1 before converting to log scale. For each test agent, probe-level log₂ CPM values were input to BMDExpress2 using the following optimized parameters: Pre-filtering was used to remove probes that cannot pass an ANOVA test (FDR p -value = 0.05) with a maximal fold-change < 2x across all concentrations. Each probe passing this pre-filter was then fit to eight different dose-response models (linear, poly2, power, Hill, exp2, exp3, exp4, and exp5). The best-fit model for each probe was selected based on the lowest Akaike information criterion (AIC). Models with a goodness of fit p -value < 0.1 and Hill models with k parameter < 1/3 the lowest positive concentration were excluded from final model selection (Akaike 1974; Harrill et al. 2021). The benchmark response (BMR) was set to 1.349 × standard deviation of sham exposure control samples, corresponding to 10% tail in a normal distribution. The BMD value is the concentration at which the winning model curve crosses the benchmark response level (BMR) (Filipsson et al. 2003; Thomas et al. 2007; Yang et al. 2007).

The overall potency of a test agent was further analyzed by aggregating the probe-level BMDs from the best fit models to estimate a biological pathway altering concentration (BPAC) based on the median BMD value for the most sensitive gene set. For the purposes of this study, we used the Molecular Signatures Database v7.2 (MSigDB_C2) collection of annotated gene sets. Briefly, probe-level curve-fits were filtered to only those meeting the following criteria: best fit model produced convergent BMD, BMDL, and BMDU values; BMD < highest measured concentration; BMDU:BMDL ratio < 40; and probe annotated as measuring a single gene. If multiple probes corresponding to the same gene had valid curve fits under this criterion, the gene-level BMD/BMDL/BMDU were taken as the average of all probes with valid curve fits. BMD bounds (i.e. BMDL and BMDU) were computed in accordance with the profile likelihood method (Banga et al. 2002; NTP 2018). The BPAC was computed as the median BMD for all associated genes passing the above filters in the most sensitive gene set. Only gene sets containing at least three valid genes and 5% gene set coverage were retained in our analysis (Harrill et al. 2021; NTP 2018). Additional analysis was conducted to determine the reason probe-level responses were not always captured in gene set results (Figure 4).

Determination of Reference TLV and NOAEL/LOAEL Values

Threshold Limit Values (TLVs) were obtained from the ACGIH® 2018 Guide to Occupational Exposure Values. LOAEL and NOAEL values were obtained by examining

the most recent risk assessment conducted by EPA or ATSDR. A complete list of examined studies and LOAEL/NOAEL values is captured in Table 4. When recording representative values in Table 3, acute or sub-chronic inhalation exposures performed in mice or rats with respiratory/pulmonary endpoints were prioritized, and the most sensitive finding was reported. The 6 h exposures are the most prevalent *in vivo* study design that can be simulated in the CCES optimized full exposure model (2 h test agent exposure, 4 h post). In the case of dichloromethane, sub-chronic 13-week respiratory LOAEL and NOAEL values were identified. Inhalation trichloroethylene studies were quite limited, but many reported changes in auditory and visual function. Immunosuppression LOAEL and NOAEL values were selected because pulmonary endpoints were assessed for immunosuppressive changes (*e.g.*, function of alveolar macrophages). Both subchronic and chronic inhalation studies with carbon tetrachloride were identified, despite no LOAEL and NOAEL values subcategorized into organ systems and all be loosely defined as hepatic changes.

Results:

Evaluation of cell viability and cytotoxicity for each test agent

We designed our exposure conditions to elicit no greater than 50% change to cell viability when compared to the sham exposure control. As described, cell viability was measured by cellular ATP levels in the CellTiter-Glo assay and cytotoxicity was measured by LDH release. In general, the same concentrations were tested in both the BEAS-2B and the pHBECs. As a baseline, we observed no statistical difference between the sham exposed cells and the incubator control cells (Supplemental figure 2). In the four VOCs tested in both cell lines, the pHBECs exhibited less change to cytotoxicity and cell viability at the highest concentration when compared to the BEAS-2B cells exposed to the same test agent.

Cytotoxicity was measured by LDH present in the basolateral compartment post-exposure by percent change relative to the sham control and the 100% lysed cell insert (Figure 2). Many test agent concentrations tested yielded no significant change in cytotoxicity compared to the sham exposure control. However, 1-bromopropane exposure at 10 ppm and carbon tetrachloride at 50 ppm induced a statistically significant increase in BEAS-2B cytotoxicity. Fifty ppm dichloromethane and 100 ppm trichloroethylene caused a significant increase in cytotoxicity in both BEAS-2Bs and pHBECs. All observed significant changes to cytotoxicity fell below a 10% change compared to the sham exposure control.

In Figure 3, when measuring percent cell viability compared to the sham exposure control, BEAS-2B cells exposed to acrolein, 1-bromopropane, dichloromethane, trichloroethylene, and carbon tetrachloride yielded a significant change at the highest tested concentration. Concentrations of 10 and 3.125 ppm acrolein caused the largest observed reduction in viability for BEAS-2B cells. Acrolein at 3.125 ppm had the most impact, reducing cell viability down to below 30%. Again, pHBECs are expectedly more robust and resistant to chemical perturbations compared to the BEAS-2B cells and only displayed a significant change to cell viability when exposed to dichloromethane and trichloroethylene exposure at the highest concentration tested, 500 ppm and 100 ppm respectively.

BMDExpress Concentration-response Analysis

In Table 2, we report median BMD and BMDL values for the top ten most sensitive gene sets meeting our criteria for each test agent and cell type combination tested. Although several major inflammatory, stress response, and cell function gene sets were altered in all VOCs tested, few common trends and overlapping targets were observed in the topmost sensitive gene sets. Ultimately, more chemical test agents need to be tested to reveal the relevance and context activation of specific gene sets. Notably, we also did not observe any overlapping gene sets between the BEAS-2B cells and pHBECs exposed to either trichloroethylene or dichloromethane. In Figure 4, gene level BMDs were calculated for all the VOCs tested in both cell types with most probes exhibiting a concentration response expression between the highest and second highest concentrations tested. Only those gene targets meeting the criteria outlined in the above methods section were considered and analyzed at the gene and gene set level. Both 1-bromopropane and carbon tetrachloride tested in pHBECs did not pass an ANOVA $p < 0.05$ FDR cutoff and were not analyzed further for gene expression changes or BMD. Although gene level BMDs were generated, no gene sets in the MSigDB_C2 collection met our minimum cut-offs for reporting a gene set BMD in the case of formaldehyde and acetaldehyde tested in BEAS-2B cells (Figure 4). We determined that most reported genes were annotated in MSigDB and therefore considered in gene set level analysis, calculated to show 95.13% of the unique entrez gene IDs considered, were also part of the unique entrez gene IDs included in the MSigDB collection. However, these cell type/test agent conditions had a relatively small number of probes with concentration-responsive transcription in the dose range tested, resulting in the lack of reported gene set BMDs for these cases. To be reported, a gene set must contain at least three concentration-responsive gene targets passing the prefilter criteria for inclusion and those gene targets must make up at least 5% of the gene set. In the example of BEAS-2B cells exposed to carbon tetrachloride, we obtained good annotation coverage with a BMD calculated for 584 genes, the majority of which (576) were annotated and mapped to 1429 different gene sets in MSigDB.

The accumulation plots shown in Figure 5 emphasize the range of response sensitivity for each test agent. Generally, the reported BPAC for each reported VOC fall between the second and third highest tested chemical concentration. Acrolein elicited the most sensitive response in a cholesterol synthesis gene set with a BPAC at 0.586 ppm. Moreover, many of the top ten sensitive gene sets recorded BMDs approaching or lower than the ACGIH TLVs, indicated by the blue dotted line in Figure 5. Trichloroethylene (Figure 5A) and carbon tetrachloride (Figure 5B) both produced a BPAC at concentrations below the TLV.

Discussion

Ultimately, the results presented in this manuscript provide a foundation for using *in vitro* ALI cultures together with human-based gene expression dose response modeling NAMs to provide quantitative PODs for VOC exposure. Evaluating *in vitro* transcriptomics driven gene set level BMDs for VOC ALI exposures provided a basis of comparison to both human occupational exposure guidelines, such as the ACGIH TLV and *in vivo* inhalation studies (Table 3). ACGIH TLVs were obtained from the 2018 TLVs and BEIs: based on the

documentation of the threshold limit values for chemical substances and physical agents & biological exposure indices (ACGIH 2018). In most cases, the gene set level BMDs from VOC ALI testing were reasonable approximations of the TLVs. Except for 1-bromopropane, calculated BPACs fell within five-fold of the TLVs for each respective test agent. A five-fold difference represents a significant correlation between our exposure generated BPAC and TLV. Previous studies examining the variability of PODs for *in vivo* experiments show higher uncertainty levels and a larger overall POD range between studies (Paul Friedman et al. 2019; Pham et al. 2020).

In the example of dichloromethane (TLV of 100 ppm), we report a very similar BPAC of 142.1 ppm for BEAS-2B and 226.7 ppm for pHBECs. The ACGIH TLV documentation on dichloromethane includes inhalation study data associated with lung cancer progression *in vivo* (NTP 1986b). Interestingly, the three most sensitive gene sets for dichloromethane exposure in pHBECs (Table 2) are all associated with cell cycle dysregulation and cancer progression (Delacroix et al. 2010; Mattioli et al. 2005). The SWEET_KRAS_TARGET gene set in particular is associated with the development of lung adenocarcinoma (Sweet-Cordero et al. 2005). There is additional overlap between the most sensitive gene sets in the dichloromethane exposed BEAS-2B data and those identified in gene methylation cohort studies (Figuroa et al. 2010). We observed additional concurrence between sensitive gene sets and previously reported toxicological effects for VOCs of interest. Acrolein induced changes in cholesterol synthesis pathways, which are known targets of oxidative stress and acrolein exposure (Moghe et al. 2015). The first and second most sensitive gene set collection in 1,3-butadiene exposed BEAS-2B cells, relates to transcription modification of known markers for myeloid leukemia NUP98 and LIF (Takeda et al. 2006). The EPA hazard summary for 1,3-butadiene highlights epidemiological studies showing association between exposure and increased incidence of leukemia (Delzell et al. 1996; Macaluso et al. 1996; Struski et al.). Chronic *in vivo* rat carbon tetrachloride inhalation toxicity studies reveal significant fatty changes and hepatic tumor progression, mirroring our most sensitive gene set pathway reported in exposed BEAS-2B cells (Nagano et al. 2007a; Villanueva et al. 2011). Finally, prevalence of renal, bladder, and lung cancer in trichloroethylene toxicity studies is reflected in the top three most sensitive gene sets we report in BEAS-2B cells exposed to trichloroethylene and the most sensitive gene set in exposed pHBECs (Heidenblad et al. 2005; Krieg et al. 2010; Maltoni et al. 1986; Olsson et al. 2007; Roversi et al. 2006).

Broad correlations exist between TLV documentation, known chemical adverse health effects, and the determined sensitive gene sets. Taken together, these relationships provide context highlighting the practicality and promise of ALI VOC exposure and transcriptomics analysis. The representative LOAEL and NOAEL values reported in Table 3 are limited to those reported in the EPA IRIS Toxicological Review database ([epa.gov/iris](https://www.epa.gov/iris)) reports. In general, the reported LOAEL and NOAEL value is higher than the BPAC calculated in our test agent exposures, however, they remain within an order of magnitude difference. BPACs for acrolein, carbon tetrachloride and trichloroethylene are all between the reported LOAEL and NOAEL values. The congruity of our transcriptome-based BPAC values with the TLV documentation and IRIS *in vivo* inhalation data provides strong evidence that our study design and analysis methods yield informative PoDs. It should be noted that,

while the BPACs calculated in this study successfully approximate the respective TLVs, these findings are not intended to be predictive of any specific adverse health outcomes. The most sensitive gene sets are broad groupings of genes with similar activities that may help guide follow-up studies but should not be viewed as definitive mechanisms of action. Limitations also exist in determining dose equivalency between ALI *in vitro* CCES and *in vivo* studies. Methods to determine dosimetric uptake *in vitro* in comparison to *in vivo* is being developed and important to assess dose equivalency across studies. Despite these challenges, the preliminary evidence that BMD analysis and PoD calculation in an ALI *in vitro* system provides an early marker of exposure is of scientific importance.

For the purposes of this study, change to viability concentration-response was not a primary endpoint and was reserved as only a cut-off for consideration in the transcriptomics data. Despite the 50% average viability threshold to be considered for TempO-Seq, our average response at the highest concentration was below 20% change in BEAS-2B and little to no change was observed in the pHBECS. In our study, preliminary dose response testing was conducted only in the BEAS-2B cells and may have represented concentrations that did not have as strong an effect on the pHBECS. Future studies should utilize cell model specific range finding to ensure an observable dose response. Even known respiratory toxicants formaldehyde and acetaldehyde did not significantly change BEAS-2B cell viability at the concentrations they were exposed. In the case of formaldehyde, the lower tested concentrations skewed both LDH and ATP measurements above those of the sham control cells. This finding indicates the challenges behind exposing airway cells to these chemicals in a humidified system under flow at such low concentrations. Future studies should endeavor to examine the possible conversion of parent chemical species within the exposure system.

It should be noted that we did attempt to quantitate a BMD for the cell viability endpoints. Ultimately, the magnitude of response in our measurements of cytotoxicity/viability were not great enough to meet the 10% relative deviation cutoff for BMD modeling as outlined in the NTP dose response modeling approach (NTP 2018). These results support the strong ability for transcriptional BMD analysis to identify adverse effects in exposures that did not result in significant changes to cell metabolism or viability, providing a more sensitive endpoint in screening level exposure endpoints. The lack of dynamic range in our tested concentrations undoubtedly contributed to the non-viable BMD models for the viability endpoints, as well as the lack of probe and gene sets assigned BMDs. A wider range of tested concentrations and viability BMD modeling should be utilized and examined in future studies. The observed probe level assigned BMDs in Figure 4 are heavily weighted toward the highest concentration tested for most of the tested VOCs. Altering the test agent concentration range and time course in future experiments could lead to tissue remodeling, uncover different expression profiles and likely reveal gene set level changes with differential BPACs for the VOCs tested. Subsequent studies are planned to evaluate 6 and 24 h post-exposure time points and repeat exposures to simulate 14-day *in vivo* inhalation study designs.

In summary, the communicated study utilized a novel inhalation exposure system to investigate VOCs which were previously incapable of being tested in traditional *in vitro*

direct-dosing methods. Second, we implemented transcriptome-based analytical techniques to describe portal of entry impacts of inhaled VOCs with many of the most sensitive gene sets correlating to known adverse effects from previous *in vivo* experiments. The findings outlined above serve as a primer to inform future *in vitro* inhalation exposure study design and risk assessment. Foremost, the BMD analysis and comparison to known LOAEL and NOAEL data, as well as ACGIH TLVs, provide a proof of concept that transcriptome-based PoD analysis can be used as a predictive tool for adverse outcomes in human exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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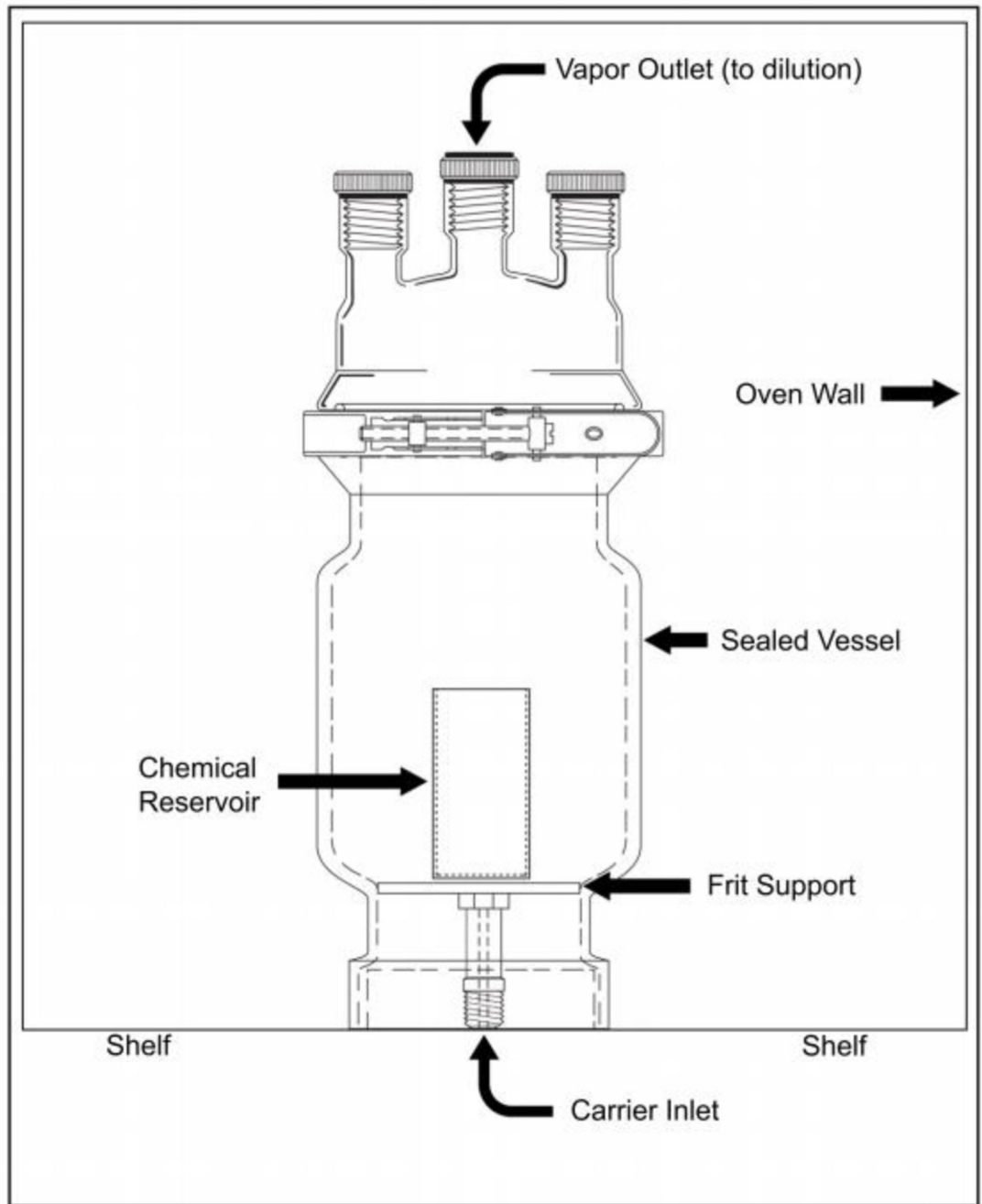
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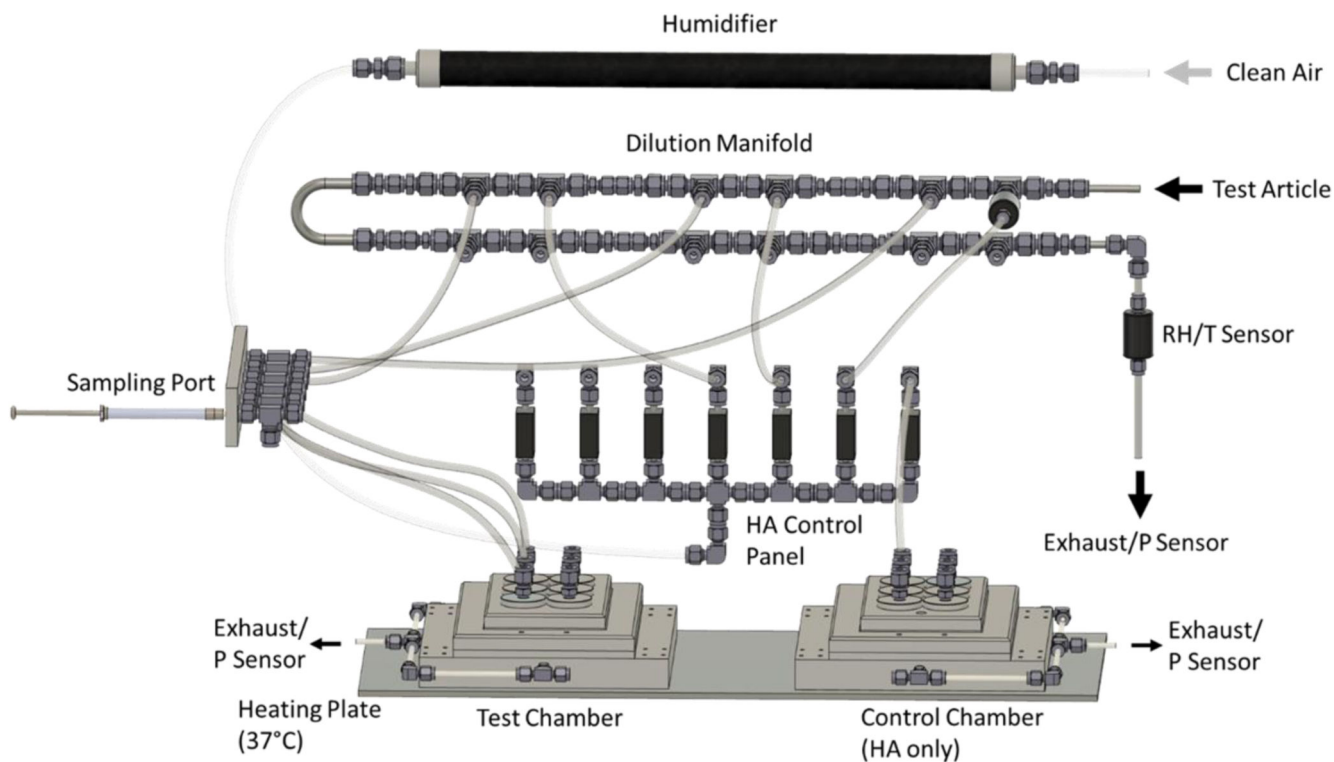
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A.





B.

Figure 1.

Schematic of CCES dilution manifold and exposure plates. A) Dynamic headspace generator design used for chemical generation and transport to the exposure manifold. B) The cell culture exposure system (CCES) includes a diffusion humidifier designed to regulate relative humidity levels in the dilution air before being passed through a dilution manifold. Each diluted chemical concentration is transported to a sampling port which allows real-time concentration analysis before being delivered to four wells containing inserts (n=4 per dose). For visual clarity, only tubing for the first three doses are shown. A separate control plate receives only humidified dilution air for the duration of the exposure. Both the VOC and sham exposure plates are enclosed in a warmed, humidified enclosure, while the incubator control remains in the 37°C 5% CO₂ cell culture incubator.

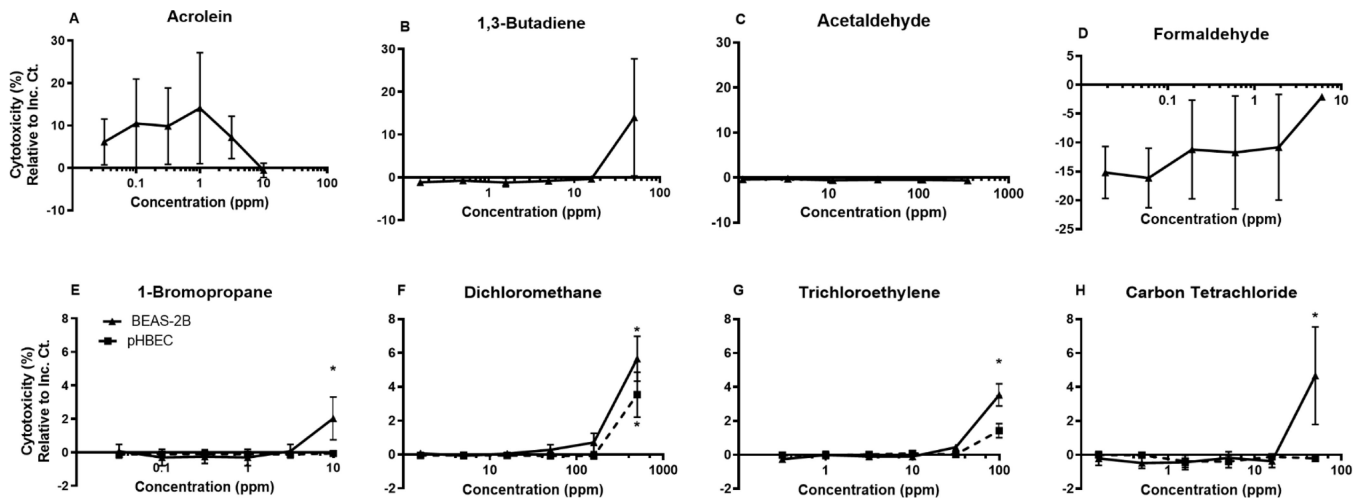


Figure 2. Dose response of each respective chemical as measured by LDH release presented as % Change in Cytotoxicity compared to the filtered air control and 100% lysed control. BEAS-2B and pHBEC cells exposed to chemicals A. Acrolein, B. 1,3-Butadiene, C. Acetaldehyde, D. Formaldehyde, E. 1-Bromopropane, F. Dichloromethane, G. Trichloroethylene, H. Carbon Tetrachloride. Mean±SEM, n=3, *p 0.05.

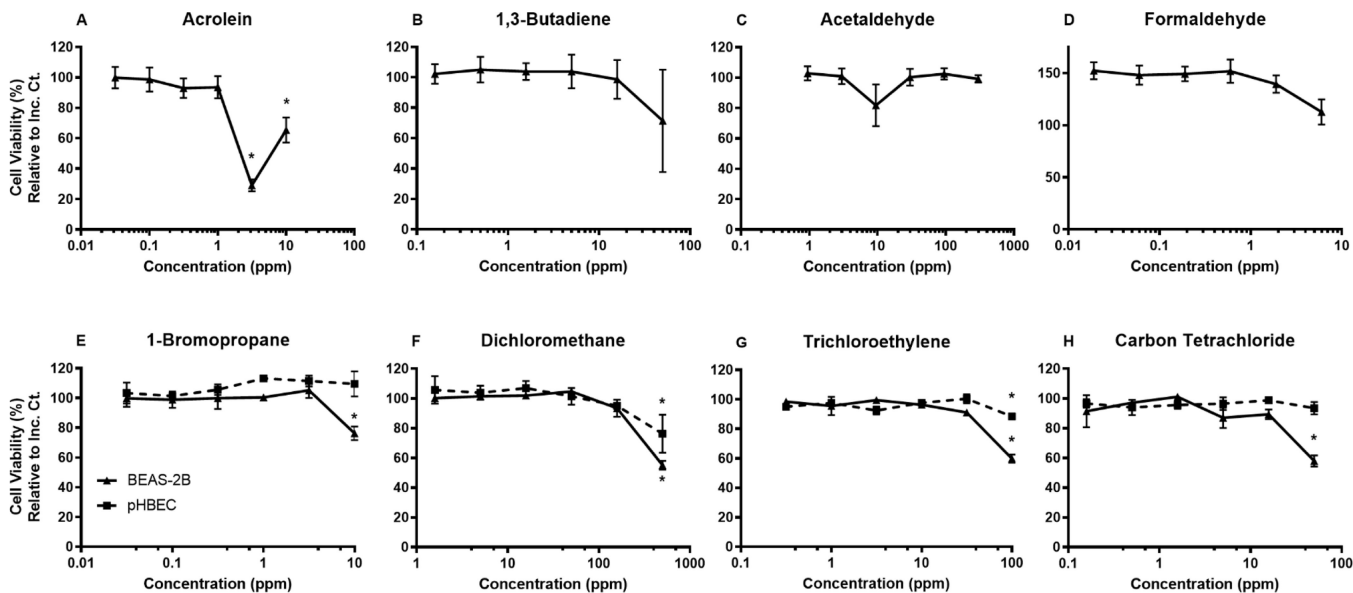


Figure 3. Dose response of each respective chemical as measured by CellTiter-Glo ATP availability and presented as % Cell Viability compared to the filtered air control. BEAS-2B and pHBEC cells exposed to chemicals A. Acrolein, B. 1,3-Butadiene, C. Acetaldehyde, D. Formaldehyde, E. 1-Bromopropane, F. Dichloromethane, G. Trichloroethylene, H. Carbon Tetrachloride. Mean±SEM, n=3, *p 0.05.

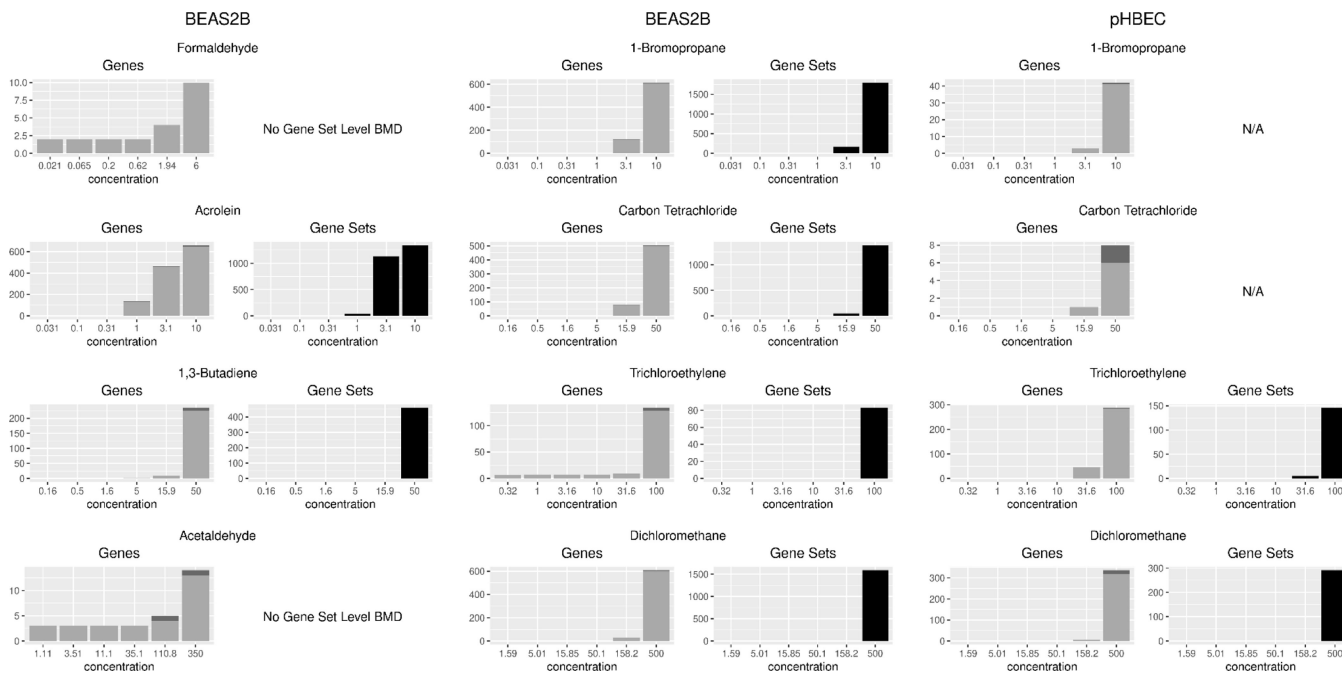


Figure 4: Bar plot of Gene and Gene Set level accumulation as a function of VOC test concentration for each cell type and chemical combination tested. Each bar represents the number of genes or gene sets that had a BMD less than or equal to the test concentration. Light grey and black shaded bars represent genes or gene sets (respectively) which meet all criteria and were analyzed while the dark grey portion of the bar represents those gene targets which were not annotated in the MSigDB gene set collection and did not contribute to gene set level BMDs. Sample size n=3 per test condition.

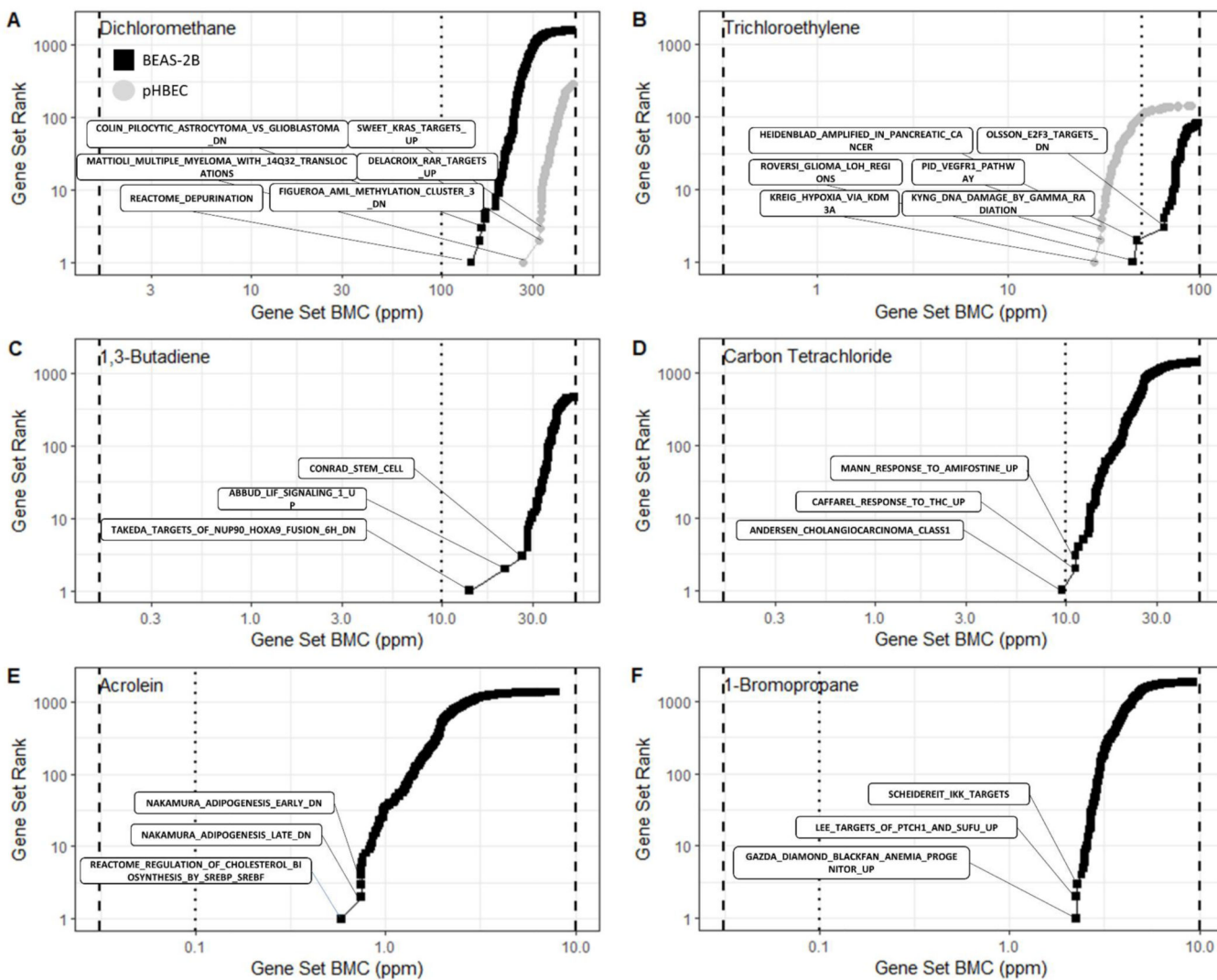


Figure 5. Accumulation plots for the best BMD values for enriched gene sets for BEAS-2B cells (black squares) and pHBEC cells (grey circles). Each point represents the Median BMD for a gene set. Black dashed lines indicate the range of concentrations tested and the dotted line indicates the ACGIH TLV.

Table 1.

Test agent generation parameters.

Chemical Name	CAS #	Vendor	Catalog Number	Gas Mixture or Lot # and Purity	GC Conditions/Methodology	Generation Method	Preparation for Method
Acrolein	107-02-8	Airgas National Welders	Custom	Acrolein in Nitrogen at 1011 PPM	Isothermal Oven Temperature: 28 °C held for 2.8 min; Column Flow: 10 mL/min; Splitless inlet at 200 °C; FID detector at 250C	Gas Cylinder	Acrolein in Nitrogen Target = 1000 PPM, Actual = 1011 PPM, Accuracy = ±2%
Acetaldehyde	75-07-0	Airgas National Welders	Custom	Acetaldehyde in Nitrogen at 2064 PPM	Isothermal Oven Temperature: 45 °C held for 1.5 min; Column Flow: 10 mL/min; Splitless inlet at 200 °C; FID detector at 250C	Gas Cylinder	Acetaldehyde in Nitrogen, Target = 2000 PPM, Actual = 2064 PPM, Accuracy = ±2%
1-Bromopropane	106-94-5	Sigma Aldrich	B78106	Lot #: WXCBC0691V Purity: 99.8%	Isothermal Oven Temperature: 45 °C held for 1.5 min; Column Flow: 10 mL/min; Splitless inlet at 200 °C; FID and ECD detector at 250C	Liquid in vial	Chemical placed in vial (25mL) with orifice which was placed into the dynamic headspace generator to carry VOCs in air stream into chamber
Formaldehyde (paraformaldehyde)	50-00-0 (30525-89-4)	Sigma Aldrich	158127	Lot #: STBB9507V Purity: 94.1%	Non-dispersive infrared analyzers	Solid in vial	Chemical placed in vial (25mL) with orifice which was placed into the dynamic headspace generator to carry VOCs in air stream into chamber
1,3-Butadiene	106-99-0	Airgas National Welders	Custom	1,3 Butadiene in Air at 511 PPM	Isothermal Oven Temperature: 27 °C held for 1.0 min; Column Flow: 10 mL/min; Splitless inlet at 200 °C; FID detector at 250C	Gas Cylinder	1,3 Butadiene in Air, Target = 500 PPM, Actual = 511 PPM, Accuracy = ±2%
Dichloromethane	75-09-2	Burdick & Jackson	300-4	Lot #: C0674 Purity: 99.9%	Isothermal Oven Temperature: 45 °C held for 2.5 min; Column Flow: 10 mL/min; Splitless inlet at 200 °C; FID and ECD detector 250C	Liquid in vial	Chemical placed in vial (25mL) with orifice which was placed into the dynamic headspace generator to carry VOCs in air stream into chamber
Trichloroethylene	79-01-6	Sigma Aldrich	251402	Lot #: SHBJ3703 Purity: 99.98%	Isothermal Oven Temperature: 60 °C held for 4 min; Column Flow: 10 mL/min; Splitless inlet at 200 °C; FID and ECD detector at 250C	Liquid in vial	Chemical placed in vial (25mL) with orifice which was placed into the dynamic headspace generator to carry VOCs in air stream into chamber
Carbon Tetrachloride	56-23-5	Sigma Aldrich	319961	Lot #: SHB0149 Purity: 99.90%	Isothermal Oven Temperature: 60 °C held for 4 min; Column Flow: 10 mL/min; Splitless inlet at 200 °C; FID and ECD detectors at 250C	Liquid in vial	Chemical placed in vial (25mL) with orifice which was placed into the dynamic headspace generator to carry VOCs in air stream into chamber

Table 2.

h chemical and cell type tested.

Gene Sets (Pathways)	Probe IDs	Median BMD (MSigDB_C2), ppm	Median BMDL (MSigDB_C2), ppm
ESTEROL_BIOSYNTHESIS_BY_SREBP_SREBF	SOLE_6736;INSIG2_21088;CYP51A1_92320	0.585727	0.39902
DIPOGENESIS_EARLY_DN	SERPINE2_27841;SERPINE2_27842;PDE6D_13043;LOX_21772	0.746618	0.4431965
DIPOGENESIS_LATE_DN	SERPINE2_27841;SERPINE2_27842;PDE6D_13043;LOX_21772	0.746618	0.4431965
A_VDR_PATHWAY	SMARCC2_12862;BAZ1B_17476;ARID1A_26387	0.746681	0.3875
HOSE_PRECISE_EFFECT_ON_RUNX1_TARGETS_IS_NOT_KNOWN	SMARCC2_12862;RUNX1_27815;RUNX1_6060;ARID1A_26387	0.746681	0.3875
ON_CANCER_GENES	NDUFS8_4528;LIMD1_17670;ATP6V0A1_11775	0.752497	0.464197
LE_MYELOMA_MS_DN	OAS3_87750;MRPS31_92464;LETMD1_12057;DHPS_91625	0.760868	0.3744815
SINE_FORMATION_AND_ARYLSULFATASE_ACTIVATION	FN3K_92980;DPH2_91691;DHPS_91625;ARSI_16411	0.7900935	0.343939
OMA_DESMOPLASIS_VS_CLASSIC_UP	UGCG_11621;PLEC_14082;LRP8_13959;FLT3LG_25439	0.828844	0.397706
GENESIS_PPARG_BOUND_36HR	EXOC6_19851;DYNLRB1_15749;CHUK_1342	0.834914	0.360823
CKFAN_ANEMIA_PREGENITOR_UP	MAFF_27478;MAFF_28315;FTH1_92999;FTH1_93000;CD83_25945	2.24587	1.8379
DE_PTCHI_AND_SUPO_UP	RND3_24211;MAGOHB_22392;IER5_15403	2.25517	1.88619
REIT_IKK_TARGETS	NFKBIA_4568;NFKB1_4566;CYLD_17180	2.26819	1.85512
PERT_LSC_R	TGHF2_27952;PPPIR10_16865;NAB1_13978	2.40606	1.98632
TRITION_NEOCORTEX_UP	RELB_28340;NFKBIA_4568;MAFF_27478;MAFF_28315;EIF2AK3_24919;CNOT2_17056	2.45342	2.0422
_DEATH_PATHWAY	NFKBIA_4568;NFKB1_4566;BIRC3_11879	2.4806	2.06161
R133_TARGETS_UP	NFAT5_16375;NFAT5_27581;MAFK_26036;CYLD_17180	2.48805	2.05714
LE_MYELOMA_PR_DN	JMJD1C_11504;FGF2_2405;CYLD_17180;ARID5B_88738;ARID5B_89998	2.50382	1.89154
ATIN_RESISTANCE_UP	TENT5A_15343;MAFF_27478;MAFF_28315;IER5_15403;FTH1_92999;FTH1_93000	2.5370175	1.7680525
_NEF_PATHWAY	TRAF1_15516;NFKBIA_4568;NFKB1_4566;BIRC3_11879	2.579205	2.144825
INUP98_HOXA9_FUSION_6HR_DN	TM4SF1_12122;KDM6B_16198;HMOX1_3041	13.9794	7.78439
F_SIGNALING_I_UP	SOC3_15641;RGS4_20411;CEBPB_10745	21.3365	16.7624
AD_STEM_CELL	KLF5_20609;KLF4_23258;CXCL5_15735	26.4384	21.1357
IA_VIA_ELK3_ONLY_UP	PPPIR15A_14098;CEBPB_10745;ADM_12873;ADM_140	28.2428	20.7323
IA_METAPLASTIC_VS_DUCTAL_UP	HMOX1_3041;CXCL8_14324;CEBPB_10745;BMP2_22143;ADM_12873;ADM_140	28.2428	20.7323

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Gene Sets (Pathways)	Probe IDs	Median BMD (MSigDB_C2), ppm	Median BMDL (MSigDB_C2), ppm
A_HIFI_TARGETS	HMOX1_3041;EDN1_2029;ADM_12873;ADM_140	28.2428	20.7323
DNOS2_TARGETS_UP	IER2_3213;HMOX1_3041;ADM_12873;ADM_140	28.2428	20.7323
RAS_TARGETS_DN	IRS2_21012;FOSL1_2463;DUSP4_1990;CXCL5_15735	28.5691	21.73905
TARGETS_KERATINOCYTE_DN	RUNX1_20078;GPRC5A_15162;DUSP4_1990	29.0199	22.4869
RESPONSE_240_MCF10A	TM4SF1_12122;PHLDA2_5123;ADRB2_151	29.6407	23.7457
ANGIOCARCINOMA_GLASSI	TMEM156_25729;RGS4_20411;PTHLH_15144;PTHLH_5582;DCDC2_14713;CXCL6_15723	9.56328	7.98027
RESPONSE_TO_THC_OP	ZFP36L1_14656;VEGFA_12596;MYC_4394;HIST1H2AC_93104;CPEB4_18785	11.2356	9.30456
USE_TO_AMIFOSTINE_UP	ZFP36L1_14656;MYC_4394;GDF15_18329	11.2356	9.30456
CANCER_COPY_NUMBER_UP	TGFB2_25683;PTGS2_5577;MYC_4394	11.6191	9.6667
MYC_TARGETS_DN	ZFP36L1_14656;PDGFB_5026;MYC_4394;CDKN2B_14415	12.3503	10.23368
SPHERICAL_VS_ADHERENT_DN	TGFB2_25683;PLAUR_23941;PLAUR_5179;NR3C1_4689	13.0934	10.60316
ROSTATE_CANCER_UP	SOX4_6686;MYC_4394;GDF15_18329	13.347	9.58186
DNOS2_TARGETS_DN	ZFP36L1_14656;SOX4_6686;SOX17_89363;NFIA_18923;HSPB8_15039	13.347	11.0524
KBI_PATHWAY	TSCI_19318;SIKI_17023;MYC_4394	13.3507	9.14916
RESPONSE_IMMORTALIZED_DN	MYC_4394;IDI_14943;EREG_21427	13.3895	10.833
LOMA_LOH_REGIONS	PPFIBP1_22884;NPFFR2_90111;GPRC5A_15162;DDHD1_10888	44.8416	29.00405
DIFFERENTIAL_TARGETS_IN_PANCREATIC_CANCER	PPFIBP1_22884;MAL2_23815;CCN3_87436	47.0682	35.7006
DIFFERENTIAL_TARGETS_DN	NFKBIA_4568;MAL2_23815;HIST1H1C_2950	65.4966	47.7346
BCL2_TARGETS_UP	RELN_21362;MACC1_23235;HIST1H1C_2950	65.4966	41.4074
DIFFERENTIAL_TARGETS_IN_PANCREATIC_CANCER	HIST2H4B_16248;HIST2H4A_33956;HIST2H2BF_93215;HIST1H2BI_92661;HIST1H2AG_93175;HIST1H2AC_93104	67.48565	54.3204
DIFFERENTIAL_TARGETS_IN_PANCREATIC_CANCER	HIST1H1C_2950;GDF15_2621;DHRS2_1857	69.8154	60.8512
DIFFERENTIAL_TARGETS_IN_PANCREATIC_CANCER	MAL_21284;FHL2_2423;EZR_25288;DAPK1_1752	72.244	51.08815
DIFFERENTIAL_TARGETS_IN_PANCREATIC_CANCER	TENT5A_15343;OASL_89164;GPRC5A_15162;CCN3_87436	72.4401	49.10325
DIFFERENTIAL_TARGETS_IN_PANCREATIC_CANCER	SUSD5_15755;MAF_13534;IGFBP3_3270;DAPK1_1752	72.6945	46.20455
DIFFERENTIAL_TARGETS_DN	ZNF532_20212;SOX4_6686;CLDN4_89537	73.9443	51.0529
DIFFERENTIAL_TARGETS_IN_PANCREATIC_CANCER	HIST2H4A_33956;HIST1H2BI_92661;HIST1H2AC_93104	142.127	118.71
DIFFERENTIAL_TARGETS_IN_PANCREATIC_CANCER	PPP1R15A_14098;HIST1H2BI_92661;HIST1H2AG_93175	158.016	130.242
DIFFERENTIAL_TARGETS_IN_PANCREATIC_CANCER	SQSTM1_6740;CHI3L1_14316;BHLHE40_689	160.996	132.484

Gene Sets (Pathways)	Probe IDs	Median BMD (MSigDB_C2), ppm	Median BMDL (MSigDB_C2), ppm
DIFFERENTIATION_GENES	KLF6_3626;JUN_89022;IRS2_21012	168.708	137.335
DIFFERENTIATION_GENES	NR3C1_4689;KLF6_3626;IRF1_21247	168.708	137.335
TOR_SIGNALING_IN_LIVER_CANCER	SMAD7_12470;IRS2_21012;HBEGF_15347;HBEGF_2892	191.462	159.963
DIFFERENTIAL_EXPRESSION	SPRY1_18786;SH3KBP1_6296;HBEGF_15347;HBEGF_2892	191.462	159.963
DIFFERENTIAL_EXPRESSION	SPRY1_18786;SH3KBP1_6296;HBEGF_15347;HBEGF_2892	191.462	159.963
DIFFERENTIAL_EXPRESSION	SOC3_15641;NFKB1_4566;IRS2_21012	198.521	154.356
DIFFERENTIAL_EXPRESSION	SOC3_15641;NR3C1_4689;HBEGF_15347;HBEGF_2892	198.521	159.963
DIFFERENTIAL_EXPRESSION	NRP2_27606;KDM3A_3541;EBP_2015	28.1584	15.5176
DIFFERENTIAL_EXPRESSION	PCSK5_27656;NRP2_27606;LRBA_90214;APIG1_21853	30.0328	16.13885
DIFFERENTIAL_EXPRESSION	VEGFA_28053;NRP2_27606;CD2AP_13562	30.6881	16.3157
DIFFERENTIAL_EXPRESSION	MAN2A1_21283;MAN1A2_89683;MAN1A1_12471;ALG14_15409	30.87085	16.32435
DIFFERENTIAL_EXPRESSION	SGMS2_22813;SGMS1_13935;KDSR_14120	31.3726	16.5192
DIFFERENTIAL_EXPRESSION	RBKS_5758;MAN1A1_12471;KIFAP3_22932;EBP_2015	32.1622	16.56735
DIFFERENTIAL_EXPRESSION	KLF2_16192;BTG1_26906;ABC A1_22891	32.2274	16.4188
DIFFERENTIAL_EXPRESSION	SIRT1_21308;RBL2_5761;BTG1_26906;BCL2L1_26447	32.29735	16.61775
DIFFERENTIAL_EXPRESSION	SIRT1_21308;SGKI_22771;RBL2_5761;BCL2L1_26447	32.48035	16.893
DIFFERENTIAL_EXPRESSION	VEGFA_28053;SLC2A1_14159;SIRT1_21308	32.5934	16.8167
DIFFERENTIAL_EXPRESSION	MAF_13534;KLF4_23258;IL6R_17769	266.73	204.336
DIFFERENTIAL_EXPRESSION	RESF1_15542;DUSP1_1978;BHLHE40_689	323.565	232.508
DIFFERENTIAL_EXPRESSION	TPM1_28000;GADD45B_2570;GADD45B_28755;CLDN4_89537;BHLHE40_689;PGLS_16643	325.962	232.508
DIFFERENTIAL_EXPRESSION	PPP1R15A_14098;MAFF_27478;MAFF_28315;KLF4_23258;INSIG1_3385;ENO2_2153;BHLHE40_689	326.754	236.46375
DIFFERENTIAL_EXPRESSION	TPM1_28000;IGF1R_3254;IER2_3213	329.401	236.301
DIFFERENTIAL_EXPRESSION	TPM1_28000;IER2_3213;GDF15_18329	329.401	236.301
DIFFERENTIAL_EXPRESSION	TPM1_28000;IER2_3213;GDF15_18329	329.401	236.301
DIFFERENTIAL_EXPRESSION	IER2_3213;FOS_2461;DUSP1_1978	329.401	236.301
DIFFERENTIAL_EXPRESSION	MAFF_27478;MAFF_28315;KLF4_23258;IER2_3213;GADD45B_2570;GADD45B_28755	329.672	238.36025
DIFFERENTIAL_EXPRESSION	MAFF_27478;MAFF_28315;FOSL1_2463;BHLHE40_689	329.943	240.4195

Table 3.

Summary of analysis showing the BPAC for each chemical tested across both cell types. Includes comparison to 2018 ACGIH TLV and reported LOAEL and NOAEL values from *in vivo* inhalation studies reviewed in the EPA IRIS toxicological reviews for each respective chemical (www.epa.gov/iris). Abbreviations used: N/A: no gene set met criteria to calculate a BPAC, NR: not reported in the literature reference. ‘--’ indicates that the chemical was not tested in pHPEC cells.

Chemical Name	BEAS-2B BPAC (ppm)	pHPEC BPAC (ppm)	Representative LOAEL (ppm)	Representative NOAEL (ppm)	TLV (ppm)
Acrolein	0.586	--	0.25	NR	0.1
1-Bromopropane	2.246	N/A	NR	6040	0.1
Formaldehyde	N/A	--	6	NR	0.3
1,3-Butadiene	13.979	--	200	NR	10
Carbon Tetrachloride	9.563	N/A	20	5	10
Acetaldehyde	N/A	--	400	150	25
Trichloroethylene	44.842	28.148	50	25	50
Dichloromethane	142.127	226.73	8400	4200	100

Table 4:

Representative studies obtained from the ATSDR and IRIS documentation with respective reported NOAEL and LOAEL values. Abbreviations: LOAEL = lowest-observed-adverse-effect level; NOAEL = no-observed-adverse-effect level; NR = not reported.

1,3-Butadiene (ASTDR 2012)						
System/Observations	Species	NOAEL (ppm)	LOAEL (ppm)	Exposure Conditions	References	
Respiratory	Rat	8000	NR	Sub-chronic: 13 wk 5 d/wk 6 hr/d	(Crouch et al. 1979)	
Respiratory	Mouse	8000	NR	Sub-chronic: 14 wk 5 d/wk 6 hr/d	(NTP 1984)	
Respiratory: alveolar epithelial hyperplasia after 40 wks	Mouse	NR	200	Sub-chronic: 13-52 wk 6 hr/d 5 d/wk	(NTP 1993)	
Respiratory: alveolar epithelial hyperplasia	Mouse	200	625	Sub-chronic: 40 wk 6 hr/d 5 d/wk	(NTP 1993)	
Respiratory Cancer: alveolar/bronchiolar adenoma/carcinoma	Mouse	NR	200	Sub-chronic: 13-52 wk 6 hr/d 5 d/wk	(NTP 1993)	
Respiratory: increased organ weight, metaplasia	Rat	1000	8000	Chronic: 105-111 wk 5 d/wk 6 hr/d	(Owen et al. 1987); (Owen and Glaister 1990)	
Respiratory: atrophy of nasal olfactory epithelium	Mouse	625	1250	Chronic: 61 wk 5 d/wk	(NTP 1984)	
Respiratory: alveolar epithelial hyperplasia	Mouse	6.25	NR	Chronic: 2 yr 6 hr/d 5 d/wk	(NTP 1993)	
Respiratory Cancer: alveolar/bronchiolar adenoma or carcinoma	Mouse	NR	625	Chronic: 61 wk 5 d/wk 6 hr/d	(NTP 1984)	
Respiratory Cancer: alveolar/bronchiolar adenoma or carcinoma	Mouse	NR	6.25	Chronic: 2 yr 6 hr/d 5 d/wk	(NTP 1993)	
Dichloromethane (USEPA 2011a)						
System/Observations	Species	NOAEL (ppm)	LOAEL (ppm)	Exposure Conditions	References	
Pulmonary: foreign body pneumonia	Rat	4200	8400	Sub-chronic: 13 wk 5 d/wk 6 hr/d	(NTP 1986a)	
Pulmonary lesions	Mouse	8400	NR	Sub-chronic: 13 wk 5 d/wk 6 hr/d	(NTP 1986a)	
Pulmonary: cara cell vacuolation	Mouse	4000	NR	Sub-chronic: 13 wk (5 d/wk 6 hr/d)	(Foster et al. 1992)	
Hepatic: hepatocyte vacuolation and necrosis; hemosiderosis in liver; renal tubular degeneration	Rat	NR	1000	Chronic: 2 yrs (5 d/wk, 6 hr/d)	(Mennear et al. 1988); (NTP 1986a)	
		NR	1000			
		1000	2000			
Hepatic: hepatocyte degeneration; renal tubule casts	Mouse	NR	2000	Chronic: 2 yrs (5 d/wk, 6 hr/d)	(Mennear et al. 1988); (NTP 1986a)	
		NR	2000			
Hepatic: hepatocyte vacuolation; hepatocyte necrosis	Rat	NR	500	Chronic: 2 yrs (5 d/wk, 6 hr/d)	(Burek et al. 1984)	
		500	1500			
Hepatic: hepatocyte vacuolation	Rat	200	500	Chronic: 2 yrs (5 d/wk, 6 hr/d)	(Nitschke et al. 1988)	
Trichloroethylene (USEPA 2011b)						

1,3-Butadiene (ASTDR 2012)						
System/Observations	Species	NOAEL (ppm)	LOAEL (ppm)	Exposure Conditions	References	
System/Observations	Species	NOAEL (ppm)	LOAEL (ppm)	Exposure Conditions	References	
Immunosuppression: ↓ pulmonary bactericidal activity assay	Mouse	2.6	5.2	Acute and Acute Repeated: 1 or 5 d (3hr/d)	(Aranyi et al. 1986)	
Immunosuppression: dose-dependent changes in bacterial clearance from lung and phagocytic function of alveolar macrophages	Mouse	25	50	Acute: Single 3 hr exposure	(Selgrade and Gilmour 2010)	
Immunosuppression	Rat	300	1000	Sub-chronic: 4 wk (5 d/wk, 6 hrs/d)	(Woolhiser et al. 2006)	
Carbon Tetrachloride: (USEPA 2010b)						
System/Observations	Species	NOAEL (ppm)	LOAEL (ppm)	Exposure Conditions	References	
Hepatic: fatty change in liver	Rat	ND	50	Sub-chronic: 10.5 mos (8 hr/d, 5 d/wk)	(Smyth et al. 1936)	
Hepatic: increased liver weight; fatty degeneration in liver	Rat	5	10	Sub-chronic: 6 mos (7 hr/d, 5 d/wk)	(Adams et al. 1952)	
Hepatic: reduced body weight gain; enlarged liver with fatty change	Rat	1	10	Sub-chronic: 13 wks (24 hr/d, 7 d/wk)	(Prendergast et al. 1967)	
Hepatic: increased liver weight; fatty change in liver	Rat	ND	10	Sub-chronic: 13 wks (6 hr/d, 5 d/wk)	(Nagano et al. 2007b)	
Hepatic: slight cytological alterations in the liver	Mouse	ND	10	Sub-chronic: 13 wks (6 hr/d, 5 d/wk)	(Nagano et al. 2007b)	
Hepatic: increased ALT, SDH; necrosis in liver	Rat	20	100	Sub-chronic: 12 wks (6 hr/d, 5 d/wk)	(Benson and Springer 1999)	
Hepatic: increased ALT, SDH; necrosis and cell proliferation in liver	Mouse	5	20	Sub-chronic: 12 wks (6 hr/d, 5 d/wk)	(Benson and Springer 1999)	
Hepatic: reduced body weight gain, lesions in the liver (fatty changes, fibrosis, cirrhosis)	Rat	5	25	Chronic: 2 yrs (5 d/wk, 6 hr/d)	(Nagano et al. 2007a)	
Hepatic: reduced survival late in study (because of liver tumors); reduced body weight gain	Mouse	5	25	Chronic: 2 yrs (5 d/wk, 6 hr/d)	(Nagano et al. 2007a)	
Acrolein: (USEPA 2003)						
System/Observations	Species	NOAEL (ppm)	LOAEL (ppm)	Exposure Conditions	References	
Respiratory: nasal necrosis of respiratory epithelium and increased proliferation	Rat	ND	0.25	Acute Repeated: 3 d (6 hr/d)	(Cassae et al. 1996)	
Respiratory: bronchial necrosis and pulmonary edema, parenchymal restriction, increase in lung collagen	Rat	ND	0.4	Sub-chronic: 62 d (6 hr/day 5 days/wk)	(Kutzman 1981) (Kutzman et al. 1985) (Costa et al. 1986)	
Acetylaldehyde: (USEPA 1991)						
System/Observations	Species	NOAEL (ppm)	LOAEL (ppm)	Exposure Conditions	References	
Respiratory: degeneration of olfactory epithelium	Rat	150	400	Acute Repeated: 5 d (6 hr/d)	(Appelman et al. 1982) (Appelman et al. 1986)	

1,3-Butadiene (ASTDR 2012)						
System/Observations	Species	NOAEL (ppm)	LOAEL (ppm)	Exposure Conditions	References	
1-Bromopropane: (USEPA 2020a)						
System/Observations						
Respiratory: pulmonary edema and emphysema	Rat	6040	NR	Acute: 4 hr	(Elf Atochem 1997)	
Respiratory: nasal lesions	Rat	250	NR	Sub-chronic: 16 d (6 hr/d, 5 d/wk)	(NTP 2011)	
Respiratory: histopathologic changes in nasal cavities	Rat	994	NR	Sub-chronic: 4 wks (6 hr/d, 5 d/wk)	(ClinTrials 1997)	
Respiratory	Rat	800	NR	Sub-chronic: 12 wks (8 hr/d, 7 d/wk)	(Tchihara et al. 2000)	
Respiratory	Rat	1800	NR	Sub-chronic: 8 wks (6 hr/d, 5 d/wk)	(Kim et al. 1999)	
Respiratory	Rat	600	NR	Sub-chronic: 13 wks (6 hr/d, 5 d/wk)	(ClinTrials 1997)	
Respiratory	Rat	1000	NR	Sub-chronic: 14 wks (6.2 hr/d, 5 d/wk)	(NTP 2011)	
Respiratory	Rat	750	NR	Sub-chronic: 6 hr/day during prenatating (70 days), mating, and until weaning of offspring	(WIL 2001)	
Respiratory: nasal and pulmonary lesions	Mouse	250	NR	Sub-chronic: 17 d (6.2 hr/d, 5 d/wk)	(NTP 2011)	
Respiratory: cytoplasmic vacuolization in the nose, larynx, trachea, and lung	Mouse	250	NR	Sub-chronic: 14 wks (6.2 hr/d, 5 d/wk)	(NTP 2011)	
Respiratory: histopathological lesions in the nasal respiratory epithelium, larynx, trachea, and bronchioles	Mouse	NR	62.5	Chronic: 105 wks (6.2 hr/d, 5 d/wk)	(NTP 2011)	
Respiratory: chronic nasal inflammation and squamous metaplasia in larynx	Rat	125	NR	Chronic: 105 wks (6.2 hr/d, 5 d/wk)	(NTP 2011)	
Formaldehyde: (USEPA 2010a)						
System/Observations						
Respiratory: histopathologic changes	Rat	NR	10	Acute: 4 hr	(Bhalla et al. 1991)	
Respiratory: histopathologic lesions to the respiratory epithelium	Mouse	NR	3.13	Acute Repeated: 5d (6 hr/d, 5 d)	(Buckley et al. 1984)	
Respiratory: histopathologic lesions to the nasal conchae, lateral wall, and ventral nasal conchae.	Rat	2	NR	Acute and Acute Repeated: 1, 2, or 4 d (6 hr/d)	(Monteiro-Riviere and Popp 1986)	
Respiratory: histopathologic lesions in the nasal turbinates and trachea	Rat	NR	15	Acute: 6 hr	(Kamata et al. 1996b) (Kamata et al. 1997)	
Respiratory: cellular necrosis to the nasal epithelium	Rat	NR	6	Acute: 6 hr	(Chang et al. 1983)	
Respiratory: phospholipid content was reduced in lung surfactant	Rat	NR	128.4	Acute: 6 hr	(Kamata et al. 1996a) (Kamata et al. 1997)	
Respiratory: biochemical changes in lung homogenates	Rat	NR	15	Acute: 6 hr	(Kamata et al. 1996b) (Kamata et al. 1997)	

1,3-Butadiene (ASTDR 2012)						
System/Observations	Species	NOAEL (ppm)	LOAEL (ppm)	Exposure Conditions	References	
Respiratory: squamous metaplasia	Mouse	4	NR	Sub-chronic: 13 wks (6 hr/d, 5 d/wk)	(Maronpot et al. 1986)	
Respiratory: squamous metaplasia, epithelial hyperplasia	Rat	1	NR	Sub-chronic: 13 wks (6 hr/d, 5 d/wk)	(Zwart et al. 1988)	
Respiratory: rhinitis, epithelial hyperplasia, and squamous metaplasia	Rat	1	NR	Sub-chronic: 4, 8, or 13 wks (6 hr/d, 5 d/wk)	(Feron et al. 1988)	
Respiratory: keratinized and nonkeratinized squamous metaplasia	Rat	1	NR	Sub-chronic: 13 wks (6 hr/d, 5 d/wk)	(Zwart et al. 1988)	
Respiratory: rhinitis and nasal lesions	Rat	1	NR	Sub-chronic: 26 wks (22 hr/d, 7 d/wk)	(Rusch et al. 1983)	
Respiratory: decrease in zinc content of lung, increase in iron content	Rat	NR	5	Sub-chronic: 4 or 13 wks (8 hr/d, 5 d/wk)	(Özen et al. 2003)	
Respiratory: increased P450 levels	Rat	0.5	NR	Sub-chronic: 1-4 days, 12 wks, or 24 wks (6 hr/d, 5 d/wk)	(Dallas et al. 1989)	
Respiratory: histological changes in tracheobronchial epithelial	Mice	NR	41	Chronic: up to 35 weeks (1 hr/d, 3 d/wk)	(Horton et al. 1963)	
Respiratory: rhinitis, hyperplasia, dysplasia, and squamous metaplasia of the nasal epithelium; atrophy of the olfactory epithelium; glandular adenitis and nasolacrimal duct hyperplasia and metaplasia.	Mice	NR	2	Chronic: 24 mo (6 hr/d, 5 d/wk)	(Swenberg et al. 1980) (Kerns et al. 1983) (CIIT 1982) (Battelle 1981)	
Respiratory: squamous metaplasia and dysplasia	Rat	NR	12.4	Chronic: 104 wks (6 hr/d, 5 d/wk)	(Holmström et al. 1989)	
Respiratory: increased rhinitis, hyperplasia, and squamous metaplasia of the nasal respiratory epithelium	Rat	NR	0.3	Chronic: 28 mo (6 hr/d, 5 d/wk)	(Tobe et al. 1985)	
Respiratory: squamous cell metaplasia and epithelial hyperplasia	Rat	NR	0.3	Chronic: 28 mo (6 hr/d, 5 d/wk)	(Kamata et al. 1997)	
Respiratory: squamous metaplasia, epithelial hyperplasia, and polyps/papillomas	Rat	NR	15	Chronic: Lifetime (6 hr/d, 5 d/wk)	(Albert et al. 1982) (Sellakumar et al. 1985)	
Respiratory: nasal lesions, squamous metaplasia and epithelial dysplasia, hyperkeratosis, goblet cell hyperplasia, and rhinitis	Rat	NR	2	Chronic: 24 mo (6 hr/d, 5 d/wk)	(Swenberg et al. 1980) (Kerns et al. 1983) (CIIT 1982) (Battelle 1981) (Morgan et al. 1986)	
Respiratory: olfactory degeneration, squamous metaplasia, epithelial hypertrophy and hyperplasia, and mixed inflammatory cell infiltrate	Rat	NR	2	Chronic: 24 mo (6 hr/d, 5 d/wk)	(Monticello et al. 1996)	
Respiratory: rhinitis, hyperplasia, and metaplasia	Rat	1	NR	Chronic: 13 or 52 wks (6 hr/d, 5 d/wk)	(Appelman et al. 1988) (Zwart et al. 1988)	