Cancun • Mexico
CORESTA
SSPT 2023

SMOKE SCIENCE and PRODUCT TECHNOLOGY Conference

SYMPOSIUM

New Approach Methods (NAMs)-II: Applications in Tobacco Regulatory Sciences

10 October 2023

New Approach Methods (NAMs) Symposium-II:
Applications in Tobacco Regulatory Sciences

Dear SSPT 2023 participants,

It is a great pleasure to introduce the New Approach Methods (NAMs) Symposium-II, in a beautiful city of Cancun in Mexico. This year's Symposium is the follow-up from the first Symposium held during the SSPT2021 Conference online, titled "Advancing New Alternative Methods for Tobacco Harm Reduction"[1] , where external experts presented the latest case examples where NAMs were used to screen and even waive *in vivo* studies in regulatory applications.

The term NAM is no longer a new vocabulary to many CORESTA participants! If I offer a general definition, NAMs are *in vitro* and in silico or computational-based methodologies in toxicology that enable clinically relevant toxicological risk assessment without needing traditional animal testing. In two years since SSPT2021, the interest and need to utilize NAMs in tobacco science become ever more relevant, with many different categories of smoke-free alternative products being introduced worldwide. Albeit they are developed to be substantially less toxic than conventional cigarettes, currently no consensus is available on how to evaluate these emerging products, where we believe that some of available NAMs offer ways to expedite toxicological screening and decision making.

In this Symposium, we have invited expert toxicologists from industry to share their case examples of applying *in vitro*- and *in silico*-based NAMs to address some of immediate gaps in product screening and assessment (NAM-TODAY; toxicity screening). We then expand into long-term applications of NAMs linking *in vitro* outcomes to clinically relevant disease progression (NAM-TOMORROW; COPD as an example). We have a total of six excellent talks from NAM experts, also the panel discussion at the end, inviting active participation from all attendees.

The Symposium is endorsed by the CORESTA Scientific Commission, the 21st Century Toxicology for Next Generation Tobacco and Nicotine Products (NGTX) Task Force, the Biomarkers (BMK) Sub-Group, and the *In Vitro* Toxicity Testing (IVT) Sub-Group, reflecting CORESTA's ongoing commitment to the 3Rs (reduce, replace, and refine animal testing).

Thank you and we look forward to seeing you all!

K. Monica Lee, PhD, DABT
Symposium Chair

[1] Toxics | Free Full-Text | Advancing New Approach Methodologies (NAMs) for Tobacco Harm Reduction: Synopsis from the 2021 CORESTA SSPT–NAMs Symposium (https://www.mdpi.com/2305-6304/10/12/760)

TUESDAY 10 OCTOBER 2023

New Approach Methods (NAMs) Symposium-II: Applications in Tobacco Regulatory Sciences

Chair: K. Monica LEE
Co-Chair: Liam SIMMS

Starting at 8:50

No.	Time	Titles	Lead, Affiliation
NAM 00 Intro	10 min.	**Introduction: New Approach Methods (NAMs): Applications in Tobacco Regulatory Sciences**	K. Monica LEE (Chair) *Altria Client Services*
Session I: NAM Today			
NAM 01	15 min. + 2 min. Q&A	**The use of NAMs in *in vitro* genotoxicity assessment of nicotine delivery products: ToxTracker assay as a case study**	Robert LEVERETTE *RAI Services Company*
NAM 02	15 min. + 2 min. Q&A	**Connecting exposure, dosimetry and toxicity responses in the preclinical evaluation of ingredients: case examples of flavoring chemicals in oral tobacco products**	Jingjie ZHANG *Altria Client Services*
NAM 03	15 min. + 2 min. Q&A	**Exposure to fresh whole smoke and aerosols: standard and novel (3D) *in vitro* models**	Liam SIMMS *Imperial Brands*
BREAK – 10 minutes			
Session II: NAM Tomorrow (NAMs without *in vivo* – clinical adverse outcomes)			
NAM 04	15 min. + 2 min. Q&A	**EpiAirway Nrf2 – oxidative stress model: Practical application of *in vitro* systems with clinical relevance**	Brian KEYSER *RAI Services Company*
NAM 05	15 min. + 2 min. Q&A	**COPD AOP-I: quantitative modeling of *in vitro* data using an adverse outcome pathway for the assessment of decreased lung function risk in humans**	Marja TALIKKA *Philip Morris International*
NAM 06	15 min. + 2 min. Q&A	**COPD AOP-II: *In vitro* assessment of mucus hypersecretion with quantitative AOP modeling**	Shigeaki ITO *Japan Tobacco Inc.*
Session III: Panel Discussion			
	20 min.	**NAMs – Applications in Tobacco Regulatory Sciences – Gaps and Opportunities**	Liam SIMMs (Co-Chair) *Imperial Brands* All speakers & panelists
Total: ~140 minutes			

NAM 00

INTRODUCTION: New Approach Methods (NAMs): Applications in Tobacco Regulatory Sciences

LEE K.M.(1) – Chair; SIMMS L.(2) – Co-Chair

(1) Altria Client Services LLC, 601 East Jackson Street, Richmond, VA 23219, U.S.A.
(2) Imperial Brands PLC, 121 Winterstoke Road, Bristol BS3 2LL, U.K.

Dr. K. Monica Lee, Ph.D., D.A.B.T., is an Associate Fellow in Regulatory Sciences at the Altria Client Services LLC. Dr. Lee leads the Biological Science Insights group, identifying, building and utilizing innovative and pragmatic in vitro and in vivo toxicological evaluation approaches in support of product development, regulatory compliance and scientific engagement. She has extensive experiences in regulatory and foundational nonclinical studies and published numerous peer-reviewed articles on the topics of reduced-risk disease models, comparative and mechanistic toxicology, in vitro-to-in vivo extrapolation and kinetic modeling. Prior to joining ALCS in 2015, she served as the BioScience Director at the JT International and Sr. Toxicologist at Battelle Toxicology Northwest and Pacific Northwest National Laboratories. Her PhD is on Pharmacology/Toxicology from the University of Georgia. Dr. Lee builds and promotes the use of in silico and in vitro-based predictive toxicological tools in support of tobacco regulatory science, proactively engaging with CORESTA members and external partners across research community. She was the chair for the first NAM symposium during SSPT-2021, leading from the concept to execution and post-symposium peer-reviewed pubilcation.

NAM 01

The use of NAMs in *in vitro* genotoxicity assessment of nicotine delivery products: ToxTracker assay as a case study

BREHENY D.(1); LEVERETTE R.(2)

(1) BAT Investments Limited, R&D Centre, Regents Park Road, Millbrook, Southampton, SO15 8TL, U.K.

(2) RAI Services Company, 401 North Main St, Winston Salem, NC 27101, U.S.A.

Genotoxicity and cytotoxicity assessments are an indispensable component in the safety assessment of tobacco and nicotine delivery products. They also form a cornerstone of the preclinical component of regulatory dossiers. In the case of genotoxicity, since no single test is capable of detecting all relevant genotoxic endpoints, a battery of *in vitro* testing techniques has been recommended by CORESTA. This comprises the Ames assay for gene mutation and the micronucleus (MN) assay or mouse lymphoma assay. Whilst the importance and usefulness of these assays are not in question, they are not without limitation. Traditional *in vitro* tests lack throughput, provide little mechanistic information, and have poor specificity in predicting *in vivo* genotoxicity. A number of NAMs have been developed in recent years to address these gaps in the current testing strategies. For example, the *in vitro* MN assay by flow cytometry (MicroFlow™) has greatly increased the throughput above the traditional version of the assay, also providing information on clastogenicity or aneugenicity. Other new assays such as the ToxTracker assay have emerged, that can both increase throughput and give additional insight into the mode of action of genotoxic compounds. The ToxTracker assay, which is based on a panel of 6 reporter-gene cell lines, has been shown to be predictive of both the Ames assay and *in vitro* micronucleus assay for single toxicants. Extensions of the ToxTracker assay protocol; ToxTracker ACE and ToxTracker AO, can be used to determine if a positive compound is aneugenic or clastogenic, and whether the DNA damage is direct or indirect via oxidative stress. The predictivity of the ToxTracker assay, coupled with mechanistic insights, means that it has potential to be used in a screening capacity and/or as a follow up to *in vitro* positives in traditional genotoxicity assays. Here we present a case study describing the use of the ToxTracker assay in the context of the assessment of cigarettes and heated tobacco products (HTPs) as an example. Findings from our industry will be discussed, along with recommendations of how this assay could be used in a toxicological risk assessment framework.

Dr. Robert Leverette is currently a Master Scientist in the Modern Oral Submissions Team at RAI Services Company. In his previous role in the Non-Clinical Studies Division at RAI Services Company, he conducted regulatory toxicological studies on next generation tobacco and nicotine products for regulatory applications, and developed whole aerosol techniques and approaches. He graduated with a PhD in Biochemistry from North Carolina State University, focusing on the processing of the U14 small nucleolar RNA from the introns of the mouse cognate heat shock gene.

NAM 02

Connecting exposure, dosimetry and toxicity responses in the preclinical evaluation of ingredients: case examples of flavoring chemicals in oral tobacco products

ZHANG Jingjie

Altria Client Services LLC, 601 East Jackson Street, Richmond, VA 23219, U.S.A.

Flavoring ingredients that are GRAS ("generally recognized as safe") in food are commonly used in oral tobacco derived nicotine (OTDN) products. While the GRAS status is not *de facto* approval for the use in oral tobacco products, the accompanying toxicological information is relevant and useful in the safety evaluation of OTDN products, considering the similarity of how the products are consumed. In addition to available regulatory limit, a weight of evidence of all available nonclinical and clinical information is used to assess the suitability of using flavoring chemicals in OTDNs and the potential health effects of the products. For some GRAS ingredients, specific *in vitro* and *in vivo* toxicity outcomes sometimes present apparently different responses – for instance, cinnamaldehyde, a common flavor in oral consumer products is known to induce positive *in vitro* genotoxicity; however, these *in vitro* hazard findings do not lead to *in vivo* sequalae based on negative long-term carcinogenicity outcomes. In this talk, we have investigated the dosimetry basis for these apparently different *in vitro* versus *in vivo* genotoxicity and carcinogenicity outcomes using cinnamaldehyde as an example flavor in OTDN products. PBPK modelling and *in vitro* to *in vivo* extrapolation (IVIVE) approaches are used to estimate the equivalent human daily exposures (EADs) and to evaluate *in vitro* toxicity findings in the *in vivo* context. Using open-source PBPK models, we estimated the C_{max} in the target organ (e.g., plasma and liver) of cinnamaldehyde under *in vivo* (rodent and human) exposure conditions and compared the estimated doses to the *in vitro* exposure ranges for cytotoxicity and genotoxicity findings. We also compared the estimated EADs from nonclinical testing to the likely use levels in human use and discuss the estimated margin of exposure in the context of known toxicological profiles of the ingredients. Using the case example, we demonstrate the relevance and opportunity of incorporating target tissue dosimetry consideration as part of nonclinical toxicity evaluation and risk assessment.

Dr. Jingjie Zhang, Ph.D., D.A.B.T., is a principal scientist in Regulatory Sciences at the Altria Client Services LLC. Dr. Zhang has managed and lead preclinical in vivo studies since she joined the Biological Science Insights group in 2016. She also investigates applications of new approach methodologies (NAMs) to product development and regulatory complicnce for porential reduced-harm tobacco products. Prior to the current position, Dr. Zhang served as a senior scientist in the Sensomics and Analytical group at Altria Client Services LLC, and applies her knowledge and skills in aerosol science to aid in the design and characterization of inhaled products. Her PhD is on Energy, Environmental, and Chemical Engineering from Washington University of St. Louis, focusing on particle physics, aerosol instrumentation and in vivo inhalation drug delivery.

NAM 03

Exposure to fresh whole smoke and aerosols: standard and novel (3D) *in vitro* models

POUR S.J.(1); SIMMS L.(2)

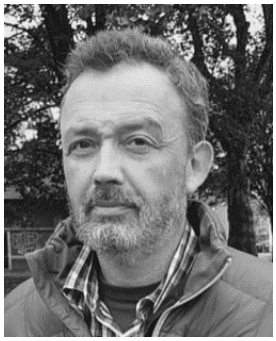
(1) Reemtsma Cigarettenfabriken GmbH (an Imperial Brands PLC Company), Albert-Einstein-Ring 7, 22761 Hamburg, Germany

(2) Imperial Brands PLC, 121 Winterstoke Road, Bristol BS3 2LL, U.K.

New Approach Methods (NAMs) describe non-animal- based methodologies that enable more human relevant toxicological risk assessments. In this talk, regulatory *in vitro* toxicology assays (CORESTA battery) are refined for NGP (Next Generation Product) testing of fresh aerosols to assess the Tobacco Harm Reduction (THR) potential of Electronic Vapour Products (EVP) and Heated Tobacco Products (HTP) compared to a reference cigarette (1R6F). Imperial's SAEIVS (Smoke and Aerosol Exposure *In Vitro* System) was used to expose cells to fresh aerosol/smoke for cytotoxicity and genotoxicity (NRU, IVM) assessment. Mutagenicity testing (Ames) was performed via bubbling of a bacterial suspension with the Vitrocell VC10 smoking robot. Using these *in vitro* systems, fresh aerosol generated by EVP and HTP were markedly less toxic than combustible cigarette smoke.

Building on these findings, *in vitro* 3D tissue models of the human upper respiratory tract (MucilAir™, Epithelix) were repeatedly exposed to whole smoke/aerosol at the air-liquid Interface (ALI) for 4 weeks, using SAEIVS. Although the EVP and HTP delivered up to 25 times more nicotine compared to the diluted cigarette smoke, minimal effects were observed for the assessed endpoints (Cytotoxicity, cilia function, barrier integrity, histology, and pro-inflammatory markers). In contrast, smoke from the reference cigarette caused significant changes across all the endpoints in a puff dependent manner. These results align with the cytotoxic and genotoxic results from the regulatory toxicology assays described above.

These results demonstrate that NAMs such as those described above can be used to provide a deeper understanding of the potentially reduced biological effects of NGP compared to cigarettes.



Dr. Liam Simms has a PhD from Durham University in the UK and has worked for two contract Toxicology laboratories conducting toxicity studies, before joining Imperial Brands in 2002 currently in the role of Principal Toxicologist. Whilst at Imperial Dr. Simms has worked in various toxicology related roles, having lead the Product Strewardship Team for several years and working on Imperials TT21C programme to introduce new in vitro techniques with a focus on the use of human cells where possible, in the area of potential harm reduction. Dr. Simms published extensive peer-reviewed articles and currently serves as the joint Coordinator for the CORESTA In Vitro Toxicity Testing (IVT) Sub-Group and the 21st Century Toxicology for Next Generation Tobacco and Nicotine Products (NGTX) Task Force.

NAM 04

EpiAirway Nrf2 – oxidative stress model: Practical application of *in vitro* systems with clinical relevance

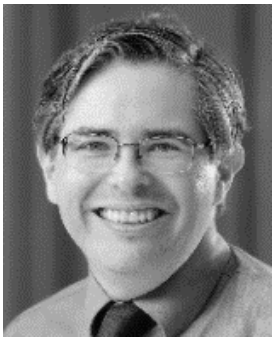
KEYSER B.M.(1); WERTMAN J.(1); HOLLINGS M.(2); JORDAN K.(1)

(1) RAI Services Company, 401 North Main St, Winston Salem, NC 27101, U.S.A.
(2) Labcorp Early Development Laboratories Ltd, Otley Road, Harrogate, Harrogate HG3 1PY, U.K.

The nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway regulates genes that contribute to the antioxidative stress response. This pathway is involved in respiratory diseases, including chronic obstructive pulmonary disease (COPD). Cigarette smoke has been shown to activate this pathway in lung. Here, we evaluated the effects of whole smoke/aerosol from two marketed combustible (nonmenthol and menthol) cigarettes (CC), two smoke-free alternative products: glo™ (heated tobacco product, HTP), and Vuse Alto® (electronic nicotine delivery system, ENDS) on cell viability and Nrf2 response in a 3D human airway model (EpiAirway™) transfected with a luciferase Nrf2 promoter. EpiAirway™ tissues were exposed at the air liquid interface to whole smoke or aerosol generated on a Vitrocell® VC10®. Whole smoke/aerosol doses were controlled using dilution airflows of 0.5 to 8 L/min for CC, and undiluted to 4 or 5 L/min for HTP or ENDS, respectively. Eighteen hours post-exposure, luciferase activity and cell viability were measured. Relative luciferase activity was expressed as fold change over the air exposed control. Post-exposure, whole smoke/aerosol deposition was quantified using chemical analysis (e.g., glycerol, nicotine, carbonyls).

Differential Nrf2 activation was observed following exposure to CC whole smoke compared to the tested HTP and the ENDS. Maximum Nrf2 fold increase occurred at the undiluted dose for the HTP and ENDS versus 3 L/min for the CC. Moreover, the minimum exposure-correlated nicotine concentration required to induce a > 2-fold increase (threshold response) in Nrf2 activation was > 30× and > 100× lower for CC than the HTPs and ENDS, respectively.

These data indicate that the tested HTP and ENDS induce significantly lower oxidative stress than the CC. Additionally, the 3D Nrf2 EpiAirway™ *in vitro* model can be used to assess and discriminate responses of a biomarker (oxidative stress) relevant to smoking-related disease pathways (e.g., respiratory and cardiovascular disease).



Dr. Brian Keyser is currently a Senior Manager in the Global Clinical Studies Division at RAI Services Company. In his previous role in the Non-Clinical Studies Division at RAI Services Company, he developed next generation in vitro lung and oral models for potentially reduced harm products and regulatory applications. He graduated with Ph.D. in Pharmacology from Tulane University in New Orleans focusing on the role of T-type voltage gated calcium channels in diabetes mellitus.

NAM 05

COPD AOP-I: quantitative modeling of *in vitro* data using an adverse outcome pathway for the assessment of decreased lung function risk in humans

TALIKKA M.; ISKANDER A.

Philip Morris International R&D, Philip Morris Products S.A., Quai Jeanrenaud 5, CH-2000 Neuchâtel, Switzerland

Adverse outcome pathways (AOPs) organize mechanistic knowledge based on empirical evidence into a sequence of cell, tissue, and organ level events that lead to an adverse outcome (AO) in humans. An AOP initiates from the interaction of a stressor with a biomolecule in a target cell, also known as molecular initiating event (MIE), progress through a series of intermediate, causally linked events termed key events (Kes) and culminate in an AO. AOP 411 is one of five AOPs connecting exposure-related oxidative stress with lung function impairment. It considers a slowing down of cilia beating frequency (CBF) following inhalation exposure and the subsequent decrease in mucociliary clearance (MCC) in the lungs.

To evaluate the potential risk of impaired lung function associated with tobacco heating system (THS) use compared with smoking, we mapped data from a series of *in vitro* studies on THS aerosol exposure, employing advanced tissue culture models. The mathematical modeling predicted a reduced risk of decreased CBF based on measurement of oxidative stress parameters and reduced risk of decreased MCC based on CBF measurement in THS aerosol- compared with cigarette smoke-exposed cultures. Finally, modeling using clinical MCC and FEV1 data predicted a 79.3 % reduced risk of decreased lung function in HTP switchers compared with smokers.

In summary, combining our *in vitro* data with publicly available clinical data allowed us to approximate the residual risk of decreased lung function in THS switchers relative to continuing smokers. Considering the causality between active smoking, reduced lung function, and COPD, the approach provides a plausible prediction of reduced COPD risk in THS switchers compared with continuing smokers.

Dr. Marja Talikka is the head of translational science at PMI. She joined the company in 2009 as Scientist exploring the conversion of biological information into computable networks, setting the foundation for the generation of a publicly available suite of network models capturing the biology of a multitude of biological processes. She also participated in a joint project with BAT and PETA to create an AOP network for decreased lung function, which is directly applicable to the assessment of smoke-free products and has since been utilized by others in the industry. More recently, Dr. Talikka has led efforts to establish an in-silico approach to the quantification of one of these AOPs to achieve meaningful risk assessment without animal experimentation. Dr. Talikka holds a PhD in biochemistry from the University of Helsinki. Her educational journey comprised research conducted at the London regional Cancer Center in Ontario, Rockefeller University in New York, and Swiss Institute for Experimental Cancer Research in Lausanne before joining PMI.

NAM 06

COPD AOP-II: *In vitro* assessment of mucus hypersecretion with quantitative AOP modeling

ITO S.; ERAMI K.; ICHIKAWA S.; MURATANI S.; MORI A.; YOSHINO K.

Scientific Product Assessment Center, Japan Tobacco Inc., Yokohama, Kanagawa, Japan

The Adverse Outcome Pathway (AOP) framework is a valuable tool for understanding the mode of action of a stressor. While traditionally used to supplement chemical risk assessment, this concept could also be useful in disease-related toxicological risk assessment. To this end, we developed *in vitro* test methods as an original version of AOP148 (AO: lung function decrease) to serve as a reference for key events. Through repeated exposure of 3D-cultured bronchial epithelial cells to whole cigarette smoke, we successfully observed goblet cell hyperplasia and mucus hypersecretion. However, since the AOP framework is based on qualitative mode of action, one needs to translate AOP into quantitative model to enable risk assessment process. To address this, we developed quantitative AOP (qAOP) models using Bayesian formalism. These models allow us to evaluate the probability of adverse outcomes occurring based on *in vitro* test results. In this talk, we will demonstrate that although data generation with next-generation-inhalable products is still ongoing, our approach holds promise for relative risk assessment among tobacco products, effectively highlighting the differences in disease risk between combustible cigarettes and NGPs. We will also discuss the importance of investigating clinical relevance, including exposure alignment between *in vitro* and *in vivo*, to enhance the reliability of our risk assessment framework.

Dr. Shigeaki Ito is leading a research project aimed at consolidating various biological assessment results obtained from in vitro, in vivo, and clinical studies to improve the interpretation of the Reduced-Risk (RR) potential of Next Generation Products. His primary responsibility now involves developing novel methodologies, including computational models and advanced in vitro test methods, to establish a clinically relevant approach for assessing the RR potential.

Dr. Shigeaki Ito obtained his Ph.D. in Agriculture from Hokkaido University in 2010, where he conducted research on anaerobic microorganisms and their characteristic enzymes. Since joining Japan Tobacco Inc. In 2010, he has consistently been in charge of conducting in vitro toxicological assessments and developing a risk assessment approach using in vitro test methods and computational modeling.