A non-animal toolbox informed by pulmonary toxicity adverse outcome pathways (AOPs): a next-generation risk assessment (NGRA) approach for human inhalation safety

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1) Background

It is important for the safety assessment of consumer spray products (e.g., antiperspirants, hairsprays, cleaning sprays) to consider the potential for ingredients to cause adverse effects in the lung under the conditions of product use. The assessment of chemical-induced lung effects has historically been achieved by performing animal testing, which has significant limitations (e.g., biological differences between rodent and human respiratory systems and ethical concerns). In this context, recent research anchored in human-relevant science has focused on developing human-relevant *in silico* and *in vitro* tools and approaches (New Approach Methodologies, NAMs) that can be employed, together with existing information, within the next-generation risk assessment (NGRA) of materials to assess the risk of lung toxicity.

This study investigated the feasibility of defining an NAM toolbox for lung toxicity assessment using two commercial 3D reconstructed human lung models to represent the upper and lower respiratory tract, namely MucilAir™-HF and EpiAlveolar™ systems, respectively. The different bioactivity readouts (from which points of departure, PoDs, are derived) are mixture of readouts directly mapped into the AOPs relevant for lung toxicity (specific) and non-specific bioactivity. To investigate the feasibility of these assays to provide protective PoDs and bioactivity exposure ratio (BER) estimates, a panel of benchmark chemicals, selected based on historical safety decisions and covering several human exposure scenarios (e.g., consumer goods products and occupational use scenarios), was tested.

2) Human-relevant strategy for selecting NAMs for lung toxicity NGRA

Eleven benchmark chemicals (**Table 1**) were tested, including inhaled materials and drugs that may cause lung toxicity following systemic exposure, covering 14 human exposure scenarios classified as low or high risk based on historical safety decisions. Directly mapped onto the AOPs relevant for lung toxicity and non-specific bioactivity, different readouts, including tissue integrity and functionality, cytokine/chemokine secretion, and transcriptomics, were investigated through a 12-day repeated exposure scenario in MucilAir™-HF and EpiAlveolar™ systems (**Fig. 1**). For calculation of BERs, the PoDs derived from the substances-induced bioactivity were combined with human exposure estimates that were obtained using multiple path particle dosimetry (MPPD) exposure modelling or literature maximum plasma concentration (C_{max}) (**Fig. 2**).

Table 1. 11 Benchmark chemicals: exposure scenarios, that are associated either with no effects in humans or have been reported to cause adverse respiratory effects.

11 benchmark chemicals investigated in **14 human low- or high risk exposure scenarios**

No.	Reference Material	Risk classification	Risk classification reasoning	Product
1	BE PVM/MA	Low	Safe use in cosmetic products	Hair spray
2	Coumarin	Low	Safe use in cosmetic products	Anti-perspirant
3	Acrylate copolymer	Low	Safe use in cosmetic products	Hair spray
4	Amorphous silica	Low	Safe use in cosmetic products	Anti-perspirant
		Low	Safe under recommended exposure limit	Occupational scenario
5	Carboxymethylcellulose sodium salt (CMC)	Low	Safe use in nasal sprays	Nasal spray
6	Benzalkonium chloride (BAC)	Low	Safe use in nasal sprays/ophthalmic products	Nasal spray
		Low	Safe use in homecare products	Cleaning spray
7	Crystalline silica	Low	Safe under permissible exposure limit	Occupational scenario
		High	Silicosis after cumulative exposure	Occupational scenario
8	Polyhexamethyleneguanidine phosphate (PHMG)	High	Serious adverse lung effects	Humidifier
9	Akemi	High	Acute lung toxicity	Tile coating product
10	Doxorubicin	High	Interstitial lung disease in cancer patients	Therapeutic dose
11	Amiodarone	High	Alveolar/interstitial pneumonitis with a subacute onset	Therapeutic dose

Tested in MucilAir™-HF only – Tested in EpiAlveolar™ only - Tested in both tissue models

Fig. 1. MucilAir™-HF and EpiAlveolar™ systems **(A)**, 3D reconstructed human lung models that represent the upper and lower respiratory tract, respectively, were used via a 12-day repeated exposure scenario **(B)**.

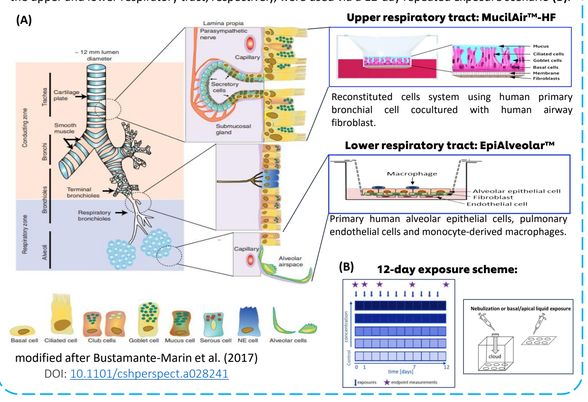
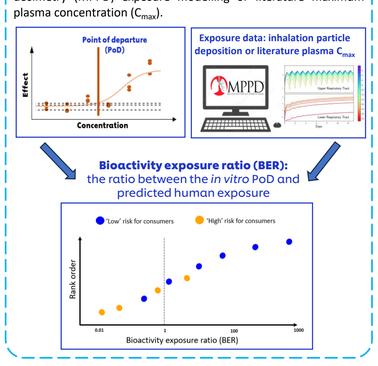


Fig. 2. Calculation of BERs. The PoDs derived from the chemicals-induced bioactivity were combined with human exposure estimates that were obtained using multiple path particle dosimetry (MPPD) exposure modelling or literature maximum plasma concentration (C_{max}).



The selection criteria of the tissue models (**Fig. 1**) involved the following:

- *In vivo*-like exposure to pulmonary toxicants: air liquid interface (ALI) exposure via 12-day exposure scheme
- Allows repeated exposure
- Stable tissue system that physiologically recapitulates many aspects of the human respiratory epithelium
- Allows measurement of biomarkers of relevant AOPs:

MucilAir™-HF
- ✓measurement for mucolytic activity and inflammation (AOP 148, 411, 424 &425)

EpiAlveolar ™
- ✓measurement for oxidative stress, fibrosis and inflammation co-culture of cells including immune competent cells/macrophages and fibroblast (AOP 173,1.25, 303,302)

3) Effects of benchmark chemicals in the lung tissue models

Main results obtained when MucilAir™-HF and EpiAlveolar™ models were exposed daily to benchmark chemicals, in three different exposure methods (aerosol, apical and/or basal liquid), over a 12-day experimental period. Several bioactivity readouts were investigated, including: measurements for tissue integrity loss (TEER) and functionality (mucociliary clearance, MCC; cilia beating frequency, CBF; and mucin secretion), cytokine/chemokine secretion with focus on those proteins involved in the inflammation (CCL2, CCL7, CCL26, CXCL10, CXCL11, ICAM-1, IL-1α, osteopontin, IFN-γ, TNF-α, IL-6, and IL-8), degradation of extracellular matrix/fibrosis (MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, TIMP-1, uPAR, uPA, serpin E1, and TGF-β1) and anti-inflammatory (IL-1ra) responses.

Upper respiratory tract: MucilAir™-HF

Test materials[1]	Exposure time/day	Tissue barrier integrity loss	Tissue functionality: Increased mucin secretion	Tissue functionality: Reduced CBF	Tissue functionality: Reduced MCC	Tissue functionality: Increased Muc5AC protein	Modulation of cytokines and chemokines: Inflammatory modulation	Modulation of cytokines and chemokines: Extracellular matrix/fibrosis modulation	Modulation of cytokines and chemokines: Anti-inflammatory modulation
Akemi[Ar]	30 min	×	×	×	×	×	×	×	×
	6 h	×	×	×	×	×	✓	✓	×
Acrylate copolymer[Ar]	30 min	×	×	×	×	×	✓	✓	×
	6 h	×	×	×	×	×	✓	✓	×
BAC[AL]	30 min	✓	×	×	×	×	✓	✓	✓
BE PVM/MA[AL]	30 min	×	×	×	×	×	✓	✓	×
	6 h	✓	×	✓	×	×	✓	✓	✓
CMC[Ar]	30 min	×	×	×	✓	×	×	×	×
Coumarin[Ar]	30 min	×	✓	×	×	×	×	×	×
	6 h	×	×	×	×	×	×	×	×
PHMG[Ar]	30 min	×	×	×	×	×	✓	×	✓
	6 h	×	✓	×	×	×	✓	×	✓

Lower respiratory tract: EpiAlveolar™

Test materials[1]	Tissue barrier integrity loss		Modulation of cytokines and chemokines: Inflammatory modulation		Modulation of cytokines and chemokines: Extracellular matrix/fibrosis modulation		Modulation of cytokines and chemokines: Anti-inflammatory modulation		Changes in GSH and GSSS levels[2]	Mitotoxicity[2]
	Lab 1	Lab 2	Lab 1	Lab 2	Lab 1	Lab 2	Lab 1	Lab 2	Lab 1	Lab 1
Akemi[Ar]	×	✓	×	✓	×	✓	×	✓	×	✓
Crystalline silica[Ar]	×	×	×	×	×	✓	×	×	×	✓
Amorphous silica[Ar]	×	✓	×	✓	×	✓	×	×	×	×
PHMG[Ar]	✓	✓	✓	✓	✓	✓	✓	✓	×	✓
Amiodarone[BL]	×	×	✓	✓	×	✓	×	×	×	×
Doxorubicin[BL]	×	✓	✓	✓	✓	✓	✓	×	×	×

The symbols × and ✓ absence or presence of bioactivity induced by the related chemical, respectively.
[1]Tissues were exposed to test materials via aerosol ([Ar]), apical liquid ([AL]) and/or basal liquid ([BL]) application.
[2]This readout was investigated by Laboratory 1 only.

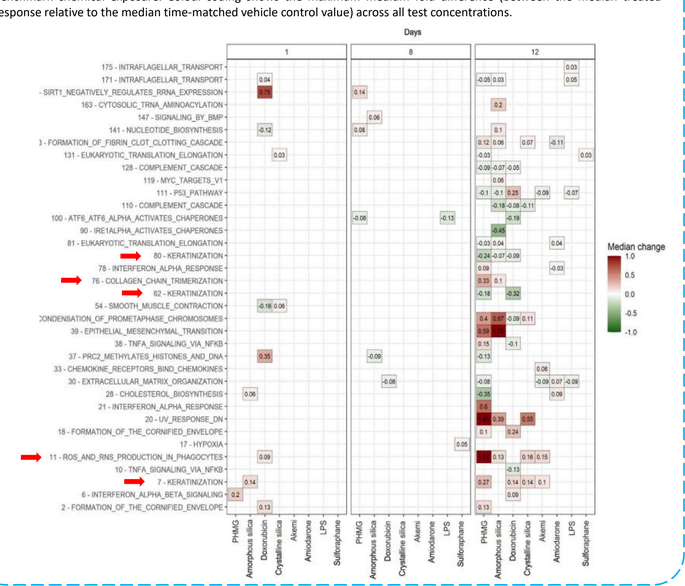
4) Transcriptomics is useful to elucidate mechanism of toxicity in the EpiAlveolar model

Here, we explored the potential utility of transcriptomics as a technology, not only for establishing a PoD but also for gaining mechanistic insights to generate hypotheses within the context of a risk assessment framework. Therefore, we set out to investigate if, by using this type of analysis, the mechanisms of lung toxicity (especially pulmonary fibrosis) associated with the benchmark chemicals could be identified. Figure 3 displays the latent variables (LVs), through Pathway-level information extractor method (Basili et al., 2022, DOI: 10.1021/acs.chemrestox.1c00444), that showed significant concentration and time-dependent responses after benchmark chemical exposure relative to the vehicle control. The number of LVs altered increased over time, with maximum effects observed at day 12 for all chemicals.

- **Pathway-level information extractor (PLIER) method[1]:**
  - ✓ Calculation of a transcriptomics POD
  - ✓ identifying patterns of co-regulated genes associated with biological knowledge (latent variables, LVs)
- Most of the LVs modulated by **PHMG, Amorphous silica, and Doxorubicin** captured **biological activity corresponding to the key factors leading to pulmonary fibrosis**:
  - ✓ inflammation, oxidative stress, epithelial mesenchymal transition which ultimately leads to excessive deposition of extracellular matrix.

In a risk assessment context this information would suggest that these chemicals could cause **pulmonary fibrosis *in vivo*** and would warrant further investigation

Fig. 3. Figure displays the latent variables (LVs) that showed significant concentration- and time-dependent response after benchmark chemical exposure. Colour-coding shows the maximum medium fold difference (between the median treated response relative to the median time-matched vehicle control value) across all test concentrations.

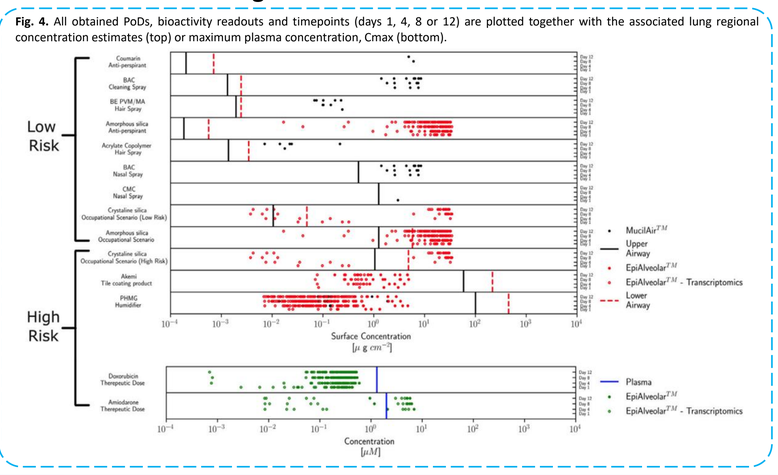


5) In general, for high-risk exposure-chemical scenarios *in vitro* PoDs were lower than the predicted exposure

Comparison of human internal exposure (upper/lower respiratory tract or plasma) and in vitro PoDs per benchmark chemical using MucilAir™-HF or EpiAveolar™ models are shown in the **Fig. 4**.

- The obtained PoDs were combined with exposure estimates to calculate BER values
- BER is able to separate the low- and high-risk benchmark exposure scenarios for 12 out of the 14 scenarios
  - ✓**Low-risk:** PoDs occurred at higher concentrations than the corresponding human exposure values. **Except:** crystalline and amorphous silica occupational scenarios
  - ✓**High-risk:** clear overlap between the PoDs and human exposure (lung deposited mass or Cmax)

Fig. 4. All obtained PoDs, bioactivity readouts and timepoints (days 1, 4, 8 or 12) are plotted together with the associated lung regional concentration estimates (top) or maximum plasma concentration, Cmax (bottom).



6) Defining a safe threshold: animal testing *versus* non-animal NAMs

Risk assessments for human inhalation toxicity based on traditional animal studies generally include a safety factor of 25 (ECHA, 2012). Therefore, a margin of safety over 25 compared to no observed adverse effects levels in animals has been judged to be protective for human health for several decades regarding local lung effects:

Traditional Margin of Safety ($MoS_{animal\ data}$ for local lung effects) > 25* → **low risk**
*Uncertainty safety factor of 25 to account for uncertainties related to interspecies (animal-to-human: 2.5-safety factor) and inter-individual (human-to human: 10-safety factor) variabilities[1]

Defining a safe BER threshold or the appropriate use of uncertainty factors remains a challenge in NGRA. A recent regulatory example, accepted by the US EPA (2021), of a non-animal risk assessment for the fungicide chlorothalonil in an occupational scenario combined in vitro PoDs from MucilAir™ readouts with dosimetry information obtained from a computational fluid-particle dynamics (CFPD) model. In this specific case, the total uncertainty safety factor, to account the response among human population, was 3 considering inter-individual toxicodynamic variability only.

In vitro Bioactivity Exposure Ratio ($BER_{NAM\ data}$) > 3 → **low risk (?)**
*Uncertainty safety factor of 3 applied in the chlorothalonil acute inhalation risk assessment to cover potential variation in sensitivity among human population (intraspecies)[2]
$BER_{NAM\ data}$ > 3 would be protective for all benchmark chemicals, particularly driven by the transcriptomics PoDs for the high-risk exposure scenarios, e.g., Amiodarone and Crystalline silica

Amiodarone - high risk therapeutic dose

Day	Min PoD	Biomarker	BER	Risk
4	6.95	Cytokine: MMP-1 (Lab 2)	3.47	Low
	0.0084	Transcriptomics: LV30	0.0042	High
8	1.31	Cytokine: ICAM-1 (Lab 1)	0.65	High
	5.20	Cytokine: ICAM-1 (Lab 2)	2.60	High
	0.0084	Transcriptomics: LV30	0.0042	High
12	0.97	Cytokine: ICAM-1 (Lab 1)	0.48	High
	5.03	Cytokine: ICAM-1 (Lab 2)	2.51	High
	0.0083	Transcriptomics: LV30	0.0041	High

Crystalline silica – high risk occupational scenario

Day	Min PoD	Biomarker	BER	Risk
1	0.032	Transcriptomics: LV131	0.071	High
4	0.0075	Transcriptomics: LV110	0.0041	High
8	34.53	Cytokine: MMP-7 (Lab 2)	11.14	Low
	0.0037	Cytokine: LV110 (Lab 2)	0.0012	High
12	30.51	Cytokine: MMP-7 (Lab 2)	6.32	Low
	0.0042	Transcriptomics: 110	0.00087	High

7) Concluding remarks

- Strategy of selecting **non-animal NAMs informed by AOPs** associated with pulmonary toxicity can provide relevant biological coverage
- **Further evaluation of the performance of NAM toolbox can build confidence in the protectiveness of the approach**: testing a wider substance dataset with varied mechanisms of action, uses, and balanced low and high-risk benchmarks
- There is a need to **establish scientific confidence** by improving the reproducibility, standardization of protocols, and *in vitro* culture methodologies
- **Benchmarking decision outcomes provides an alternative to the traditional validation of NAMs:** apical effects in rodent studies *versus* NAMs in the context of making protective safety decisions

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[1]ECHA (2012). Guidance on information requirements and chemical safety assessment: chapter R.8: characterisation of dose [Concentration]-Response for human health.

[2]EPA (2021). Document ID: EPA-HQ-OPP-2011-0840-0080. Available at https://www.regulations.gov/document/EPA-HQ-OPP-2011-0840-0080

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