In Vitro Toxicity Testing Sub-Group (IVTSG) Annual Report

Hamburg, Germany

October 8, 2019

SG Composition

- **SG Coordinator:** Kei Yoshino (JT)
- **SG Secretary:** David Thorne (BAT)
- **SG Membership**
  - Altria Client Services, BAT, Battelle, Charles River Laboratories, CNTC, Covance, Enthalpy Analytical, Imperial Brands, JTI, JTI/Oekolab, JUUL Labs, KT&G, Labstat, Millipore Sigma, PMI, RAI, Vitrocell, JT, Charles River Laboratories, ITC, Zhejiang University
- **SC Liaison:** Kei Yoshino (JT)

Objectives

- **Objective 1: To compile and review information on *in vitro* toxicity testing and apply learnings to further biological research.**
- **Objective 2: To organize and conduct periodically proficiency testing of tobacco and tobacco related products.**

Meetings

Recent Two Meetings

March 8, 2019: Baltimore, US

- 27 delegates attended the meeting
- Meeting was hosted by Japan Tobacco and Altrial Client Services

October 6, 2019: Hamburg, Germany

- 34 delegates attended the meeting

Upcoming Meetings

TBD

- Meeting will be hosted by JT or Vitrocell

❖ Completed project

➢ "Rational and Strategy for In Vitro Testing of Combustible Tobacco Products" (IVT Technical Report)

- Contributors: Lee K.M. (ALCS), Jordan. K.G. (RAI), Wieczorek. R (Imperial Brands), Moennikes O. (PMI), Clements. J (Covance), Hashizume. T (JT), Miller J. (JTI), Weber E. (JTI Oekolab)

REPORTS current

Rationale and Strategy for In Vitro Toxicity Testing of Combustible Tobacco Products

IVT Technical Report

September 2019 *Ref. IVT-225-CTR*

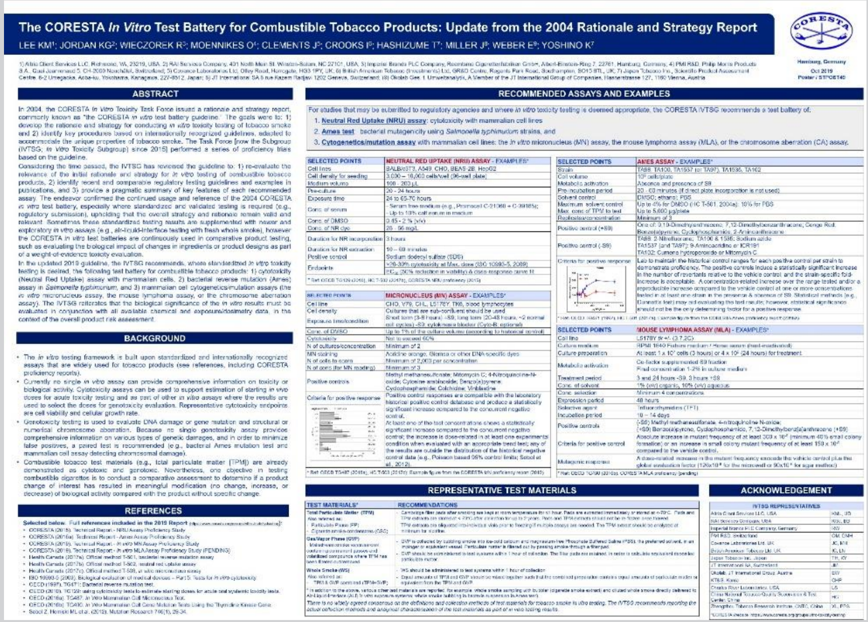
In 2004, the first guideline report was published covering the rationale and strategy for conducting *in vitro* toxicity testing of tobacco smoke and to identify key procedures based upon internationally recognized guidelines, adapted to accommodate the nature and unique properties of tobacco smoke.

In 2018, the CORESTA In Vitro Toxicity Testing Sub-Group decided to review the 2004 guideline in order to: 1) re-evaluate the relevance of the initial rationale and strategy of *in vitro* testing of combustible tobacco products, 2) identify recent and comparable regulatory testing guidelines and examples in publications, and 3) provide a pragmatic summary of key features of each recommended assay.

The review effort revealed the continued usage and reference of the CORESTA *in vitro* test battery, especially where standardized and validated testing is required, upholding that the overall strategy and rationale remains valid and relevant. Sometimes these standardized testing results were supplemented with newer and exploratory *in vitro* assays. However, the *in vitro* tests recommended in 2004 continue to be used in a comparative product testing, such as to evaluate the biological impact of changes in ingredients or product designs against reference tobacco products as part of weight-of-evidence toxicity evaluation.

This report is an update of the 2004 report.

- **Poster Presentation (STPOST 49)**
  - Presented by Lee K.M. (ALCS)



Update of the on-going projects

Whole Smoke (Publication)

- Lead: David Thorne (BAT)
- Michael Hollings (Covance), Tobias Krebs (Vitrocell) and Robert Leverette (RAI) volunteered to review whole smoke publication for NGP gap analysis. This will be followed by an author review and prior to submission the journal - submit for review to authors by end of the year.

In Vitro Micronucles Inter-laboratory Study

- Lead: E. Weber (JTI Oekolab)
- Draft report under review by the SC

Update of the on-going projects (continued)

MLA Inter-laboratory Study

- Lead: D. Smart (PMI)
- Additional Data to be input (Nicotine analysis)

NRU Inter-laboratory Study

- Lead: K. Yoshino (JT)
- Draft report circulated before end of October

Ames Inter-laboratory Study

- Lead: R. Wieczorek (Imperial Brand) and E. Weber (JTI Oekolab)
- Draft report circulated before end of October

Projects

Upcoming projects

Rational and Strategy for in vitro Toxicity Testing of e-Vapor Products

- Lead: U. Doshi (ALCS)
- Outline under discussion

Inter-laboratory Study

- Based on the members needs

Inter-laboratory Study

- Ames Assay-

Ames

Objectives

- To conduct an inter-laboratory proficiency testing programme on two test items and the Kentucky References 3R4F and 1R6F using a common experimental design for the AMES Test.
- Assessment of the discriminatory power of the test towards different tobacco products.

Responsibilities

- Coordinator: Roman Wieczorek (Imperial Brands)
- Co-Coordinator: Elisabeth Weber (JTI Ökolab)
- Statistical analysis: Alexander Hauleithner (JTI Ökolab)

Ames

Test Design

- **Labs use their own protocols**
- **Basic requirements were defined in the study plan (based on OECD TG 471)**
  - Conditioning and smoking of test items according to ISO International Standards
  - At least 3 replicates per test item, concentrations as per lab protocol
  - Salmonella typhimurium TA98 / TA100 mandatory, ± S9
  - Negative and positive controls
- **Test Pieces: 100 % FC, 100 % Bly, 3R4F, and 1R6F**

Participating Labs (10 labs)

- **Altria-CRL, CNTQS&TC, Covance, Enthalpy, IB-Reemtsma, JT, KT&G, Labstat, JTI Oekolab, PMI**

❖ Ranking in mutagenic rate (TA98 +S9)

LOT 1 (100% FC) | LOT 3 (KR 3R4F)
LOT 2 (100% BY) | LOT 4 (KR 1R6F)

LAB ID	[lower mR]		Ranking in mutagenic rate				[higher mR]
LAB A	LOT 1	<	LOT 4	=	LOT 3	<	LOT 2
LAB B	LOT 1	<	LOT 4	=	LOT 3	<	LOT 2
LAB C	LOT 1	<	LOT 3	=	LOT 4	<	LOT 2
LAB D	LOT 1	<	LOT 4	=	LOT 3	<	LOT 2
LAB E	LOT 1	<	LOT 3	=	LOT 4	<	LOT 2
LAB F	LOT 1	<	LOT 4	=	LOT 3	<	LOT 2
LAB G	LOT 1	<	LOT 4	=	LOT 3	<	LOT 2
LAB H	LOT 3	=	LOT 4	=	LOT 1	=	LOT 2
LAB I	LOT 1	<	LOT 4	=	LOT 3	<	LOT 2
LAB K	LOT 1	<	LOT 4	=	LOT 3	<	LOT 2

❖ Ranking in mutagenic rate (TA100 +S9)

LOT 1 (100% FC) | LOT 3 (KR 3R4F)
LOT 2 (100% BY) | LOT 4 (KR 1R6F)

LAB ID	[lower mR]		Ranking in mutagenic rate				[higher mR]
LAB A	LOT 1	<	LOT 4	=	LOT 3	=	LOT 2
LAB B	LOT 1	=	LOT 3	<	LOT 2	=	LOT 4
LAB C	LOT 1	=	LOT 2	=	LOT 4	=	LOT 3
LAB D	LOT 1	<	LOT 4	=	LOT 3	=	LOT 2
LAB E	LOT 1	=	LOT 3	=	LOT 2	=	LOT 4
LAB F	LOT 1	=	LOT 3	=	LOT 4	=	LOT 2
LAB G	LOT 1	=	LOT 4	=	LOT 2	=	LOT 3
LAB H	LOT 1	=	LOT 3	=	LOT 2	=	LOT 4
LAB I	LOT 1	<	LOT 3	=	LOT 4	=	LOT 2
LAB K	LOT 1	=	LOT 3	=	LOT 2	=	LOT 4

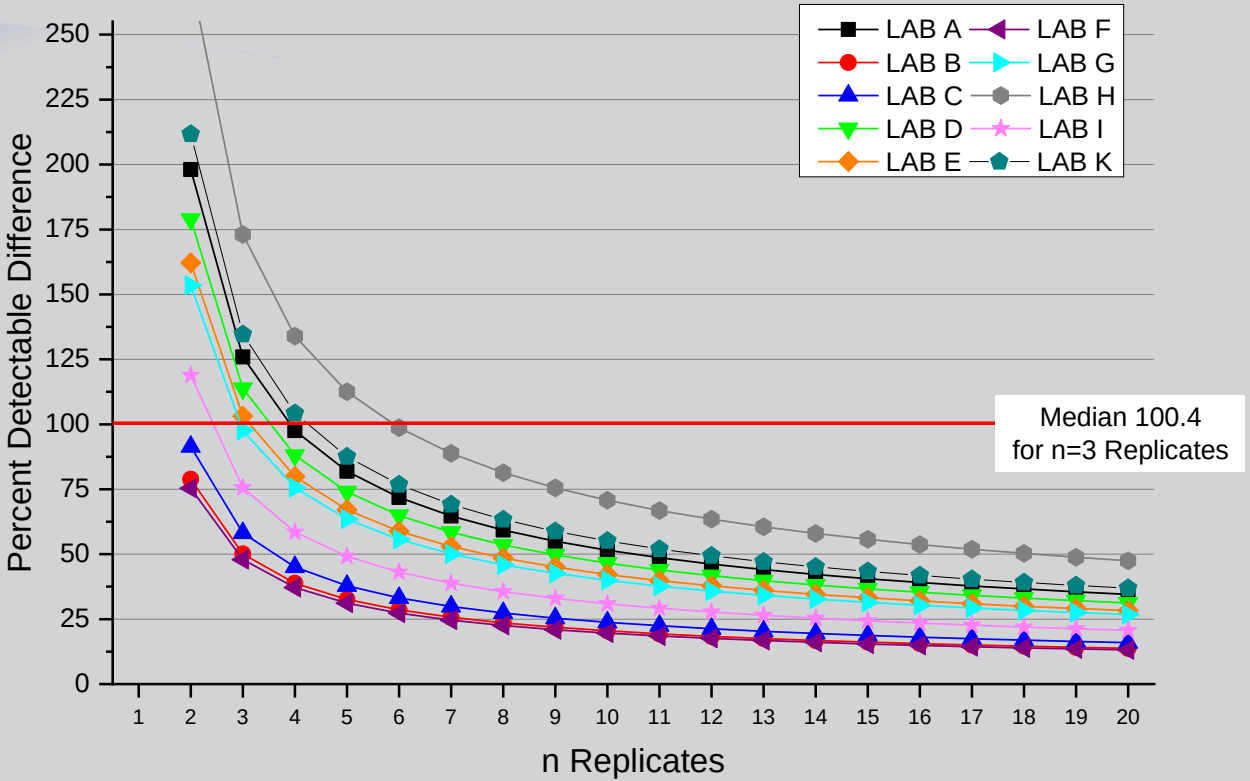
❖ Ranking in mutagenic rate (TA97a +S9)

LOT 1 (100% FC) LOT 3 (KR 3R4F)
LOT 2 (100% BY) LOT 4 (KR 1R6F)

LAB ID	[lower mR]		Ranking in mutagenic rate				[higher mR]
LAB A	*not tested*						
LAB B	**LOT 1**	<	**LOT 4**	=	**LOT 3**	<	**LOT 2**
LAB C	**LOT 1**	<	**LOT 2**	=	**LOT 4**	=	**LOT 3**
LAB D	**LOT 1**	<	**LOT 3**	=	**LOT 4**	<	**LOT 2**
LAB E	*not tested*						
LAB F	**LOT 1**	<	**LOT 3**	=	**LOT 4**	=	**LOT 2**
LAB G	*not tested*						
LAB H	*not tested*						
LAB I	*not tested*						
LAB K	*not tested*						

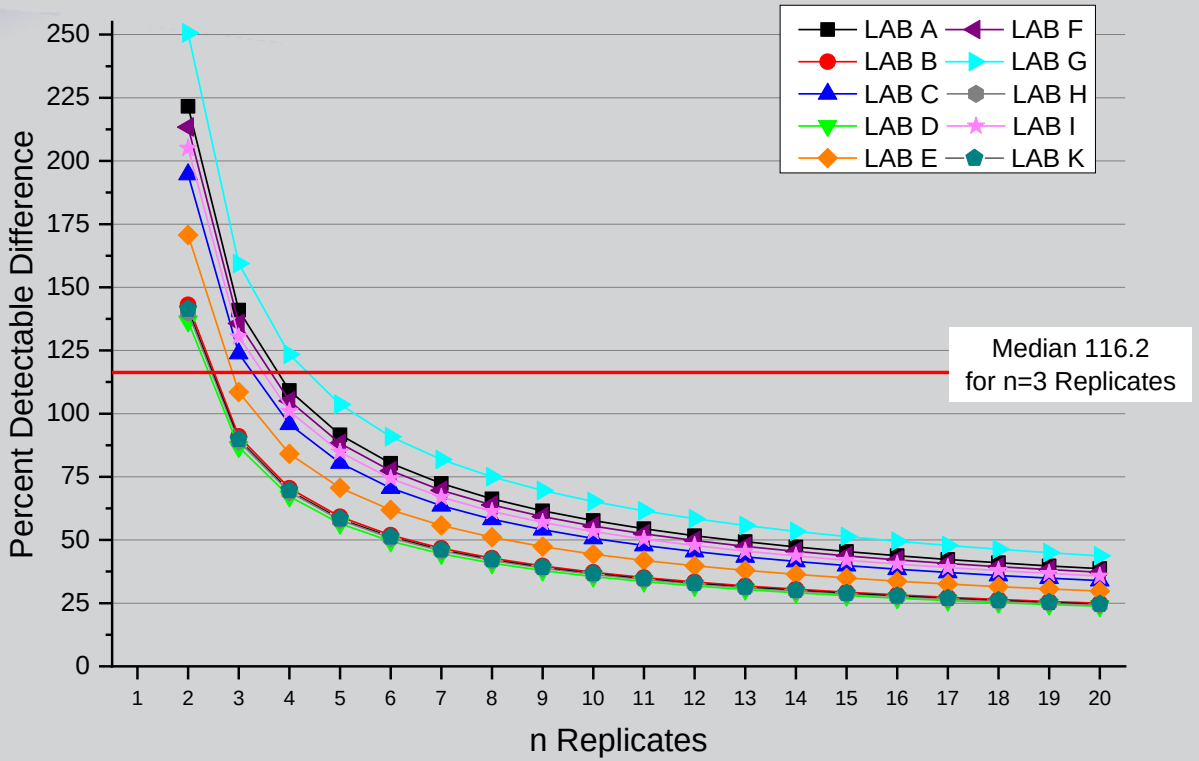
Minimum Detectable Difference (MDD)

❖ **TA98 +S9**



Minimum Detectable Difference (MDD)

- TA100 +S9



Summary

- 4 Samples (100% FC, 100% BY, KR 3R4F and KR 1R6F) were tested in AMES assay.
- 10 Laboratories reported data.
- Test were performed for S. typhimurium strains TA98, TA100 and TA97a in presence and absence of S9.
- TA98 and TA97 showed in comparison to TA100 significant higher increase in revertants.
- In TA98+S9, laboratories could significantly discriminate FC and BY samples, the Reference Cigarettes could not be discriminated.
- If the samples differ ~100-120% in mutagenic rate in the presence of S9, the participating labs can detect that difference (median) in a t-test with 3 replicates.

Independent Genetox Workshop in US

“Recommendations for the Generation & Use of In Vitro Assay Data for Tobacco Product Regulations”

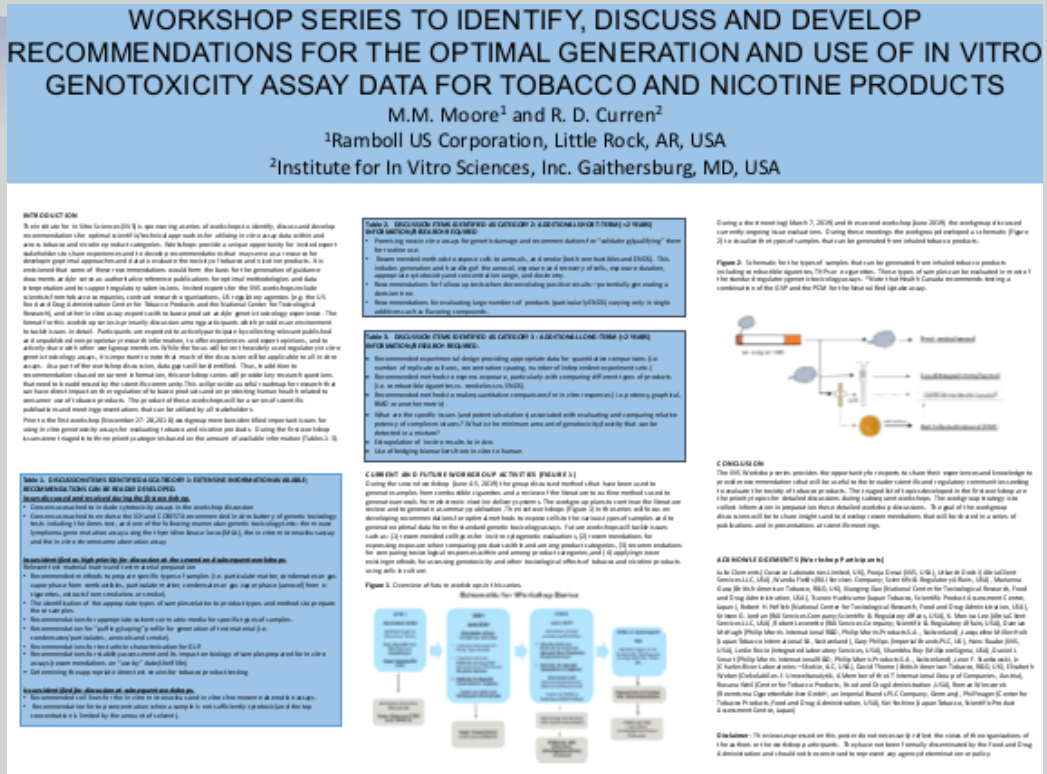
Independent Genetox Workshop in US

- **Background**
  - Proposed by Dr. Martha Moore (Ramboll Environ)
  - Proposal to undertake a series of discussion workshop (similar to the IWGT approach). Representatives for all the relevant “stakeholders”
  - Identify key issues, discuss and reach consensus on key issues and publish a series of papers presenting the consensus
  - Focus on regulatory issues including those specific to US FDA (and therefore could complement the CORESTA IVTSG efforts)
- **Host organization**
  - IIVS (Institute for In Vitro Sciences): Non-profit organization in US

Independent Genetox Workshop in US

- **Participants:**
  - Tobacco Companies (2-3 key individuals from each organization)
  - CROs
  - FDA: CTP & NCTR
- **Goals for the first meeting (November 27-28, 2018)**
  - Outline “all” the key issues & Prioritize into three priority buckets
- **Relationship of this workshop to CORESTA**
  - To be an independent exercise. K. Yoshino will serve as a pipeline to CORESTA.
  - Draft discussion topics reviewed/discussed by the IVT members.

Independent Genetox Workshop in US



WORKSHOP SERIES TO IDENTIFY, DISCUSS AND DEVELOP RECOMMENDATIONS FOR THE OPTIMAL GENERATION AND USE OF IN VITRO GENOTOXICITY ASSAY DATA FOR TOBACCO AND NICOTINE PRODUCTS

M.M. Moore[1] and R. D. Curren[2]
[1]Ramboll US Corporation, Little Rock, AR, USA
[2]Institute for In Vitro Sciences, Inc. Gaithersburg, MD, USA

(TSRC, 2019)

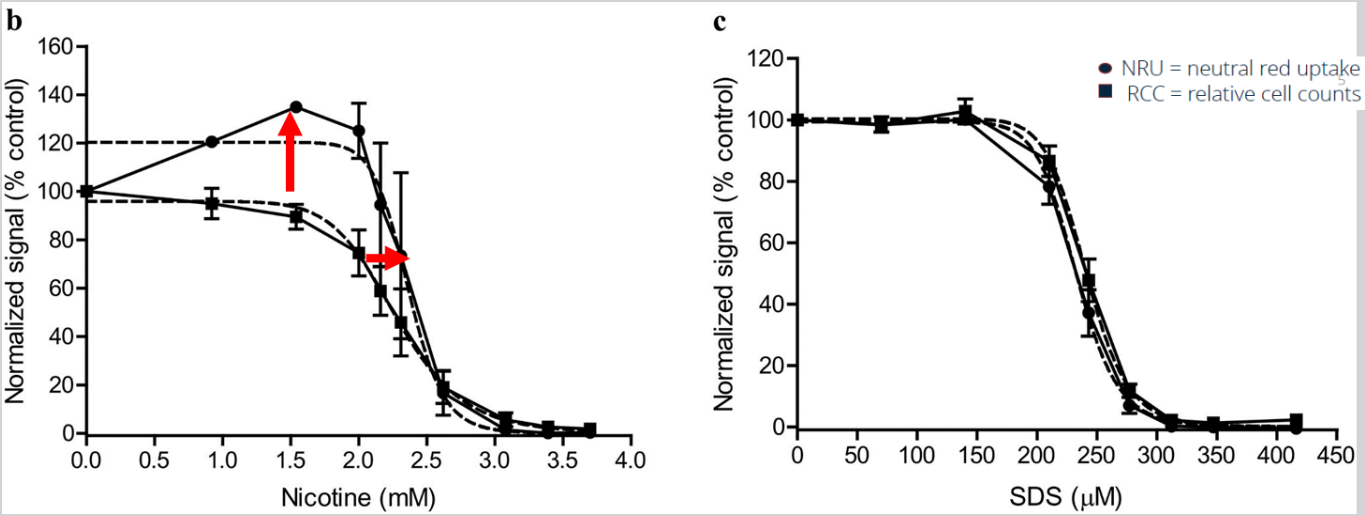
Independent Genetox Workshop in US

- **Second meeting (June 4 – 5, 2019)**
  - **Updates on the grant and comments from FDA**
  - **Ames strains**
  - **Sample preparation**
  - **Issues/considerations for the preparation of samples from combustibles**
  - **E-cig aerosol trapping**
- **Seek feedback form Dr. Moore regarding Poster Presentation**
- **Third meeting is expected to be held next spring**

NRU Protocol investigation with BEAS-2B & Balbc/3T3 cells

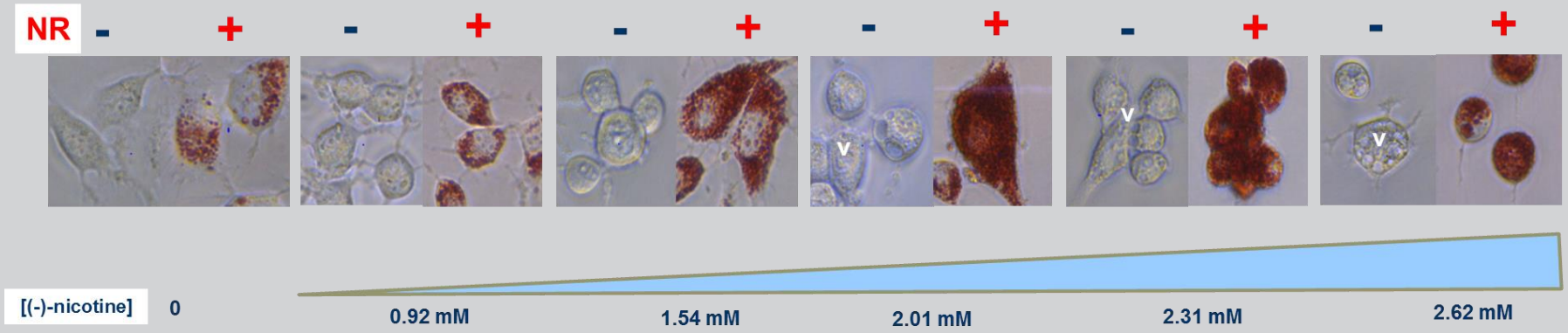
NRU Protocol investigation (Background)

- **Apparent increase in neutral red uptake following exposure to eliquids containing (-)-nicotine (PMI presentation, CORESTA Kunming 2018)**
  - **Balb/c 3T3 cells treated for 24 h to eliquids + nicotine**
  - **Increase in NR uptake apparent vs non-nicotinated eliquid controls**



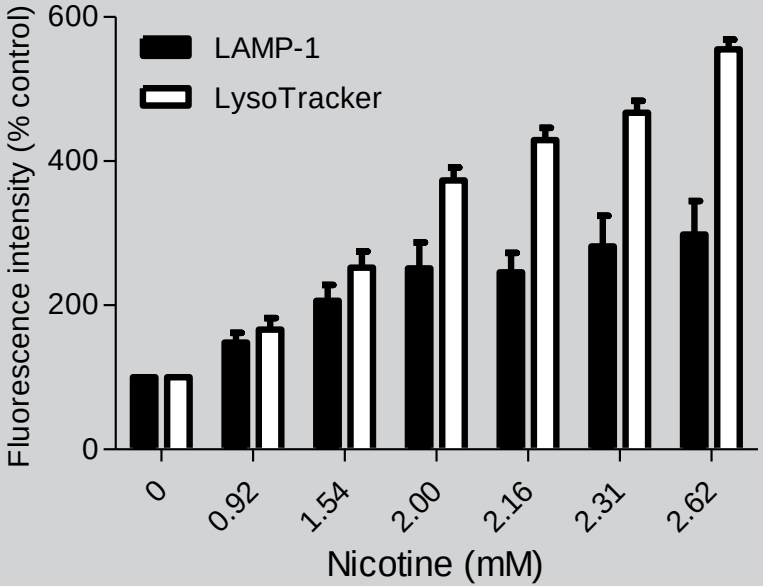
NRU Protocol investigation (Background)

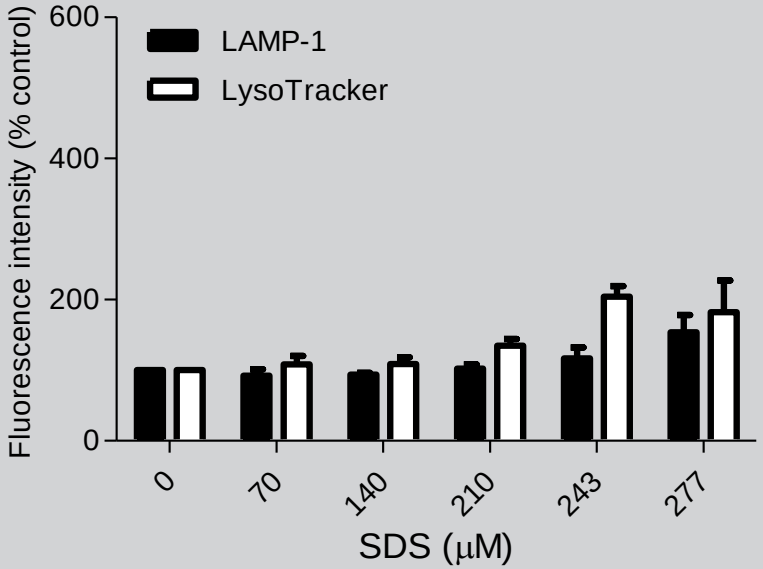
- **Macroscopic changes apparent in nicotine treated cells**
  - **Enhanced NR uptake coincident with macroscopic changes to cell ultrastructure**



NRU Protocol investigation (Background)

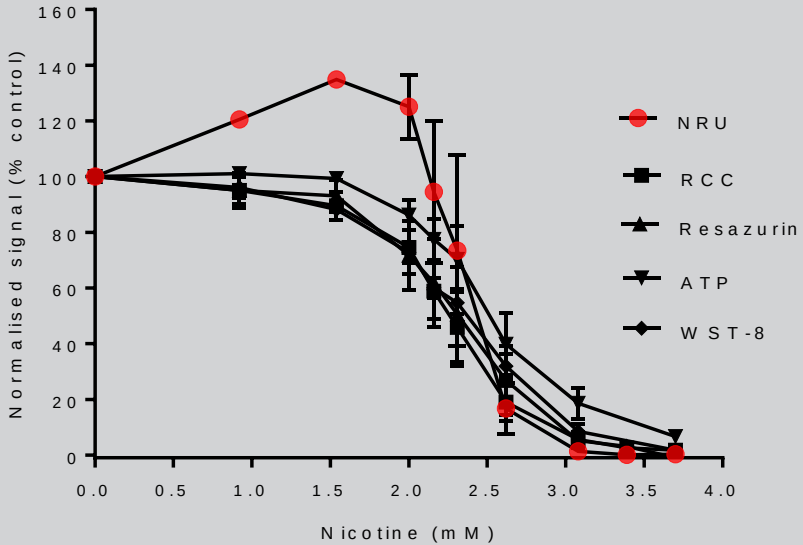
❖ Lysosome analysis via FACS confirms perturbation by (-)-nicotine





NRU Protocol investigation (Background)

High-throughput compatible approaches to determine cytotoxicity in Balb/c 3T3 cells



- Nicotine cytotoxicity was successfully evaluated with all assays using metabolic measures of viability
- No nicotine-induced artefacts were detected
- Agreement between the direct (RCC) and metabolic assay processes estimation of the cytotoxicity EC50

NRU Protocol investigation (Background)

- **OECD Draft guidance No. 129 : Guidance Document On Using Cytotoxicity Tests To Estimate Starting Doses For Acute Oral Systemic Toxicity Tests : ENV/JM/MONO(2010)20**
  - **Caution for the chemical affecting lysosome was already noticed.**
  - **1.3.3 Range of Substances Amenable to the In Vitro NRU Test Methods … The toxicity of substances that specifically affect lysosomes may be overestimated because they may affect NRU binding…**

NRU Protocol investigation

		Lab 1				Lab 2	Lab 3	Lab 4		Lab 5	Lab 6	Lab 7	Lab 8	Lab 9
Assay process	Cell line	CHO-WBL	CHO-WBL	Balb/c3T3	Balb/c3T3	CHO-K1	Balb/c3T3	BEAS-2B	HepG2	CHO-K1	CHO-K1	CHO-WBL (IVGT)	Balb/c3T3	CHO-K1
	Cell density for seeding	1.25 x 10^4 cells/mL	1.25 x 10^4 cells/mL	1.0 x 10^5 cells/mL	1.0 x 10^5 cells/mL	5.0 x 10^4 cells/mL	3.0 x 10^3 cells/mL	4.0 x 10^4 cells/mL	6.7 x 10^4 cells/mL	1.0 x 10^4 cells/mL	1.0 x 10^5 cells/mL	5.0 x 10^4 cells/mL	5.0 x 10^4 cells/mL	5.0 x 10^4 cells/mL
	Medium Volume	200 uL/well	200 uL/well	100 uL/well	100 uL/well	200 uL/well	100 uL/well	150 uL/well	150 uL/well	200 uL	100 uL	200 uL	100 uL	200 uL
	Pre-culture	23 - 24 hrs.	22 - 24 hrs.	24 hrs.	24 hrs.	20 - 25.5 hrs.	24 -27 hrs.	20 - 21 hrs.	20 - 21 hrs.	24 hrs.	27 hrs.	22 -26 hrs.	24 - 25 hrs.	24 hrs.
	Exposure time	24 hrs.	48 hrs.	48 - 49 hrs.	48 -49 hrs.	24 - 25 hrs.	23 - 24 hrs.	69 - 70 hrs.	69 - 70 hrs.	24 hrs.	24 hrs.	24 hrs.	23 - 24 hrs.	24 hrs.
	Conc. Of serum	10%	10%	10%	0%	10%	10%	0%	0%	10%	10%	10%	10%	10%
	Conc. Of DMSO	0.5%	0.5%	1.0%	1.0%	0.25 - 2%	0.5%	0.42%	0.45%	2.0%	1.5%	2.0%	0.33%	2.0%
	Conc. Of NR dye	50 ug/mL	50 ug/mL	25 ug/mL	25 ug/mL	10%	25 ug/mL	66 mg/mL?	66 mg/mL?	33 ug/mL	50 ug/mL	50 ug/mL	50 ug/mL	15 ug/mL
	Duration for NR incorporation	3 hrs.	3 hrs.	3.5 hrs.	3.5 hrs.	3 hrs.	3 hrs.	3 hrs.	3 hrs.	3 hrs.	3 hrs.	3 hrs.	3 hrs.	3 hrs.
	Duration for NR extraction	7 - 10 min.	5 - 10 min.	20 min.	20 min.	10 min.	5 - 10 min.	15 min.	15 min.	30 min.	10 min.	6 - 8 min.	7 - 11 min.	10 - 12 min.
Positive response	Positive control			SLS		SLS	SLS		SDS	SLS	SDS	SDS	SDS	SDS
	Criteria for positive response			< 70 %		non.	< 70 %		non.	non.	non.	non.	non.	non.
Sensitivity	Dose at where relative abs. is less than 10% (with 3R4F TPM)	150 ug/mL	150 ug/mL	> 150 ug/mL	150 ug/mL	150 ug/mL	80 -90 ug/mL	**30 ug/mL**	**30 ug/mL**	120 ug/mL	120 ug/mL	120 ug/mL	130 ug/mL	120 ug/mL

- **Cell line: CHO-WBL, Balb/c3T3, CHO-K1, BEAS-2B, HepG2, CHO-WBL(IVGT)**
- **Cell density for seeding**
- **Exposure time**
- **DMSO Conc.**
- **Dose at where relative abs. is less than 10% (with 3R4F TPM)**

NRU Protocol investigation

- **Confirmation studies voluntarily conducted by PMI, JT, and IB-Reemtsma**
  - **The same findings confirmed with Balb/c 3T3 but not with BEAS-2B cells. Vacuole seen in cytoplasm of both cells at the concentration showing 10-20% cytotoxicity.**
  - **Study conditions have impacts on "bi-phasic response" in NRU assay**
- **NRU should be considered when testing e-liquids to ensure appropriate data. Where possible other cytotoxic methods should be employed or considered.**
- **The group will address this further based on members interests.**

Collaboration between BMK SG and IVT SG

Collaboration between BMKSG and IVTSG

- Areas of science and technologies in between “human” and “in vitro”
  - ‘Omics
  - Organ-on-a-chip
  - Human Relevant Dose
  - Subpopulations (donor variation)
  - IVIVE (in vitro – in vivo extrapolation)
- “Guidance Documents” (under development in NGTX TF) covers all the above.
  ⇒ NGTX locates between IVT and BMK.
- The coordinators of BMK, NGTX, and IVT will discuss together and propose future action plan.

Acknowledgement

Hosts of the Spring Meeting

2011 : ITG (Hamburg)

2012 : Covance (Harrogate)

2013 : BAT (Brockenhurst)

2014 : JTI (Vienna)

2015 : PMI (Neuchâtel)

2016 : Battelle + RAI (New Orleans)

2017 : ALCS (Baltimore)

2018 : BAT (Southampton)

2019 : JT + ALCS (Baltimore)

“Speak Up!”

Betsy R. Bombick in RAI

Pierre-Marie Guitton