National Institute of Environmental Health Sciences
Division of Translational Toxicology

Comparison of the toxicological effects of highly volatile inhaled components of artificial butter flavoring using in vivo rodent and in vitro human approaches

WM Gwinn, MF Cesta, AR Pandiri, P-L Yao, MD Stout, and GK Roberts
Division of Translational Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

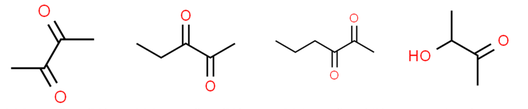
Abstract

Artificial butter flavoring (ABF) is used in the food and beverage industry, including in the processing and production of microwave popcorn. Highly volatile organic components of ABF include 2,3-butanedione (BD or diacetyl), 2,3-pentanedione (PD), 2,3-hexanedione (HD), and/or acetoin. Inhalation exposure of microwave popcorn plant workers to BD vapors has been associated with the development of small airway fibrosis in the form of obliterative bronchiolitis (OB). PD has been used as a major substitute for BD in some ABF but has been shown to exhibit similar toxicological potency with regards to the induction of airway fibrosis. Other substitutes in ABF include HD. Whole-body inhalation exposure to ≥150 ppm BD or PD for approximately 2 weeks has been shown to recapitulate OB-like airway fibrotic lesions in rats similar to those in exposed workers. Furthermore, in vitro human air-liquid interface (ALI) airway epithelial culture models have been applied to help elucidate the mechanisms of airway fibrosis induced by these chemicals. The objective herein was to review the current understanding of the in vivo and in vitro toxicological effects of these four major components of ABF, especially in the context of the lung, including a presentation of new in vivo NTP study data for the acute and sub-chronic inhalation toxicity testing of acetoin, for which there is no inhalation toxicity data, and the sub-chronic inhalation toxicity testing of PD. Whole-body inhalation exposure to ≥50 ppm PD for 3 months induced adverse respiratory tract and ocular effects in rats and mice. The pulmonary effects of PD included airway inflammation along with squamous metaplasia and/or hyperplasia (with atypical lesions in mice) of the tracheal, bronchial, and/or bronchiolar epithelium. The ocular effects included acute inflammation, ulceration, and/or epithelial hyperplasia of the cornea. Minimal toxicological effects were observed after acute (2-week) or 3-month inhalation exposure to acetoin. Overall, the in vivo and in vitro data suggest that the potential adverse human health effects of BD and PD with regards to lung toxicity are of greater concern compared to HD and acetoin.

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 obliterative bronchiolitis (OB), mostly in workers in the microwave popcorn packaging and flavoring industry exposed to 2,3-butanedione (BD, also commonly called diacetyl). OB 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. Acetoin and 2,3-pentanedione (PD) are also highly volatile components 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) and has been shown to exhibit toxicological potency similar to BD in the induction of airway epithelial injury with OB-like fibrotic lesions in rats, following acute (2-week) inhalation (whole-body) exposure, that are similar to the OB lesions observed in occupational exposures. Another potential ABF replacement is 2,3-hexanedione (HD); however, it too is structurally similar to both BD and PD.

To date, the inhalation toxicity of PD has been reported for approximately 2-week (and shorter) whole-body exposure studies in rats with a main focus on adverse respiratory tract effects. Therefore, **the objective was to evaluate the 3-month inhalation toxicity of PD vapors within the respiratory tract and other target organs (including the eyes) in Wistar Han rats and B6C3F1/N mice, including airway/lung lesion formation following 3 months of whole-body exposure. In addition, the 2-week and 3-month inhalation toxicity of acetoin, which has not been previously investigated, was evaluated in rats and mice. An additional objective was to provide an overall review of the current understanding of the in vivo and in vitro toxicological effects (in rodent models and for human air-liquid interface (ALI) airway approaches) of these four major components of ABF (BD, PD, HD, and acetoin) with regards to tracheal and lung effects in the context of inhalation exposure and OB.**



Experimental Design (PD and acetoin tox studies)

In the 2-week (acute) studies, male and female Wistar Han rats and B6C3F1/N mice were exposed via inhalation (whole-body exposure, WBE) to acetoin vapors at concentrations of 0 (air), 6.25, 25, 100, 400, or 800 ppm for 6 hours per day, 5 days per week, for 2 weeks plus 2 (rats) or 3 (mice) additional exposure days for a total of 12 (rats) or 13 (mice) exposures over a period of 16 (rats) or 17 (mice) days. In the 3-month (sub-chronic) studies, male and female rats and mice were exposed via inhalation (WBE) to acetoin vapors at concentrations of 0 (air), 50, 100, 200, 400, or 800 ppm, or to PD vapors at concentrations of 0 (air), 6.25, 12.5, 25, 50, or 100 ppm, for 6 hours per day, 5 days per week, for 13 to 14 weeks. **Complete necropsies were performed on all animals. Endpoints were body and organ weights, clinical observations, hematology and clinical chemistry, histopathology, and genetic toxicology.**

Results

Histopathology tables are shown for parts of the respiratory tract (trachea and lung). **There were no exposure-related adverse (including histopathologic) effects in rats or mice exposed by inhalation to acetoin for either 2 weeks or 3 months (data not shown).** The following findings were observed in rats and/or mice exposed by inhalation to PD (at 50 and/or 100 ppm) for 3 months (data not shown). Clinical observations were abnormal breathing, sneezing, and eye abnormality. Nasal effects included suppurative inflammation, olfactory epithelial atrophy, hyperplasia, squamous metaplasia, necrosis, and regeneration of the respiratory epithelium, turbinate atrophy and necrosis, and necrosis and perforation of the septum. Laryngeal effects included chronic active inflammation, necrosis, ulceration, hyperplasia and squamous metaplasia (atypical in mice; see **Figure 1**), and regeneration. **Exposure-related histopathologic effects in other organs (besides the respiratory tract and eyes) were not observed.**

3-month PD effects in trachea (rats)

Wistar Han rats	0 ppm	6.25 ppm	12.5 ppm	25 ppm	50 ppm	100 ppm
Male						
Trachea	10	10	10	10	10	10
Inflammation, acute	0**	0	0	1 (1.0)	0	3 (1.0)
Epithelium, hyperplasia	0**	0	0	0	0	8** (2.5)
Epithelium, metaplasia, squamous	0**	0	0	0	0	3 (1.7)
Epithelium, regeneration	0**	1 (1.0)	1 (1.0)	0	8** (1.4)	10** (2.8)
Female						
Trachea	10	10	10	10	10	10
Inflammation, acute	0*	0	0	0	0	2 (1.0)
Epithelium, hyperplasia	0**	0	0	1 (2.0)	0	4* (3.0)
Epithelium, metaplasia, squamous	0*	0	0	0	0	2 (1.0)
Epithelium, necrosis	0*	0	0	0	0	2 (1.0)
Epithelium, regeneration	0**	3 (1.0)	0	0	7** (1.6)	10** (3.0)

*p ≤ 0.05 by the Cochran-Armitage (trend) or Fisher's exact (pairwise) test (vs. 0 ppm); **p ≤ 0.01

3-month PD effects in trachea (mice)

B6C3F1/N mice	0 ppm	6.25 ppm	12.5 ppm	25 ppm	50 ppm	100 ppm
Male						
Trachea	10	10	10	10	10	10
Inflammation, suppurative	0**	0	1 (2.0)	0	0	5* (1.6)
Inflammation, chronic active	0**	0	0	0	0	5* (1.2)
Epithelium, metaplasia, squamous, atypical	0**	0	0	0	1 (2.0)	10** (3.3)
Epithelium, regeneration	0**	0	0	0	8** (2.9)	0
Female						
Trachea	10	10	10	10	10	10
Inflammation, suppurative	0**	0	0	0	0	5** (1.6)
Inflammation, chronic active	0**	0	0	0	0	3 (2.0)
Epithelium, hyperplasia, atypical	0**	0	0	0	0	3 (2.0)
Epithelium, metaplasia, squamous, atypical	0**	0	0	0	1 (1.0)	8** (3.1)
Epithelium, regeneration	1 (1.0)	0	0	0	10** (2.8)	0

3-month PD effects in lung (rats)

Wistar Han rats	0 ppm	6.25 ppm	12.5 ppm	25 ppm	50 ppm	100 ppm
Male						
Lung	10	10	10	10	10	10
Inflammation, eosinophil	3** (1.0)	3 (1.0)	1 (1.0)	5 (1.0)	3 (1.0)	10** (2.0)
Bronchiole, epithelium, hyperplasia	0**	0	0	0	3 (1.0)	10** (2.2)
Bronchus, epithelium, hyperplasia	0**	0	0	0	0	5* (1.6)
Bronchus, epithelium, regeneration	0**	0	0	0	1 (1.0)	9** (2.8)
Bronchus, epithelium, metaplasia, squamous	0**	0	0	0	0	3 (1.3)
Female						
Lung	10	10	10	10	10	10
Fibrosis, focal	0**	0	0	0	0	2 (1.0)
Inflammation, eosinophil	4** (1.3)	3 (1.0)	4 (1.0)	3 (1.0)	5 (1.0)	10** (1.7)
Alveolus, infiltration, cellular, polymorphonuclear	0**	0	0	0	0	2 (1.0)
Bronchiole, epithelium, hyperplasia	0**	0	0	0	0	7** (1.1)
Bronchus, epithelium, hyperplasia	0**	0	0	0	0	4* (1.5)
Bronchus, epithelium, regeneration	0**	0	0	0	0	8** (1.8)
Bronchus, epithelium, metaplasia, goblet cell	0**	0	0	0	0	3 (2.3)

3-month PD effects in lung (mice)

B6C3F1/N mice	0 ppm	6.25 ppm	12.5 ppm	25 ppm	50 ppm	100 ppm
Male						
Lung	10	10	10	10	10	10
Bronchiole, infiltration, cellular, polymorphonuclear	0**	0	0	0	0	5* (1.0)
Bronchiole, epithelium, degeneration	0**	0	0	0	0	4* (1.5)
Bronchiole, epithelium, hyperplasia	0**	0	0	0	1 (1.0)	4* (1.0)
Bronchus, infiltration, cellular, polymorphonuclear	0**	0	0	0	0	9** (1.1)
Bronchus, inflammation, chronic	0**	0	0	1 (1.0)	6** (1.2)	9** (1.2)
Bronchus, epithelium, degeneration	0**	0	0	2 (1.0)	9** (3.0)	8** (2.5)
Bronchus, epithelium, hyperplasia	0	0	0	0	2 (2.5)	0
Bronchus, epithelium, hyperplasia, atypical	0	0	0	0	7** (2.1)	0
Bronchus, epithelium, necrosis	0**	0	0	0	0	3 (2.0)
Bronchus, epithelium, metaplasia, squamous, atypical	0**	0	0	0	0	10** (2.6)
Bronchus, epithelium, regeneration	0**	1 (1.0)	0	1 (1.0)	8** (3.5)	7** (2.3)
Female						
Lung	10	10	10	10	10	10
Bronchiole, infiltration, cellular, polymorphonuclear	0**	0	0	0	0	3 (1.0)
Bronchus, infiltration, cellular, polymorphonuclear	0**	0	0	0	0	9** (1.3)
Bronchus, inflammation, chronic	0**	0	0	0	4* (1.0)	9** (1.6)
Bronchus, epithelium, degeneration	0**	0	0	0	9** (1.8)	8** (2.8)
Bronchus, epithelium, metaplasia, squamous, atypical	0**	0	0	0	0	10** (2.1)
Bronchus, epithelium, necrosis	0**	0	0	0	0	2 (1.0)
Bronchus, epithelium, regeneration	0**	0	0	0	9** (2.4)	9** (2.1)
Bronchus, epithelium, ulcer	0**	0	0	0	0	2 (1.0)

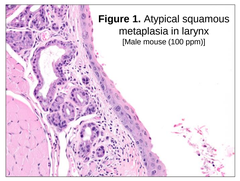


Figure 1. Atypical squamous metaplasia in larynx
[Male mouse (100 ppm)]

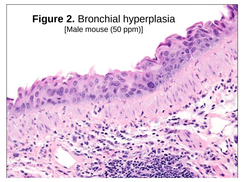


Figure 2. Bronchial hyperplasia
[Male mouse (50 ppm)]

3-month (significant) PD effects in eyes

- **Male rats:** acute inflammation, neovascularization, and epithelial vacuolation of the cornea (100 ppm)
- **Female rats:** acute inflammation of the ciliary body (50 ppm) and cornea (50 and 100 ppm)
- **Female mice:** acute inflammation, mineralization, ulceration, and epithelial hyperplasia (100 ppm)

Conclusions (3-month PD respiratory tract effects)

- Significant adverse respiratory tract effects in rats and mice after **3-month inhalation exposure to PD at concentrations of 50-100 ppm** included inflammation, necrosis, ulceration, degeneration, squamous metaplasia, hyperplasia, and/or regeneration of the nasal, laryngeal, tracheal, bronchial, and/or bronchiolar epithelium.
  - ➢ In mice, hyperplasia and squamous metaplasia of the laryngeal, tracheal, and bronchial epithelium were considered atypical (potentially preneoplastic).
  - ➢ No bronchial/bronchiolar (OB-like) fibrotic lesions were induced in rats or mice.
    - ❖ Likely due to the PD exposure concentration being too low (<150 ppm) for rats (based on comparisons to previous shorter-term exposure studies) and species-specific differences in mice
    - ❖ Bronchial/bronchiolar adverse effects in the lung are most relevant to OB (distal bronchi/bronchioles are the target sites for OB).

Review of the tracheal and lung effects of BD, PD, HD, and acetoin

2,3-Butanedione (14 studies)

McGraw et al., 2022
WBE; 1 hr/d for 5 d; female C57BL/6J mice
Lung effects (200 ppm): Minimal histopathology (day 19)

Ghio et al., 2021
ITI (125 mg/kg bw, 1 dose); male SD rats
Lung effects: Bronchial/bronchiolar inflammation and fibrotic polypoid lesions (day 7)

House et al., 2021
WBE; 6 hr/d for 5 d; male SD rats
Lung effects (200 ppm): Mixed, granulocytic inflammation with bronchial lymphoid aggregates; increased CD4+CD25+ T cells (day 12 and 19) which are Foxp3+ (day 12); **bronchial wall thickening and ECM deposition (day 12 and 19)**
BALF: ↑ lymphocytes, neutrophils, and IL-17a

Wang et al., 2021
WBE; 6 hr/d for 5 d (14-d recovery); male SD rats
Lung effects (200 ppm): fibrotic (OB-like) lesions (day 19)
BALF: ↑ neutrophils
[Increased ubiquitin-C expression and proteasome activity]

Morgan et al., 2016
WBE; 6 hr/d, 5 d/wk for 2 wk (+/- 2-wk recovery); male WH rats
Lung effects (150-200 ppm): Bronchial necrosis, ulceration, squamous metaplasia, and hyperplasia; bronchial and alveolar chronic active inflammation; bronchiolar hyperplasia; bronchial (OB-like) fibrosis; interstitial fibrosis (recovery group)

Hubbs et al., 2016
WBE; 6 hr; C57BL/6N WT or *Dcxr*-KO mice
Lung effects (100-300 ppm): bronchial (vacuolar) degeneration with apoptosis
[Increased bronchial ubiquitin- role of α-carbonyl group in protein damage- autophagy]

Hubbs et al., 2012
WBE; 6 hr; male SD rats
Tracheal and lung effects (240 ppm): Tracheitis and bronchitis

Palmer et al., 2011 (also Kelly et al., 2014)
ITI (125 mg/kg bw, 1 dose); male SD rats
Lung effects: Peribronchiolar and mixed intraluminal inflammation (day 1 and/or 3); bronchiolar erosion and regeneration (day 1 and/or 3); bronchial regeneration and mild hyperplasia (day 3); bronchial/bronchiolar (OB-like) fibrosis (day 3 and 7)
BALF/lung: ↑ neutrophils, CINC-1 (**Kelly et al., 2019**), and AREG (**Kelly et al., 2014**)

Hubbs et al., 2008
WBE; 6 hr or 4 x 15 min exposures over 6 hr; male SD rats
Tracheal effects (≥224 ppm): Necrosis, loss of cilia, denuded BM, suppurative to fibrinosuppurative inflammation (with neutrophils)
Lung effects (≥294.6 ppm): Bronchial necrosis, denuded BM, nuclear pyknosis, suppurative to fibrinosuppurative inflammation

Morgan et al., 2008
WBE, nose-only exposure, or OPA; male C57BL/6 mice
WBE (6 hr/d for 4 d or 6 hr/d, 5 d/wk for 12 wk); nose-only (1 hr/d, 5 d/wk for 4 wk or 15 min twice per day, 5 d/wk for 2 wk); or OPA (400 mg/kg bw, 1 dose)
Lung effects (WBE, 100-400 ppm; nose-only exposure, 400-1200 ppm): Peribronchial/peribronchiolar lymphocytic inflammation; bronchial atrophy, necrosis, denudation, and regenerative hyperplasia with karyomegaly; focal fibrohistiocytic (proliferative) lesions with minimal inflammation (OPA, day 4)

Hubbs et al., 2002
WBE; 6 hr; male SD rats
Lung effects (285-371 ppm): Multi-focal, necrotizing bronchitis (with denuded BM)
BALF: ↑ neutrophils
[Exposure to complex mixture (diacetyl - marker of exposure concentration)]

NTP Technical Report (2018) [TR-593; sub-chronic]
WBE; 6 hr/d, 5 d/wk for 3 mo; WH rats and B6C3F1/N mice
Tracheal effects (50-100 ppm, mice): Atypical squamous metaplasia, hyperplasia, degeneration, and regeneration; chronic active inflammation
Lung effects (100 ppm, rats): Bronchial necrosis, hyperplasia (including atypical), and regeneration; bronchiolar hyperplasia
Lung effects (100 ppm, mice): Bronchial atypical hyperplasia/squamous metaplasia and regeneration; chronic inflammation and infiltration of PMNs

NTP Technical Report (2018) [TR-593; chronic]
WBE; 6 hr/d, 5 d/wk for 2 yr; WH rats and B6C3F1/N mice
Tracheal effects (50 ppm, rats): Atrophy, necrosis, hyperplasia, squamous metaplasia, and regeneration; chronic active inflammation; submucosa fibrosis
Tracheal effects (50 ppm, mice): Necrosis, hyperplasia, squamous metaplasia, and regeneration; chronic active inflammation; submucosa fibrosis and carina submucosa fibrosis
Lung effects (50 ppm, rats): Suppurative and granulomatous inflammation, peribronchial chronic active inflammation; bronchial atrophy, hyperplasia, and regeneration; bronchiolar and alveolar hyperplasia; bronchial submucosa fibrosis; interstitial fibrosis
Lung effects (50 ppm, mice): Bronchial regeneration; suppurative inflammation of the lung, pleura, and mediastinum; chronic active inflammation of the mediastinum; bronchial submucosa fibrosis
[Some evidence of nasal cancers including squamous cell carcinoma (rats) and adenocarcinoma (mice)]

WBE, whole-body exposure; ITI, intratracheal instillation; OPA, oropharyngeal aspiration

2,3-Pentanedione (5 studies)

NTP Toxicity Report (2023) [TOX-98 to be released; sub-chronic]
WBE; 6 hr/d, 5 d/wk for 3 mo; WH rats and B6C3F1/N mice
Tracheal and lung effects (50-100 ppm): (POSTER)

Morgan et al., 2016
WBE; 6 hr/d, 5 d/wk for 2 wk (+/- 2-wk recovery); male WH rats
Lung effects (150-200 ppm): Bronchial necrosis, ulceration, squamous metaplasia, and hyperplasia; bronchial and alveolar chronic active inflammation; bronchiolar hyperplasia; bronchial (OB-like) fibrosis; interstitial fibrosis (recovery group)

Morgan et al., 2015
WBE; 6 hr/d, 5 d/wk for 2 wk; male WH rats
Lung effects (200 ppm): Bronchial ulceration, inflammation, hyperplasia, and regeneration; bronchial (OB-like) fibrosis
[↓ inflammatory cytokines/chemokines in non-fibrotic bronchi of exposed rats. ↑ TGF-β2 (and downstream genes) and genes for collagens and ECM proteins in fibrotic bronchi. Altered expression of genes for peptidases and peptidase inhibitors.]

Morgan et al., 2012
WBE; 6 hr/d, 5 d/wk for 2 wk; WH rats and B6C3F1 mice
Lung effects (200 ppm, rats): Bronchial necrosis, ulceration, inflammation, squamous metaplasia, and regeneration; bronchial/bronchiolar (OB-like) fibrosis
BALF: ↑ PMNs and MCP-1, MCP-3, CRP, FGF-9, fibrinogen, and OSM
Lung effects (100-200 ppm, mice): Bronchial necrosis, ulceration, inflammation, and regeneration; **no fibrosis**
BALF: ↑ PMNs and fibrinogen

Hubbs et al., 2012
WBE; 6 hr, male SD rats
Tracheal and lung effects (318 ppm): Tracheitis and bronchitis

2,3-Hexanedione (2 studies)

NTP Research Report 10 (Morgan et al., 2019)
WBE; 6 hr/d, 5 d/wk for 2 wk; B6C3F1 mice
Tracheal effects (100-200 ppm): Squamous metaplasia, hyperplasia, degeneration, and regeneration; chronic active inflammation
Lung effects (100-200 ppm): Bronchial hyperplasia (including atypical) and degeneration; chronic active inflammation
[3-month sub-chronic study was not conducted.]

Morgan et al., 2016
WBE; 6 hr/d, 5 d/wk for 2 wk (+/- 2-wk recovery); male WH rats
Lung effects (200 ppm): Bronchial hyperplasia and minimal squamous metaplasia; minimal bronchiolar hyperplasia; **minimal bronchial fibrosis**

Acetoin (2 studies)

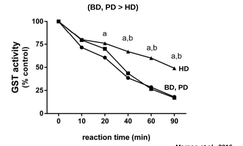
NTP Toxicity Report (2023) [TOX-98 to be released; acute and sub-chronic]
WBE; 6 hr/d, 5 d/wk for 2 wk or 3 mo; WH rats and B6C3F1/N mice
Tracheal and lung effects: None exposure-related (POSTER)

Toxicological potency in trachea/lung (inhalation)

BD, PD [Fibrosis*] **> HD** [non-fibrotic effects] **> Acetoin** [no effects]

***Dose and species-dependent (rat) for OB-like fibrotic lung lesions**

Correlates with chemical reactivity (arginine)
(BD, PD > HD)



Morgan et al., 2016

Summary (adverse tracheal and lung effects in vivo)

Histopathologic Effect	2,3-Butanedione	2,3-Pentanedione	2,3-Hexanedione	Acetoin
Tracheal inflammation	√ (inhalation*; rats or mice)	√ (inhalation; rats or mice)	√ (inhalation; mice)	NA
Lung inflammation	√ (ITI, OPA, or inhalation; rats or mice)	√ (inhalation; rats or mice)	√ (inhalation; mice)	NA
Tracheal atrophy, necrosis, ulceration, erosion, denudation, and/or degeneration	√ (inhalation; rats or mice)	NA	√ (inhalation; mice)	NA
Lung atrophy, necrosis, ulceration, erosion, denudation, and/or degeneration	√ (ITI, OPA, or inhalation; rats or mice)	√ (inhalation; rats or mice)	√ (inhalation; mice)	NA
Tracheal hyperplasia	√ (inhalation; rats or mice)	√ (inhalation; rats or mice [A])	√ (inhalation; mice)	NA
Lung hyperplasia	√ (OPA or inhalation; rats [A] or mice [A])	√ (inhalation; rats or mice [A])	√ (inhalation; rats or mice [A])	NA
Tracheal squamous metaplasia	√ (inhalation; rats or mice [A])	√ (inhalation; rats or mice [A])	√ (inhalation; mice)	NA
Lung squamous metaplasia	√ (inhalation; rats or mice [A])	√ (inhalation; rats or mice [A])	NA	NA
Tracheal regeneration	√ (inhalation; rats or mice)	√ (inhalation; rats or mice)	√ (inhalation; mice)	NA
Lung regeneration	√ (ITI or inhalation; rats or mice)	√ (inhalation; rats or mice)	NA	NA
Tracheal submucosa fibrosis	√ (inhalation [2 yr]; rats or mice)	NT	NT	NT
Bronchial submucosa fibrosis	√ (inhalation [2 yr]; rats or mice)	NT	NT	NT
Bronchial/bronchiolar (OB-like) fibrosis	√ (ITI or inhalation [≥2 wk]; rats)	√ (inhalation [≥2 wk]; rats)	NA	NA
Interstitial fibrosis	√ (inhalation [>2 wk or 2 yr]; rats)	√ (inhalation [>2 wk]; rats)	NA	NA

*Histopathologic effects shown reported in at least one study in rats and/or mice exposed by inhalation, ITI, or OPA (inhalation: 6 hr, < 2 wk, ≥ 2 wk, 3 mo, and/or 2 yr; ITI or OPA: 1 dose).
[A], including atypical lesions; NA, not applicable; NT, not tested in 2 yr bioassay

Review of in vitro ALI airway culture models

ALI cultures derived from primary human tracheobronchial epithelial (TBE) cells

Reference	Exposure	Histopathologic effects	Cytokines, chemokines, and GFs	Other end-points
Langel et al., 2022	BD (25 mM*); vapor cup (1 hr) [day 0, 2, and 4]	Loss of cilia and flat dysplastic epithelium (↓acetylated tubulin); relocalization of keratin 5+ basal cells	↑IL1B transcript	Cilia injury/dysregulation; ↑hypoxia- and sterile inflammation-associated pathways and ↓expression of anti-viral IFN-stimulated genes like RSAD2 (transcriptomics)
Wang et al., 2021	BD (25 mM); vapor cup (1 hr) [2 days]	NA	NA	↑CD14 (associated with TLR4-mediated LPS signaling) and HAS2, ITGA2, VEGF2, WNT7A, and PTGS2 transcripts (positive regulation of epithelial cell migration GO pathway); ↑HA
McGraw et al., 2020	BD (25 or 50 mM); vapor cup (1 hr)	↑Basal cell inury (intra- and intercellular clearing with cytoplasmic hypopigmentation in the basal/suprabasal layers)	NA	Basal cell markers: ↓keratin 5 (58 kDa); ↑cross-linked keratin 5 (116 kDa); ↓ΔNp63α
				↑K48-linked ubiquitin; ↑co-IP of ubiquitin C and keratin 5
				↑Proteasome 20S (day 3); ↑keratin 5 (58 kDa) and keratin 5 (116 kDa) with MG132 (day 3)
				↑LDH and cleaved caspase 3/7 release and ↓TEER (50 mM)
Thimraj et al., 2019	BD (1, 3, 10, or 30 ppm); chamber (30 min)	NA	↑TNF and ↓IL6 transcripts; ↑AREG protein	↑NFKB transcript
				↑GSTA1 and PTGES2 transcripts (oxidative stress markers)
				↑MMP9 transcript and protein (tissue injury/repair marker)
				↓Anti-protease SLPI transcript (30 ppm) but ↑SLPI (1 ppm); ↑SLPI protein (10 ppm)
				No LDH release
Kelly et al., 2019	BD (25 mM); vapor cup (1 hr)	NA	↑IL-8 protein (EGFR-dependent)	NA
Gwinn et al., 2017	BD (25 mM) or HD (10 mM*); vapor cup (1 hr) [day 0, 2, and 4]	↓cilia and goblet cells and ↑apoptosis/necrosis, atropy, erosion/denudation, and basal cell effects (basal/suprabasal spongiosis) by BD but not HD [FT model]	↑IL-6, IL-8, TGFa, MCP-3, TNFa, IL-1a, and sIL-1Ra and ↓fractalkine proteins by BD but not HD except fractalkine [FT model]; ↑IL-8, TGFa, IL-1a, and sIL-1Ra by BD [non-FT model]	↑MMP-1, MMP-3, and TIMP-1 and ↓MMP-2, MMP-7, and TIMP-2 proteins by BD but not HD except TIMP-2 [FT model]; ↑MMP-1 and TIMP-1 and ↓MMP-2 and MMP-7 [non-FT model]
				↑LDH release by BD but not HD [FT model]
Foster et al., 2017	BD (25 mM); vapor cup (1 hr) [day 0, 2, and 4]; multiple donors (4)	Loss of cell-cell and cell-BM adhesion and/or presence of squamoid features (donor-dependent); ↓RSPH4A in ciliated cells and ↑ in basal cells; ↑TGM1 and RPTN in basal region	NA	Altered expression of proteins and phosphopeptides suggestive of loss of cilia and increased squamous differentiation (proteomics); hyperphosphorylation and cross-linking of basal cell keratins
				↓RSPH4A protein (cilia-specific marker); ↑TGM1 and RPTN proteins (markers of squamous differentiation)
Brass et al., 2017	BD (25 mM); vapor cup (1 hr) [day 0, 2, and 4]; multiple donors (4)	See above (Foster et al., 2017)	↓FBLN3 and DDB1 and ↑ECM1 and GDF15 transcripts	Altered expression of proteins associated with matrix remodeling, including degradation, assembly, and new matrix organization and of modifiers of EGFR-signaling (analysis by secretomics of apical rinse and basolateral medium)
Zaccone et al., 2015	BD and PD (≥25 ppm); chamber (6 hr)	No effect on cell morphology (25 ppm)	NA	Inhibited active ion transport
				Metabolites acetoin and 2-hydroxy-3-pentone detected in basolateral medium (DCXR activity)
				Cell death (60 and 100-360 ppm); no changes in transepithelial R values (25 or ≥60 ppm)
Kelly et al., 2014	BD (25 mM); vapor cup (1 hr) [day 0, 2, and 4]	NA	↑ AREG protein	NA
	BD (20 and 40 mM); applied apically (30 min)	NA	↑ AREG protein	NA

*25 mM BD and 10 mM HD applied to vapor cup equivalent to ~1000 ppm
BM, basement membrane; FT, full-thickness (model includes donor-matched primary fibroblasts in ECM); DCXR, dicarbonyl and L-xylulose reductase; NA, not applicable

Summary (in vitro human ALI airway approaches)

- Multiple published studies have used **TBE-ALI models** to assess human-relevant inhalation toxicity of BD vapors in vitro. [One published study also tested PD and one also tested HD (in addition to BD).] **The toxicological effects of BD in vitro include those to:**
  - ➢ Basal epithelial cells (keratins and cell-cell/cell-basement membrane adhesion)
    - ❖ Keratin 5 effects have been shown to be linked to hemidesmosome-associated protein effects (e.g., ITGβ4) using an in vitro human (non-ALI) airway model (Kim and McGraw, 2022)
  - ➢ Cilia
  - ➢ Pro-inflammatory signaling (e.g., cytokines/chemokines)
  - ➢ EGFR-signaling (e.g., AREG)
  - ➢ Protein damage/proteasome (protease) pathways
  - ➢ Tissue injury/repair and ECM remodeling pathways (e.g., MMPs and TIMPs)