Human In Vitro Models for Respiratory Toxicology: Evaluation of Goblet Cell Hyperplasia, Mucus Production, and Ciliary Beating Assays

S. Frentzel[1], M. Aragon[2], J. Hoeng[1], S. Ito[3], S. Ishikawa[3], J. Budde[4], A. Maione[5], P. Hayden[5], W. Fields[6], B. Keyser[6], L. Haswell[7], D. Azzopardi[7] and H. Behrsing[2].

[1]Phillip Morris International, Neuchatel, Switzerland; [2]IIVS, Inc., Gaithersburg, MD; [3]Japan Tobacco, Yokohama, Japan; [4]ITL-Reemtsma Cigarettenfabriken GmbH, Hamburg, Germany; [5]MatTek, Inc., Ashland, MA; [6]RAI Services Company, Winston-Salem, NC and [7]British American Tobacco, R&D, Southampton, United Kingdom.

ABSTRACT

Robust non-animal models and assays for pulmonary toxicology are required to make competent product development and risk assessments for new materials requiring toxicity testing. Three *in vitro* assays (goblet cell hyperplasia [GCH], ciliary beat frequency [CBF], and MUC5AC quantitation) were evaluated for performance and reproducibility. To assess these assays, 6 laboratories contributed data using a common protocol utilizing IL-13 as an inducer of adverse mucociliary-relevant tissue changes. MatTek EpiAirway™ and Epithelix MucilAir™ 3D tissue models were used to evaluate endpoints using histology for GCH, software-based applications, Cilia FA and SAVA, for CBF, and ELISA assay for MUC5AC. Continuous 10 ng/mL IL-13 (GCH, MUC5AC) exposures or one hour 10 µM procaterol (CBF) exposures prior to day 7 and 14 time-points were included as positive controls. Quality control endpoints (e.g. adenylate kinase tissue content and trans-epithelial electrical resistance) were also evaluated. Multi-fold increases (ranging from 2.6 to 33-fold, and 1.5 to 238-fold) in MUC5AC-stained goblet cells were measured in both tissue models after exposure with IL-13 after 7 and 14 days induction, respectively. For CBF, procaterol caused a significant increase, and IL-13 elicited a significant decrease as expected. However, the MUC5AC ELISA did not yield consistent results when frozen apical rinse samples were thawed and assayed. These results suggest these non-animal test systems may provide consistent, human-relevant data corresponding to key events involved in respiratory disease. A streamlined protocol using these controls will be applied toward additional testing. These assays, utilized in a pragmatic manner with other *in vitro* assays have the potential to be included in a Reduced Risk Product assessment framework.

INTRODUCTION

The Family Smoking Prevention and Tobacco Control Act of 2009 established the FDA Center for Tobacco Products (CTP) and gave the agency regulatory authority over the marketing, manufacture and distribution of tobacco products, including those termed "modified risk". On December 8-10, 2014, IIVS organized a workshop conference, "Assessment of *in vitro* COPD Models for Tobacco Regulatory Science" to bring together stakeholders representing regulatory agencies, academia, industry and animal protection to address the research priorities articulated by the FDA CTP. Specific topics were covered to assess the status of current *in vitro* technologies as they are applied to understand the adverse pulmonary events resulting from tobacco product exposure. The four topics covered were, 1) Inflammation and Oxidative Stress, 2) Ciliary Dysfunction and Ion Transport, 3) Goblet Cell Hyperplasia and Mucus Production and 4) Parenchymal/bronchial Tissue Destruction and Remodeling. Breakout group sessions were held for three of the four core topics and were intended for consolidating current views on assay endpoints, test systems, and related technologies that should be considered for standardization, and identifying areas that require additional research and/or development. Conclusions drawn from the breakout groups resulted in three *in vitro* assays; GCH, mucus production, and CBF being identified that have merit for further exploration.

To evaluate these assay-endpoints, IIVS held a technical workshop in June of 2015. A group of assay-specific experts met to discuss technical considerations for the three assays under review. Utilizing the most promising technical approaches, a common protocol was developed to test inter-lab reproducibility of assay-endpoint results derived from using two reconstructed airway models of the human respiratory tract. Participating labs included IIVS, Inc., British American Tobacco, R&D (BAT), Japan Tobacco (JT), National Center for Toxicological Research (NCTR), Phllip Morris Intl. (PMI). and ITL-Reemtsma Cigarettenfabriken GmbH (ITL). Multicellular, pseudostratified human reconstructed airways (MatTek's EpiAirway™ and Epithelix's Mucilair™) were exposed to reference materials known to perturb the expression of goblet cells and mucus production, or alter the CBF of these tissues. Results from the 2-week study were compared and discussed. Herein, data from 5/6 labs (those that were able to contribute to this presentation) is shown to highlight the utility of these non-animals methods and assays as a potential means to evaluate test articles, such as next generation tobacco and nicotine products.

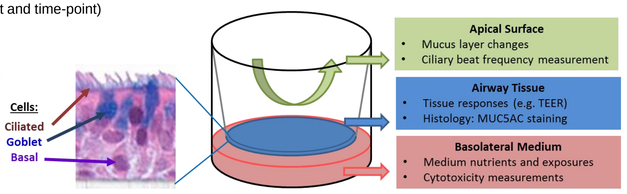
MATERIALS & METHODS

Test System: Human Reconstructed Airway (RHuA) grown at the Air-Liquid-Interface (ALI) (n=5 per treatment and time-point)

MatTek EpiAirway™ and Epithelix Mucilair™

Reference materials:

1. IL-13 (induction of mucous producing goblet cells) 10 ng/mL
2. Procaterol (β2 adrenoreceptor agonist for CBF stimulation) 10 µM



Procedure:

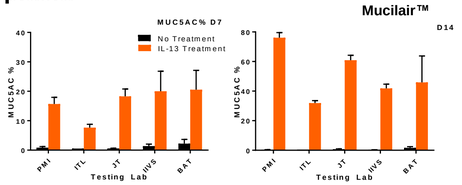
- All laboratories coordinated orders with one or both tissue manufacturer to ensure receiving RHuA from the same donor cells. Cells were obtained from the bronchial region of a healthy donor.
- Labs received vendor QC'd RHuA tissues and allowed 2-3 days (or longer if necessary) of acclimation prior to use. Qualification for use in the study included vendor–specified trans-epithelial electrical resistance (TEER) QC values (MatTek: 300 Ω*cm^2; Epithelix: 200 Ω*cm^2). Following the apical rinse, adenylate kinase (leakage marker) was measured in the basolateral medium and apical rinse as additional QC assessment.
- On the following day (day 0), CBF measurements (10x objective, 4 fields/tissue) were made at room temperature (RT) or 37ºC using using SAVA and/or CiliaFA software. Subsequent CBF readings were taken when tissues were re-fed.
- Tissues were re-fed on Monday, Wednesday, and Friday. Treatments groups included 1) untreated (medium only) or 2) medium + IL-13.
- At days 7 (1st set of tissues) and 14 (2nd set of tissues), select treatment groups received 10 µM procaterol (in basolateral medium) 1 hr before CBF measurements were made. At harvest, all tissues had an apical rinse collected (and stored at ≤ -60ºC) for MUC5AC quantification.
- Harvested tissues for histology were fixed in formalin and sent to the respective manufacturers for processing, MUC5AC staining and quantitation of % MUC5AC (+) cells.
- Samples collected for MUC5AC quantitation were stored frozen until thawed for analysis using an ELISA assay (e.g. CUSABIO (Code: CSB-E10109h, Uniprot NO.: 98088)) for human mucin-5AC.

RESULTS

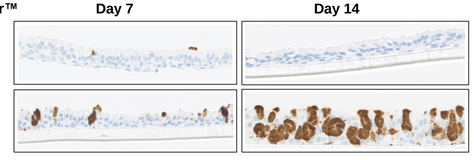
Goblet Cell Hyperplasia

MUC5AC staining (Goblet) Cells (% of all cells)

	Epithelix MucilAir				MatTek EpiAirway			
	Control		IL-13		Control		IL-13	
	D7	D14	D7	D14	D7	D14	D7	D14
IIVS	1.37	0.30	20.02	41.81	3.18	2.30	15.15	10.12
PMI	0.85	0.32	15.67	76.08	2.10	2.10	13.46	15.75
BAT	2.22	1.64	20.56	45.93	*not tested*			
JT	0.54	0.63	17.84	61.55	*not tested*			
ITL	0.54	0.53	7.64	31.93	9.47	24.57	24.46	37.45



- IL-13 treatment induced GCH for all laboratories; albeit, the level of induction varied between labs and for both tissue manufacturers.
- Different levels of baseline goblet cell expression greatly impact fold-induction calculations when assessing IL-13 treatment



RESULTS cont.

Mucus Production (MUC5AC)

MUC5AC ELISA (IIVS)

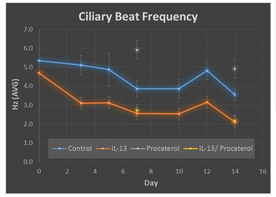
Harvested	Treatment	Rinse Day	EpiAirway™ AVG	EpiAirway™ SD	Mucilair™ AVG	Mucilair™ SD
D7	Untreated	7	6.5	1.2	28.7	6.7
D7	IL-13	7	4.5	1.7	24.6	2.5
D14	Untreated	7	7.8	3.7	15.6	2.8
D14	IL-13	7	3.9	1.3	12.4	3.3
D14	Untreated	14	6.3	0.8	6.3	0.8
D14	IL-13	14	5.0	1.1	5.0	1.1

- MUC5AC measurements were mostly at the limit of detection (LOD).
- Non-LOD measurements do not reflect anticipated exposure-specific changes.

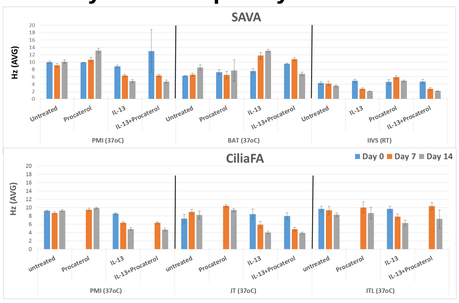
- MUC5AC ELISA did not yield interpretable results for any labs.
- The assay was considered unsuccessful and several possible explanations were entertained:
  - Lack of homogeneous sample mixing
  - Use of frozen stored (≤ -60ºC) samples (whereby freezing compromised the sample integrity)
  - Lack of goblet cell MUC5AC secretion

Ciliary Beat Frequency

IIVS SAVA data



- As expected, IL-13 treatment reduces CBF, while procaterol increases CBF



- A general comparison of SAVA and Cilia FA applications was made. (representative data from five labs)
- Labs generating CiliaFA data at 37ºC noted that measurements above ~10 Hz were erroneous.
- SAVA measurements appeared consistent without issues at higher Hz CBF measurements.

CONCLUSIONS

The Inter-lab comparison of assay data using a common protocol identified strengths and weaknesses of all three endpoints.

- **GCH:** Both commercially available tissues were responsive to IL-13 induction of goblet cell hyperplasia. Induction of GCH was variable across labs and across vendors; the latter was possibly due to donor cell properties.
- **MUC5AC:** Despite consistent standard curves by the ELISA manufacturer's kits, all labs had uninterpretable results. The use of ELISAs to quantify MUC5AC from RHuA samples must be revisited, including storage conditions of samples prior to measurement.
- **CBF:** Assessments with the CiliaFA software was problematic with measurements above ~10Hz; however, the SAVA software appeared to perform consistently. RHuA displayed different levels of % Active Area (motile points).

Additional testing of technical facets related to assay endpoints will allow optimization of testing procedures. Elements include: the number of CBF fields required per insert, ELISA performance for RHuA sampling, impact of RHuA acclimation length, etc.

REFERENCES

- Bérubé K, Aufderheide M, Breheny D, et al. (2009) In vitro models of inhalation toxicity and disease. The report of a FRAME workshop. Alternatives to Laboratory Animals 37(1):89–141.
- Adamson, J., L. E. Haswell, et al. (2011). In Vitro Models of Chronic Obstructive Pulmonary Disease (COPD). Bronchitis. D. I. MartÃn-Loeches, British American Tobacco.

ACKNOWLEDGMENTS

The authors thank the following groups for their help, contributions and support:

- Altria Client Services (including the abstract and poster review by Dr. Mike Oldham) and RAI Services Company for their contribution to support IIVS' laboratory work.