National Institute of Environmental Health Sciences
Division of Translational Toxicology

Alterations in Secreted Biomarkers Induced by Exposure of Air-Liquid Interface (ALI) Airway Tissues to 2,3-Pentanedione Vapor

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Introduction

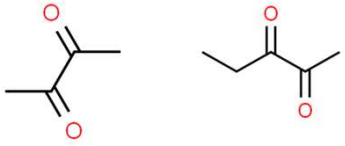
Occupational exposure to volatile components of artificial butter flavoring (ABF) via inhalation has been reported to be associated with airway fibrosis in the form of bronchiolitis obliterans (BO), mostly in workers in the microwave popcorn packaging and flavoring industry exposed to 2,3-butanedione (BD, also commonly called diacetyl). BO is a potentially fatal lung disease that is frequently found in lung transplant patients and is characterized by bronchiolar wall inflammation and fibrosis resulting in constrictive bronchiolitis with restricted airflow. 2,3-pentanedione (PD) is also a highly volatile component of ABF. PD has been used as a major substitute for BD in some ABF due to concerns about the respiratory toxicity of BD. However, PD is structurally similar to BD (both are alpha-diketones) (Fig. 1) and has been shown to exhibit toxicological potency similar to BD in the induction of airway epithelial injury with BO-like fibrotic lesions in rats, following acute (2-week) inhalation exposure, that are similar to the BO lesions observed in occupational exposures (Morgan et al. 2012 and 2016).

In addition, organotypic in vitro human air-liquid interface (ALI) airway epithelial culture models have been previously used, mostly with BD, to help elucidate the mechanisms of airway injury and fibrosis induced by these chemicals. In a proof-of-concept study, PD was selected as a test article for the characterization and optimization of a VITROCELL 48 2.0 plus exposure system (Fig. 2) together with human and rat ALI airway tissues to evaluate PD vapor-induced airway toxicity in vitro (and between species). The toxicity endpoints selected for analysis are relevant to previously reported in vivo rat (BD and PD) and in vitro human ALI (BD) airway findings as well as key events in an Adverse Outcome Pathway (AOP 280: "α-diketone-induced bronchiolitis obliterans") [Fig. 3].

Rationale for test article selection

PD has been well-characterized in vivo and is relatively straightforward to work with from a chemistry perspective in terms of the generation of stable vapor atmospheres. Also, there are currently very little in vitro human ALI airway toxicity data for PD (only one published study – Zaccone et al. 2015), but there have been multiple studies conducted with BD (e.g., Gwinn et al. 2017 and McGraw et al. 2020) which can be used to guide study design and anticipated findings since one would expect similar in vitro toxicological effects for PD based on the in vivo data in rodents.

Figure 1. Chemical structure

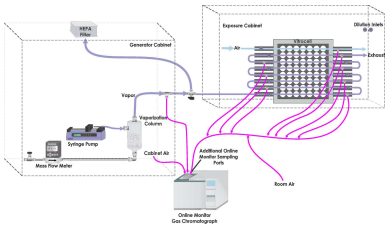


2,3-Butanedione (BD) **2,3-Pentanedione (PD)**

Source: ChemSpider

In Vitro Exposure System

Figure 2. Schematic of the Exposure System

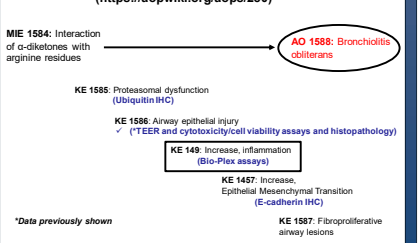


System set-up for the PD studies

- 56 wells total (8 concentrations x 7 replicates per concentration)

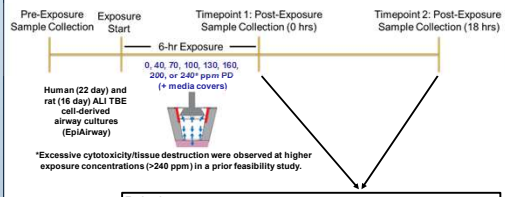
Adverse Outcome Pathway 280

Figure 3. AOP 280: "α-diketone-induced bronchiolitis obliterans" (https://aopwiki.org/aops/280)



Experimental Design

ALI airway (EpiAirway) tissues from MatTek (Ashland, MA), derived from primary normal human (single donor) or rat (Sprague Dawley) tracheobronchial epithelial (TBE) cells, were exposed for 6 hrs to PD vapor at multiple concentrations or clean air only (control) using the VITROCELL 48 2.0 plus exposure system (Fig. 2). The range of exposure concentrations and duration (6 hrs) selected were similar to those tested in previous in vivo inhalation studies with PD conducted by the DTT (e.g., Morgan et al. 2012 and 2016 and NTP TOX-98). In a preliminary study, 6-hr exposure of the ALI airway cultures to air only was found to cause no adverse effects. Apical wash and basolateral (baso) culture media samples and tissues (including lysates) were assessed for test article concentrations (by GC-MS) and PD-induced toxicological effects at 0 and ~18 hrs after exposure including measurements of transepithelial electrical resistance (TEER), lactate dehydrogenase (LDH) and adenylate kinase (AK) release, secreted biomarkers using Bio-Plex assays [45-plex (human) and 23-plex (rat)], and histopathology [hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining and immunohistochemistry (IHC)]. **The TEER, LDH, AK, and histopathology (H&E) data were previously shown. Herein, the Bio-Plex assay data and methods of analysis are reported.**



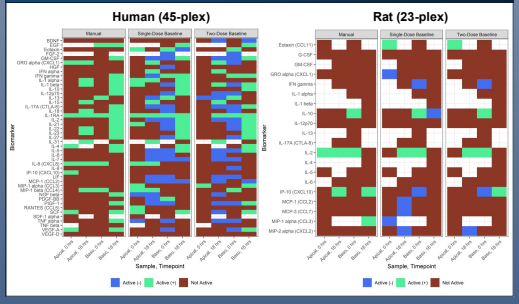
End-points

- ✓ External (apical wash and baso media) and internal (lysates) PD concentrations
- ✓ Airway injury/cytotoxicity/cell viability (TEER; LDH and AK assays of apical wash)
- ✓ Airway injury/histopathology (H&E and PAS staining)
- ➢ **Secreted biomarkers in apical wash and baso media (human and rat Bio-Plex assays)**
- • IHC (e.g., ubiquitin and E-cadherin)

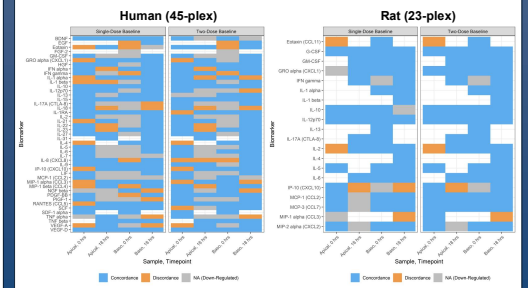
Analysis Methods (Bio-Plex Assay Data)

- Two methods were used to flag biomarkers as "active" (i.e., exhibiting a significant concentration-effect relationship).
  1. Manual review and graphing of the data with statistical analysis comparing the PD-exposed groups to each other and the 0-ppm control group by one-way ANOVA and Tukey's test (* indicates $p<0.05$ vs. 0-ppm).
  2. A more systematic modeling (curve-fitting) approach based on ToxCast hit-call criteria (US EPA) using a single-dose (0 ppm) or two-dose (0 and 40 ppm) baseline. All graphs show "Log_2 (Fold-Change of Baseline Median) vs. Concentration (ppm)". The criteria were as follows:
     - ➢ Fitted Hill or Gain-Loss model had a lower AIC than the constant model.
     - ➢ At least one median response and the top of the fitted curve exceeded a cutoff defined as $6 \times bmad$ ($bmad$ = baseline median absolute deviation).
     - ➢ **Added criteria:** AC_{50} within range of tested concentrations (AC_{50} is the concentration at 50% of maximal modeled activity).
     - ➢ **Added criteria:** Biomarkers with 50% or more of the values marked as out-of-range (OOR) were manually reviewed.
- Analyses were conducted separately for each biomarker based on species, post-exposure timepoint, and sample type (apical wash or baso media).
  - ➢ Of these, 28 biomarkers were flagged differently by the two approaches when considering only the positive concentration-effect relationships.
  - ➢ This discordance was likely due to higher variability at baseline or across the range of exposure concentrations or the influence of outliers/OOR values.

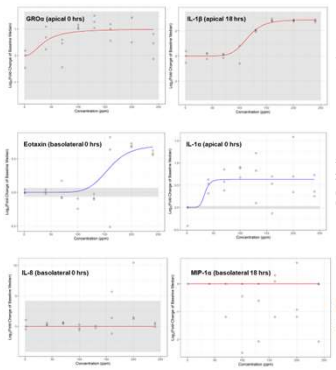
Summary of Active Biomarkers



Comparison (vs. Manual Review Method)



Potential Reasons for the Discordance

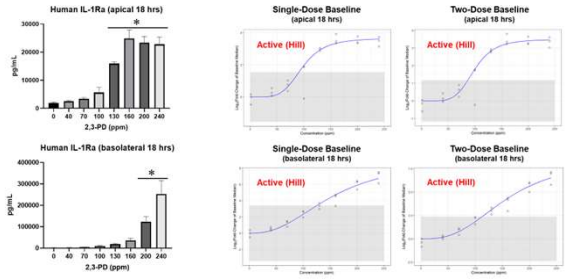


Scenario 1: *There is a possible dose-response relationship but higher variability at baseline.* These two examples for human were flagged as not active (Hill model) using the systematic approach (single-dose baseline) but active using the manual review method.

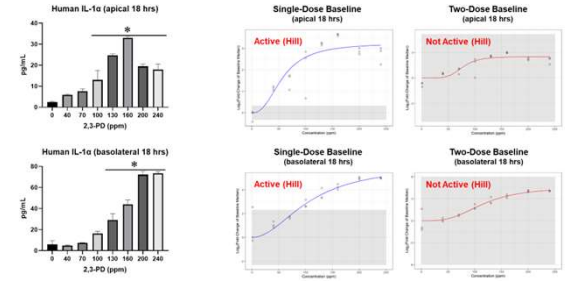
Scenario 2: *There is a possible dose-response relationship but higher variability across the range of exposure concentrations.* These two examples for human were flagged as active (Hill model) using the systematic approach (single-dose baseline) but not active using the manual review method.

Scenario 3: *The influence of outliers and OOR values.* These two examples (human IL-8 and rat MIP-1α) were flagged as not active (constant) using the systematic approach (single-dose baseline) but active using the manual review method. [For MIP-1α, all values at the two lowest concentrations were OOR and <LLOQ.]

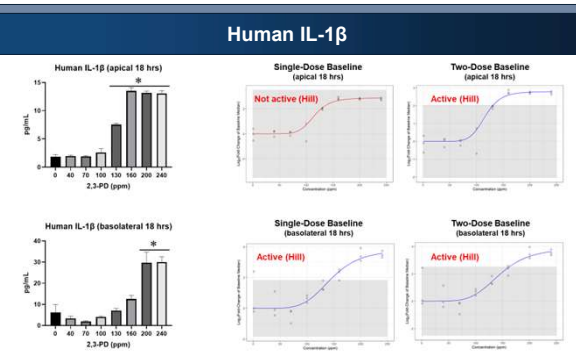
Human IL-1Ra



Human IL-1α



Human IL-1β



Bio-Plex Results Summary

- For human, there were significant concentration-dependent alterations in many immunoregulatory and inflammatory cytokines/chemokines and growth factors relevant to KE 149 (AOP 280) **in apical wash and/or basolateral media samples (at 0- and/or ~18-hours post-exposure)** based on the different analytical approaches applied (for positive concentration-effect relationships only) including:
  - ➢ Up-regulation of the pro-inflammatory IL-1 pathway (↑ IL-1α (single-dose baseline only), IL-1β, and IL-1Ra).
  - ➢ ↑ IL-2, -4, -5, -10, -13, -15, -21, -22, -23, -27, and -31 and EGF, GM-CSF, GROα, and SCF; and IL-8 and IL-12p70 (single-dose baseline only).
  - ➢ IL-1α, IL-1Ra, IL-8, and TGFα (an EGFR ligand) were previously shown to be increased after exposure of human ALI airway tissues to the related chemical BD (Gwinn et al. 2017).
- For rat, there were far fewer significantly upregulated biomarkers compared to human.
  - ➢ ↑ IL-2 and IL-10; and IP-10 (two-dose baseline only).
  - ➢ Possibly because the rat ALI cultures (age day 16) were not fully matured and differentiated (ciliated).