Development and Validation of in vitro Human Inhalation Toxicity Tests for Volatile Liquids, Mists, and Sprays

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Introduction

In vivo animal models are currently accepted by regulatory authorities for acute respiratory toxicity (ART) testing. However, animal tests have been discredited as predictors of human responses on physiological and ethical grounds. The goal of this work was to develop physiologically relevant ART tests utilizing the EpiAirway™ tissue model, to demonstrate correlation to OECD accepted GHS categorization, and investigate interlaboratory reproducibility.

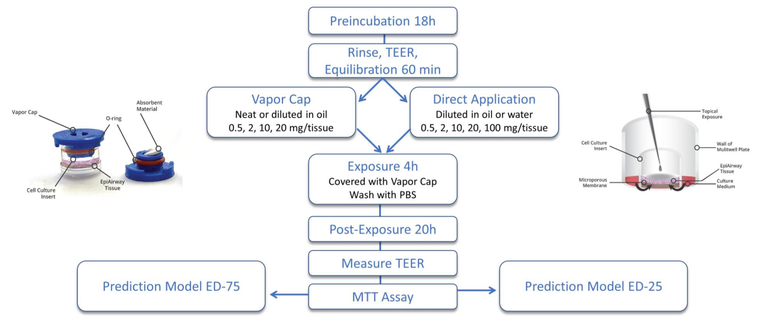
Test articles (TA, n=53) were applied to EpiAirway tissues produced at MatTek (USA) and IVLSL (Slovakia) with two ART protocols, the Direct Application Protocol (DAP) for exposure to mists/sprays, and the Vapor Cap Protocol (VCP) for exposure to vapors/volatile liquids. In both protocols, tissues were exposed for 4h to 4 fixed doses (diluted in corn oil or water) to mimic in vivo rat exposure; followed by 20h post-incubation. The effects on tissue viability (MTTassay) and barrier properties (Transepithelial Electrical Resistance, TEER) were determined. The effective doses which reduced tissue viability by 25% (ED-25) or by 50% (ED-50) were mathematically interpolated for the DAP and VCP methods, respectively, and correlated to the GHS categories.

In the DAP, TAs were applied to the apical surface. Using the MTT assay, the DAP discriminated between GHS Cat.1&2/3&4/5 & NC with a Sensitivity/Specificity/Accuracy (S/S/A) of 63.5/76.1/69.8% (MatTek) and 63.8/76.1/70.0%(IVLSL). Utilizing the changes in TEER, the DAP discriminated between GHS categories with a S/S/A of 65.9/76.7/71.3% (MatTek) and 64.1/76.6/70.3% (IVLSL). The correlation coefficient between the two laboratories was R2=0.91 for MTT and 0.76 for TEER.

In the VCP, TAs were applied to an absorbent material in a special cap that forms a tight seal above the tissue allowing exposure to TA vapor. Using the MTT assay, the VCP discriminated between GHS categories with S/S/A of 70.8/83.2/77.0 (MatTek) and 71.9/83.2/77.5% (IVLSL). Utilizing the changes in TEER, the VCP discriminated between GHS categories with a S/S/A of 64.4/78.5/71.5 (MatTek) and 67.1/80.1/73.6 (IVLSL). The correlation coefficient between the laboratories was R^2=0.96 for MTT and 0.93 for TEER.

UN GHS Acute Toxicity Estimate (ATE) values and categories					
Exposure route	**Category 1**	**Category 2**	**Category 3**	**Category 4**	**Category 5**
Oral (mg/kg bodyweight)	ATE ≤ 5	5 < ATE ≤ 50	50 < ATE ≤ 300	300 < ATE ≤ 2000	2000< ATE≤ 5000
Dermal (mg/kg bodyweight)	ATE ≤ 50	50 < ATE ≤ 200	200 < ATE ≤ 1000	1000 < ATE ≤ 2000	
Gases (ppmV)	ATE ≤ 100	100 < ATE ≤ 500	500 < ATE ≤ 2500	2500 < ATE ≤ 20000	2000< ATE≤ 5000
Vapours (mg/l)	ATE ≤ 0.5	0.5 < ATE ≤ 2.0	2.0 < ATE ≤ 10.0	10.0 < ATE ≤ 20.0	
Dusts and Mists (mg/l)	ATE ≤ 0.05	0.05 < ATE ≤ 0.5	0.5 < ATE ≤ 1.0	1.0 < ATE ≤ 5.0	

Figure 1: Acute Toxicity Classifications. GHS Hazard statements are from https://pubchem.ncbi.nlm.nih.govand GHScriteria for acute toxicity is from:https://www.chemsafetypro.com/Topics/GHS/GHS_classification_criteria_acute_toxicity_category.html



Vapor Cap: ED-50					Direct Application: ED-25				
PM-A: GHS Classification			PM-B: I/NI		PM-A: GHS Classification			PM-B: I/NI	
	mg/tissue	mg/cm2		mg/tissue mg/cm2		mg/tissue	mg/cm2		mg/tissue mg/cm2
Cat.1&2	≤ 2.5	≤ 4.2	I	< 10 < 16.7	Cat.1&2	≤ 5	≤ 8.3	I	< 20 < 33.3
Cat.3&4	2.5 – 15	4.2 – 25			Cat.3&4	5 – 20	8.3 – 33.3		
Cat.5&NC	≥ 15	≥ 25	NI	≥ 10 ≥ 16.7	Cat.5&NC	≥ 20	≥ 33.3	NI	≥ 20 ≥ 33.3

Figure 2: Vapor Cap and Direct Application Protocols. Schematics of the procedures and prediction models.

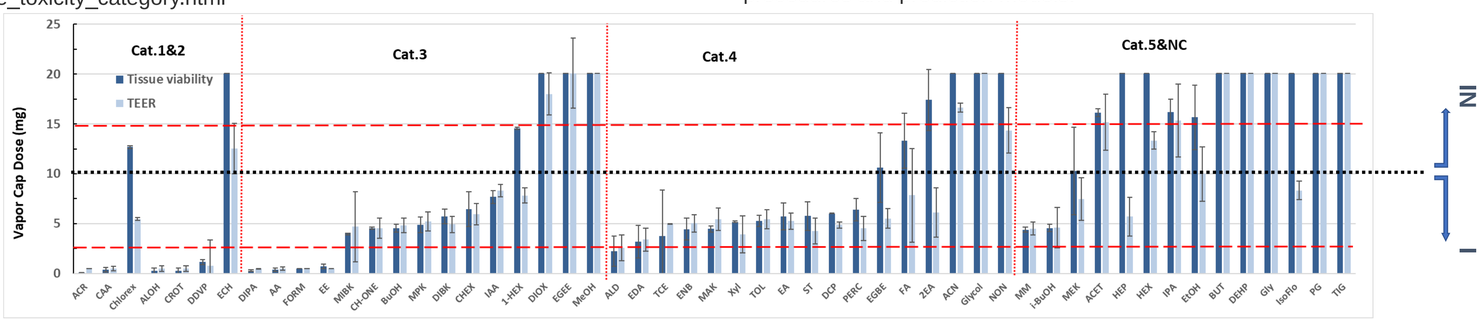


Figure 3: Vapor Cap Protocol. Correlation to the GHShazard classification (tested at MatTek Life Sciences). End points: Tissue viability and barrier function. Cutoffs: VCP PM-A (horizontal dashed red lines)and PM-B (horizontal dottedblack line).
Note: Vapor Pressure of underpredicted chemicals from Cat.1&2(at 25°C): Chlorex–1.6 mmHg; 2-chloroethanol –4.9mmHg.

VCP PM-A (red):		VCP PM-B (blue):	
ED-50 < 2.5mg	-> Cat.1&2	ED-50 < 10mg	-> Cat.1-4 (I)
2.5mg ≤ ED-50 < 15mg	-> Cat.3&4	ED-50 ≥ 10mg	-> Cat.5&NC (NI)
ED-50 ≥ 15mg	-> Cat.5&NC		

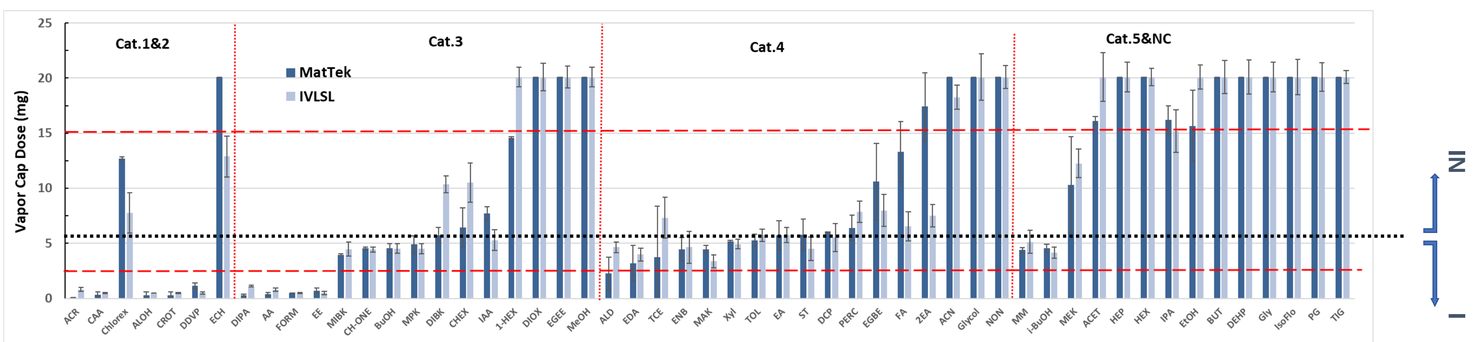


Figure 4: Inter-laboratory Validation of the Vapor Cap Protocol. Tissue Viability: Correlation to the GHS hazard classification (VCPPM-A [horizontal dashed red lines] and PM-B [horizontal dotted black line]). The concordance correlation coefficient between two laboratories for the tissue viability is R^2=0.96 and for the tissue barrier is R^2=0.93 (not shown).

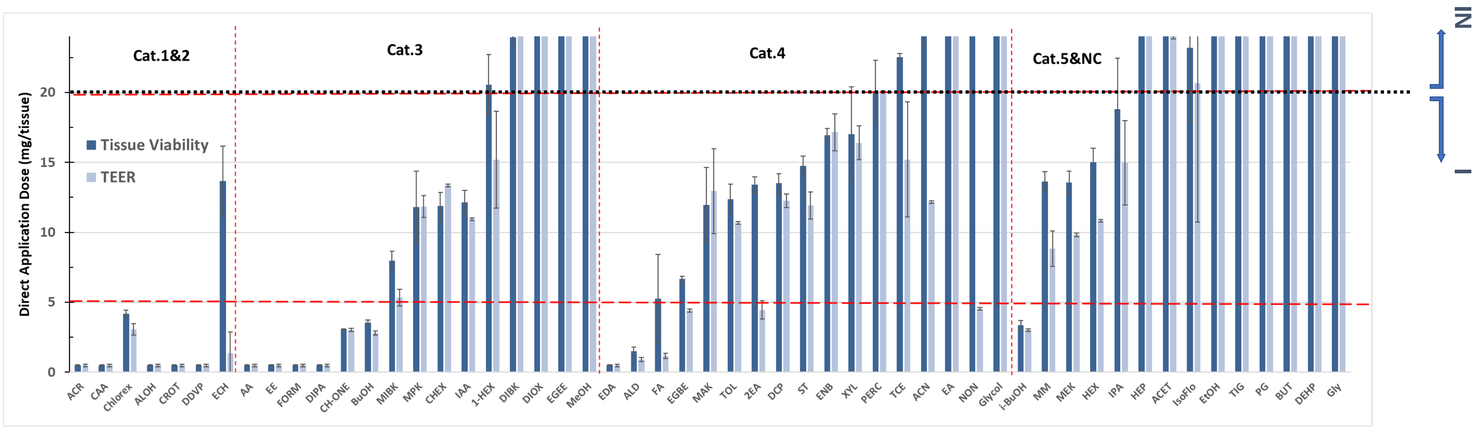


Figure 5: Direct Application Protocol. Correlation to the GHS hazard classification (tested at MatTek Life Sciences). Endpoints: Tissue viability and barrier function. Cutoffs: DAPPM-A (horizontal dashed red lines) and DAP PM-B (horizontal dotted black line).

VCP PM-A (red):		VCP PM-B (blue):	
ED-25 < 5mg	-> Cat.1&2	ED-25 < 20mg	-> Cat.1-4 (I)
5mg ≤ ED-25 < 20mg	-> Cat.3&4	ED-25 ≥ 20mg	-> Cat.5&NC (NI)
ED-25 ≥ 20mg	-> Cat.5&NC		

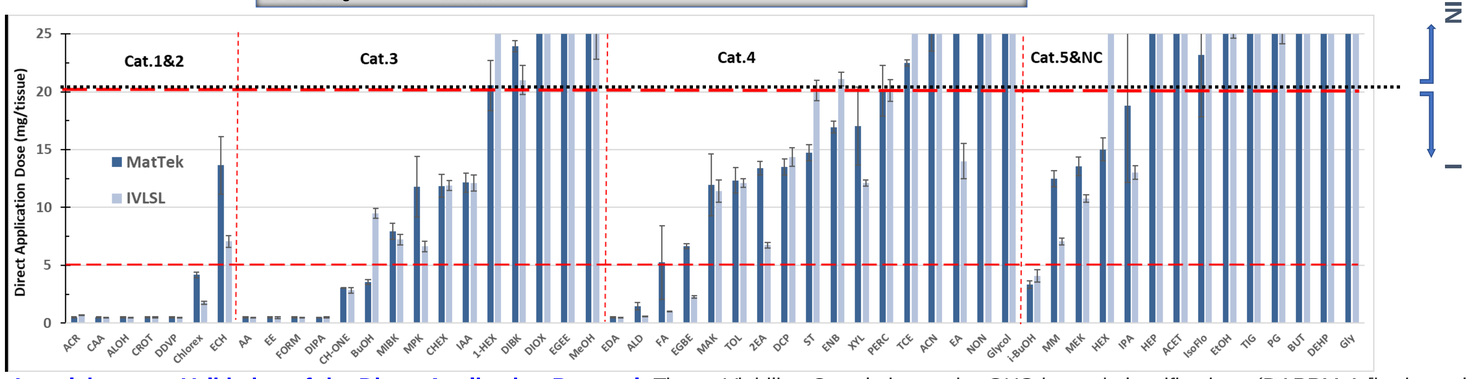


Figure 6: Inter-laboratory Validation of the Direct Application Protocol. Tissue Viability: Correlation to the GHS hazard classifications (DAPPM-A [horizontal dashed red lines] and DAPPM-B [horizontal dotted black line]). The concordance correlation coefficient between two laboratories for the tissue viability is R^2=0.88 and for the tissue barrier is R^2=0.76 (not shown).

Methods

Tissue model: EpiAirway™ is an *in vitro* reconstructed *in vivo*-like tracheobronchial tissue model (MatTek Corporation) produced using primary human bronchial epithelial cells under GMP conditions. The tissue is grown at the air-liquid interface, develops pseudostratified morphology and a functional barrier, and comprises multiple cell types, including basal, club, ciliated, and goblet cells.

Vapor Cap Protocol: This protocol utilized vapor caps fitted with an absorbent material (filter/ glass fiber disc) and a silicone ring (to create a tight seal). Test chemicals were applied onto the filter. The total volume was calculated to deliver 20 mg/tissue of neat material; the same volume was applied for all doses (diluted in corn oil) to deliver 0.5, 2, 10, and 20 mg/tissue. Tissue viability was assessed using the MTT assay (following a post-exposure incubation). A dose-response curve was constructed and the ED-75 (dose/tissue required to reduce tissue viability by 75% relative to NC) was calculated by mathematical interpolation.

Direct Application Protocol: Test chemicals were diluted in water or corn oil prior to the application. 0.1 mL of four dilutions was applied to the apical surface of the EpiAirway tissues to deliver 0.5, 2, 10, 20, and 100 mg/tissue. A dose-response curve was constructed and the ED-25 (dose/tissue required to reduce tissue viability by 25% relative to NC) was calculated by mathematical interpolation.

Tissue viability assay (MTT assay) was determined by colorimetric MTT assay, a yellow tetrazole that is reduced to purple formazan by the mitochondria of metabolically active, living cells. Purple formazan crystals were extracted and analyzed spectrophotometrically at 570 nm. *%Viability =OD sample / OD NC x 100, where OD sample*

Transepithelial Electrical Resistance (TEER): TEER was measured with an EVOMX volt-ohmmeter and an EndOhm-12 chamber (World Precision Instruments). EpiAirway tissues with TEER over 300 Ω*cm2 were used in the study. Relative tissue barrier was determined using the following equation: %TEER =TEER sample / TEER NC x 100.

Vapor Cap Protocol ED-50	VCP PM –A: GHS classification				VCP PM –B: I/NI			
	MatTek	IVLSL	MatTek	IVLSL	MatTek	IVLSL	MatTek	IVLSL
End Point	Tissue Viability	TEER	Tissue Viability	TEER	Tissue Viability	TEER	Tissue Viability	TEER
Sensitivity (%)	70.8	64.4	71.9	67.1	79.5	82.1	82.1	84.6
Specificity (%)	83.2	78.5	83.2	80.1	78.6	64.5	78.6	64.3
Accuracy (%)	77.0	71.5	77.5	73.6	79.0	73.2	80.3	74.5
Positive predictivity (%)	63.7	58.3	65.8	60.0	91.2	56.5	91.4	86.8
Negative predictivity (%)	66.9	68.3	68.8	68.4	57.9	56.3	61.1	60.0

Direct Application Protocol ED-25	VCP PM –A: GHS classification				VCP PM –B: I/NI			
	MatTek	IVLSL	MatTek	IVLSL	MatTek	IVLSL	MatTek	IVLSL
End Point	Tissue Viability	TEER	Tissue Viability	TEER	Tissue Viability	TEER	Tissue Viability	TEER
Sensitivity (%)	63.5	65.9	63.8	64.1	71.8	82.1	69.2	71.8
Specificity (%)	76.1	76.7	76.1	76.6	64.3	64.3	71.4	64.3
Accuracy (%)	69.8	71.3	70.0	70.3	68.0	73.2	70.3	68.0
Positive predictivity (%)	52.4	53.5	52.1	52.9	84.8	86.5	87.1	84.8
Negative predictivity (%)	52.8	53.0	51.2	48.2	45.0	56.3	45.5	45.0

Summary

1. Both protocols, Vapor Cap and Direct Application demonstrated:
   - High predictivity in discrimination of respiratory Irritants/Non-irritants (>90%)
   - High accuracy in discrimination of respiratory Irritants/Non-irritants (>80%)
   - High inter-laboratory reproducibility for EpiAirway produced at MatTek Life Sciences (USA) and MatTek IVLSL (Slovakia);
   - Enable high throughput screening
2. Application of test materials dissolved or resuspended in water or oils (DAP) is suitable for exposure to dust and mists. Exposure to evaporated test materials within the sealed cell culture insert (VCP) is suitable for volatile materials.
3. For both methods,VCP and DAP:
   - PM-A can discriminate between hazard categories (Cat.1&2/3/4/5&NC);
   - PM-B can discriminate between respiratory Irritants (I) and Non-irritants (NI).
4. Both protocols provide robust and efficient, physiologically relevant, organ-specific in vitro tests that can improve the predictivity of human responses, reduce the number of animals being used to assess respiratory toxicity, and help establish confidence for regulatory applications.