National Institute of Environmental Health Sciences
Division of Translational Toxicology

In Vitro Evaluation of Inhalation Toxicity Induced by 2,3-Pentanedione Vapor Using a VITROCELL 48 2.0 Plus Exposure System and Air-Liquid Interface (ALI) Airway Model

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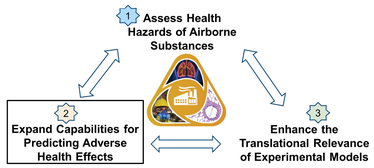
DTT OIE Program

The Occupational and Inhalation Exposures (OIE) program is one of three programs in the Exposure-Based Research strategic area of focus of the Division of Translational Toxicology (DTT) at NIEHS. The purpose of the Exposure-Based Research programs is to solve contemporary public health problems related to environmental and occupational exposures and improve our ability to carry out substance-based hazard evaluations that are more translational, innovative, and responsive. The OIE program focuses primarily on the cause of adverse health effects to the respiratory tract and other organ systems after inhalation exposure and has 3 main objectives (Fig. 1). Currently, as part of **objective 2**, the OIE program is evaluating novel/alternative technologies (i.e., in vitro airway models and lung microphysiological systems) to investigate human relevant inhalation (respiratory) toxicity.

Figure 1. OIE program objectives



https://www.niehs.nih.gov/research/atniehs/dtt/strategic-plan/exposure/occupational

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.

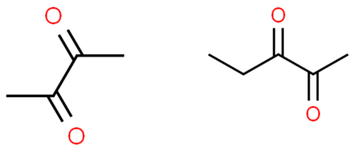
2,3-pentanedione (PD) is also a highly volatile component of ABF. PD has been used as a major substitute for BD in some ABF due to concerns about the respiratory toxicity of BD. However, PD is structurally similar to BD (both are alpha-diketones) (Fig. 2) and has been shown to exhibit toxicological potency 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.

In addition, 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. 3) together with human and rat ALI airway cultures to evaluate PD vapor-induced airway toxicity in vitro (and across 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. 4].

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 2. Chemical structure

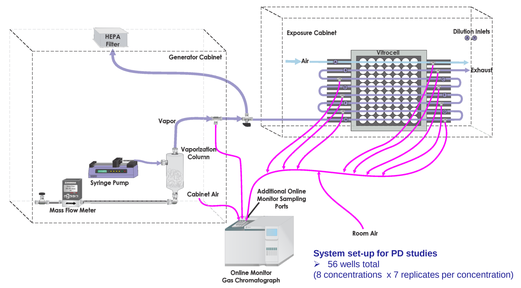


2,3-Butanedione (BD) **2,3-Pentanedione (PD)**

Source: ChemSpider

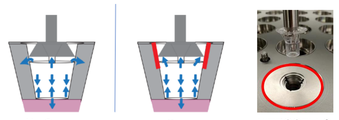
In Vitro Exposure System

Figure 3. Schematic of the Exposure System



System set-up for PD studies
- 56 wells total
(8 concentrations x 7 replicates per concentration)

Exposure System Optimization (Media Covers)

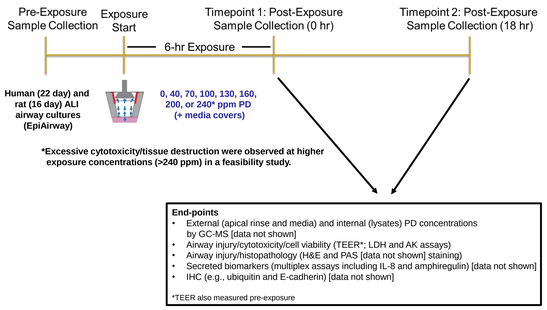


30-45% PD decrease with cover (in basolateral culture media)

Experimental Design

Organotypic ALI airway (EpiAirway) tissues from MatTek (Ashland, MA), derived from primary normal human (single donor) or rat (Wistar) tracheobronchial epithelial cells, were exposed for 6 hr to PD vapor at multiple concentrations or filtered clean air only (control) using the VITROCELL 48 2.0 plus exposure system (Fig. 3). The range of exposure concentrations and duration (6 hr) as well as rat strain selected were similar to those tested in previous in vivo inhalation studies with PD (e.g., Morgan et al. 2012 and TOX-98). In a preliminary study, 6-hr exposure of the ALI cultures to air only was found to cause no adverse effects.

Apical rinse and basolateral culture media samples and tissues (including lysates) were assessed for test article concentrations and PD-induced toxicological effects at 0 and approximately 18 hr after exposure including measurements of transepithelial electrical resistance (TEER), lactate dehydrogenase (LDH) and adenylate kinase (AK) release, secreted biomarkers, and histopathology [hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining and immunohistochemistry (IHC)].



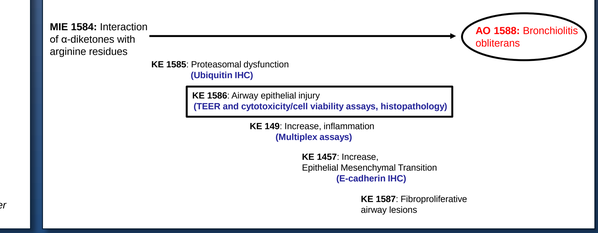
*Excessive cytotoxicity/tissue destruction were observed at higher exposure concentrations (>240 ppm) in a feasibility study.

End-points
- External (apical rinse and media) and internal (lysates) PD concentrations by GC-MS [data not shown]
- Airway injury/cytotoxicity/cell viability (TEER*; LDH and AK assays)
- Airway injury/histopathology (H&E and PAS [data not shown] staining)
- Secreted biomarkers (multiplex assays including IL-8 and amphiregulin) [data not shown]
- IHC (e.g., ubiquitin and E-cadherin) [data not shown]

*TEER also measured pre-exposure

Adverse Outcome Pathway 280

Figure 4. AOP 280: “α-diketone-induced bronchiolitis obliterans” (https://aopwiki.org/aops/280)

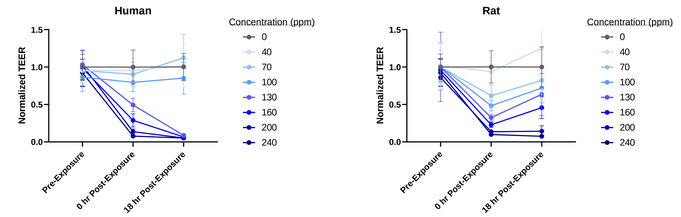


PD Exposure Data Summaries

	Human		Rat	
Target Concentration (ppm)	Mean Concentration (ppm)	Percent Target ± RSD	Mean Concentration (ppm)	Percent Target ± RSD
Exposure Chamber	<LOD	NA	<LOD	NA
Filtered Air	<LOD	NA	<LOD	NA
40	40.2 ± 0.6	101 ± 2	40.8 ± 1.3	102 ± 3
70	69.5 ± 1.0	99 ± 1	72.6 ± 7.9	104 ± 11
100	99.5 ± 1.3	100 ± 1	103 ± 6.2	103 ± 6
130	131 ± 1.6	101 ± 1	133 ± 8.6	102 ± 6
160	162 ± 2.8	101 ± 2	164 ± 5.9	102 ± 4
200	203 ± 2.8	101 ± 1	203 ± 6.5	102 ± 3
240	242 ± 3.5	101 ± 1	241 ± 12.2	100 ± 5

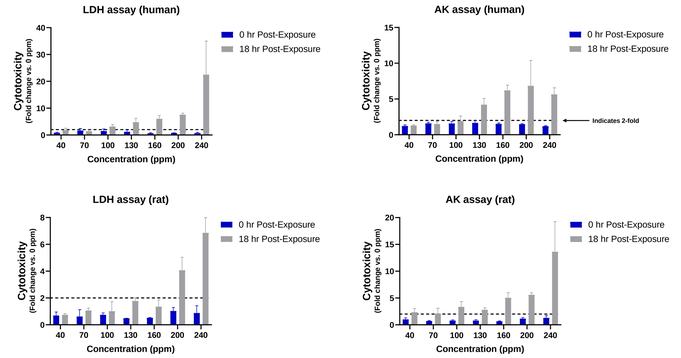
Mean ± SD shown
LOD = 2.09 ppm

PD-induced Alterations in TEER



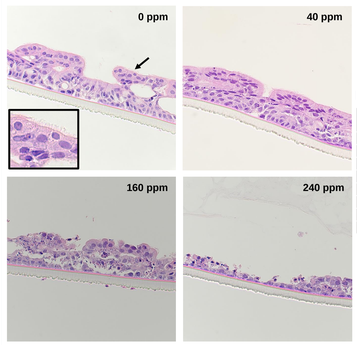
Mean ± SD shown
(n = 12 biological replicates per concentration for Pre- and 0 hr and n = 6 for 18 hr)

PD-induced Cytotoxicity (LDH and AK Activity in Apical Samples)



Mean ± SD shown
(n = 3 biological replicates per concentration)

PD-induced Histopathologic Effects (Human, 0 hr)

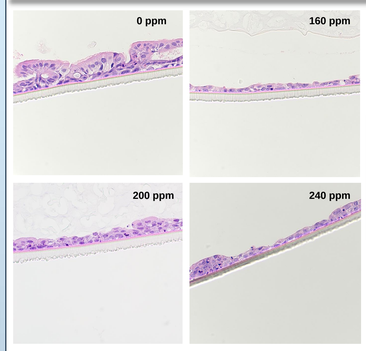


Human (0 hr post-exposure)

Concentration (ppm)	Cell layers	Degeneration & necrosis	Loss of cilia	% Cells denuded (focal areas)
0	5 to 7	0	-	0
40	5 to 7	0	-	0
70	5 to 7	0/1	-	0
100	5 to 7	1	-	0
130	4 to 6	2	Y	0
160	3 to 5	2	Y	0
200	1 to 5	3	Y	0
240	0 to 4	3	Y	5 to 10

Severity scoring (for degeneration & necrosis)
0 = within normal limits; 1 = minimal (< 5%); 2 = mild (5-10%); 3 = moderate (11-25%); 4 = marked (>25%)

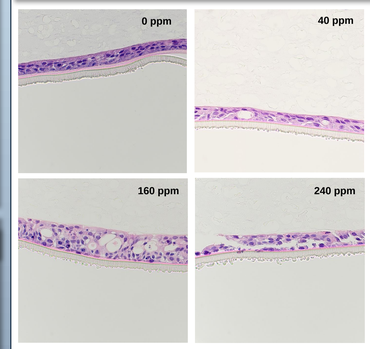
PD-induced Histopathologic Effects (Human, 18 hr)



Human (18 hr post-exposure)

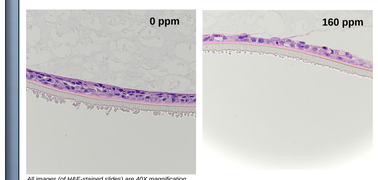
Concentration (ppm)	Cell layers	Degeneration & necrosis	Loss of cilia	% Cells denuded (focal areas)
0	5 to 7	0	-	0
40	5 to 7	0	-	0
70	5 to 7	0	-	0
100	5 to 7	1	-	0
130	4 to 7	1	Y	0
160	2 to 5	2	Y	0
200	0 to 5	3	Y	< 5
240	0 to 3	3	Y	50 to 80

PD-induced Histopathologic Effects (Rat, 0 and 18 hr)



Rat (0 hr post-exposure)

Concentration (ppm)	Cell layers	Degeneration & necrosis	Loss of cilia	% Cells denuded (focal areas)
0	3 to 5	0	-	0
40	3 to 5	0	-	0
70	3 to 5	0/1	-	0
100	3 to 5	1	-	0
130	0 to 4	2	Y	0
160	0 to 4	2	Y	< 10
200	0 to 3	3	Y	< 10
240	0 to 3	3	Y	40 to 60



Rat (18 hr post-exposure)

Concentration (ppm)	Cell layers	Degeneration & necrosis	Loss of cilia	% Cells denuded (focal areas)
0	3 to 5	0	-	0
40	3 to 5	0	-	0
70	3 to 5	0	-	0
100	2 to 4	1	Y	0
130	0 to 4	2	Y	< 10
160	0 to 3	3	Y	60 to 80
200*	0 to 2	3	Y	80 to 90
240*	0 to 1	3	Y	90 to 100

All images (of H&E-stained slides) are 40X magnification

**Images not shown (membranes ~ completely denuded)*

Conclusions

Exposure of human and rat ALI airway cultures to PD (6 hr) induced concentration-dependent changes in the following toxicological parameters relevant to in vivo rat (BD and PD) and in vitro human ALI (BD) airway findings as well as key events in AOP 280. Airway epithelial injury is thought to be an initiator of bronchial/bronchiolar fibrosis.

↓ **TEER**
- ≥ 130 ppm (0 and 18 hr) in human; ≥ 70 ppm (0 and 18 hr with some recovery at 70-160 ppm) in rat

↑ **Cytotoxicity measurements (18 hr only)**
- LDH and AK release (above 2-fold vs. 0 ppm): ≥ 130 ppm in human; ≥ 200 ppm in rat

↑ **Histopathologic effects (degeneration & necrosis, loss of cilia, and denudation)**
- ≥ 130 ppm (0 and 18 hr) in human; ≥ 130 ppm (0 hr) and ≥ 100 ppm (18 hr) in rat

Additional end-points (in-progress)
- External/internal PD concentrations
- Secreted biomarkers
- Histopathology (PAS staining and IHC)

Further optimization of rat ALI model (e.g., age of cultures) is needed.

Based on the results of this proof-of-concept study with PD, this VITROCELL exposure system/ALI airway model has the potential to be used to investigate human-relevant inhalation (respiratory) toxicity in vitro, and applications may include providing screening level assessments to help predict the adverse airway/lung effects of inhaled substances and/or to help select compounds for further toxicity testing.

References

- Gwinn et al. Airway injury in an in vitro human epithelium-fibroblast model of diacetyl vapor exposure: diacetyl-induced basal/suprabasal spongiosis. Inhal Toxicol. 2017 Jun;29(7):310-321.
- McGraw et al. Airway basal cell injury after acute diacetyl (2,3-butanedione) vapor exposure. Toxicol Lett. 2020 Jun 1;325:25-33.
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