Characterization of aerosol exposure, metabolism and pharmacokinetics for toxicological assessment using in vitro organotypical airway models

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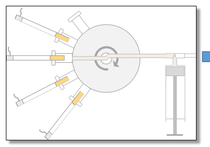
Introduction and Objectives

Direct exposure to inhaled mainstream cigarette smoke is known to cause smoking-related damage in the human lung. Aerosol exposure of human three dimensional (3D) organotypical airway epithelial tissue cultures, growing at an air-liquid interface, is a well-established in vitro model enabling a Systems Biology-based Reduced Risk Product (RRP) assessment. Migration kinetics and metabolism of deposited aerosol compounds are important parameters in understanding their bioavailability. To better understand the role of xenobiotic metabolism after cigarette smoking, human subcellular liver and lung fraction models (microsomes, S9) have been established to assess metabolite profiles and reactive metabolites relevant for toxicological assessment. The integration of exposure characterization results with a systems toxicology approach to measure the biological impact of exposure is informative to the product assessment strategy and has the potential to highlight modes of action associated with RRP exposure.

Methods

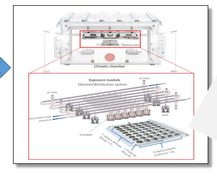
Aerosol Generation and Exposure of Organotypical Tissue Cultures at the Air-Liquid Interface (ALI)

Smoke Generation



Smoking machine: SM2000, Philip Morris International
Puff profile: Health Canada regimen (2 puffs/min. of 55 mL and 2 sec. of aspiration and 8 sec. exhaust)

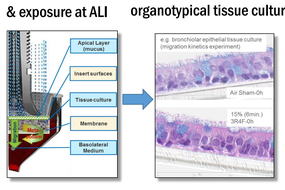
Tissue Exposure System



Exposure system: VITROCELL® 24/48 simultaneous exposure of 48 cell culture inserts with up to 7 different smoke dilutions with humidified air and exposing 6 inserts per dilution.

Cell culture inserts & exposure at ALI

Aerosol/Smoke exposed organotypical tissue cultures



Organotypical Tissue Cultures:
- Bronchiolar, Nasal, Buccal (human)

Surrogate Matrix:
- Phosphate Buffered Saline (PBS)

Figure 1: Aerosol Generation and Exposure of Organotypical Tissue Cultures at the Air-Liquid Interface.

Analytical Methods

Nicotine in Aerosol:
GC-FID after trapping of the aerosol by Extrelut 3NT columns, connected to VITROCELL®

Carbonyls (8) in PBS:
LC-MS/MS after trapping of the aerosol in phosphate buffered saline (PBS) in the wells of the Cultivation base module, derivatization with 2,4-dinitrophenylhydrazine (DNPH)

Aerosol Characterization GC×GC-TOFMS:
2-Dimensional gas chromatography:
- Agilent 7890A + LN2 Modulator + secondary oven
- Injection: Cool-on-column, 0.1µL
- Column 1: 30m DB-5ms
- Column 2: 2.2m DB-17ht

	primary oven	secondary oven
initial	30°C (2min)	35°C (2min)
rate	5°C/min	5.2°C/min
final	320°C (15min)	340°C (14.5min)

Mass Spectrometry (TOFMS):
- LECO Pegasus 4D
- Ionization: EI, 70eV
- Scan range: 35-700 Da
- Data acquisition rate: 200 spectra/s

Characterization of Exposure, Metabolism and Kinetics LC-HRAM-MS:

Traditional method:
- LC-pump: Thermo Accela 1250
- Column: Hypersil GOLD™ aQ (150x2.1 mm, 1.9µ)

Time [min]	A [%] 0.1% formic acid (pH2.7)	B [%] 0.1% formic acid in acetonitril
0	100	0
3.0	100	0
18.0	10	90
24.0	0	100

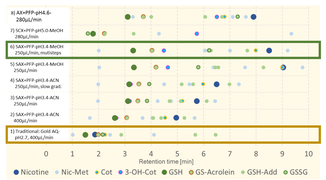
- Flow: 400µL/min, 40°C, Inj.: 5µL

Mass Spectrometry (HRAM-MS): Thermo QExactiveTM, HESI(+/-)
- Full scan: 80 – 800 Da, res.: 70000

New multi-column method:
- Columns:
  1) Biobasic SAX (50x2.1 mm, 5µ)
  2) Kinetex PFP (150x2.1mm, 2.6µ)

Time [min]	A [%] 0.1% formic acid / 10mM NH4formiate (pH3.4)	B [%] 0.1% formic acid / 1mM NH4formiate in MeOH
0	98	2
4.0	90	10
8.0	60	40
10.0	0	100

- Flow: 250µL/min, 40°C, Inj.: 1.5µL
- Full scan: 145 – 650 Da, res.: 70000



Optimized multi-column separation:
- High abundant compounds (e.g. nicotine, GSH, GS-acrolein) well separated from low abundant metabolites (e.g. 3-hydroxycotinine)
- Polar compounds well separated from chromatographic dead-volume

Traditional reversed phase method:
- Poor retention for polar compounds
- Co-elution for most compounds of interest

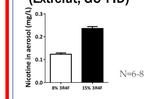
Figure 2: Chromatographic approaches for separation of nicotine, red./ox. glutathione (GSH/GSSG), GSH adducts of acrolein, crotonaldehyde and benzoquinone and nicotine metabolites (nicotine-N-oxide, cotinine-N-oxide, cotinine, 3-hydroxycotinine, nicotine-N-glucuronide, cotinine-N-glucuronide)

Results

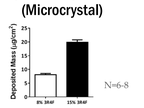
Vitrocell Characterization

Aerosol deposition for the reference cigarette 3R4F and for THS2.2*

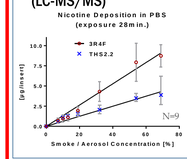
A) Nicotine in aerosol (Extrelut, GC-FID)



B) Mass deposition (Microcrystal)



C) Nicotine in PBS (LC-MS/MS)



D) Carbonyls in PBS (LC-MS/MS)

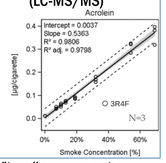


Figure 3: Characterization of the Vitrocell exposure system: A) nicotine in the aerosol, B) total mass deposited on Quartz Crystal Microbalances (QCM's), C) nicotine deposition in PBS for cigarette whole smoke or THS2.2 aerosol for a complete dose range, D) carbonyls, e.g. acrolein deposition in PBS

Non-targeted screening of smoke deposited at a microcrystal insert (GC×GC-TOFMS)

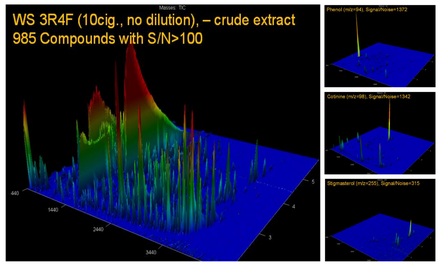


Figure 4: Comprehensive, non-targeted screening of whole smoke (WS) of a reference cigarette 3R4F deposited on a microcrystal insert of a QCM demonstrates the complexity in chemical composition of deposited particles, smoke compounds such as e.g. phenol, cotinine and stigmasterol are shown as an example

**THS2.2 = Tobacco Heating System 2.2*

Exposure Characterization

Exposure of organotypical airway epithelial tissue cultures to aerosol from THS2.2 or smoke from the reference cigarette 3R4F

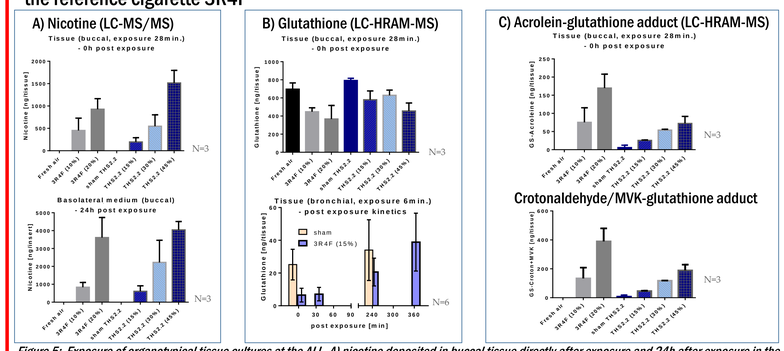


Figure 5: Exposure of organotypical tissue cultures at the ALI , A) nicotine deposited in buccal tissue directly after exposure and 24h after exposure in the basolateral medium, B) glutathione level in exposed tissues for different exposure doses and time points, C) glutathione adducts of electrophilic smoke compounds acrolein and sum of crotonaldehyde and methylvinylketone

Metabolism

Cell-free Model

In-vitro Model

Case study: Metabolism of furan-type compounds using human lung and liver microsomes / S9 (cytosolic fraction)

Analytical challenge: multiplicity of initial adducts is formed, which are also subject to subsequent metabolic reduction

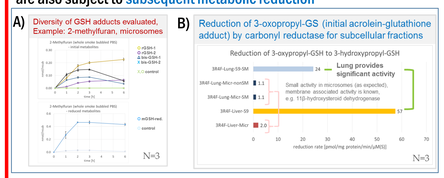


Figure 6: A) Bioactivation of furan and simple alkylfurans followed by conjugation to glutathione and subsequent metabolic reduction. B) Rate of reduction of the initial acrolein glutathione adduct for different subcellular fractions

Results of furans case study (summary):
- Human lung microsomes provide CYP2E1 activity
- Human lung microsomes are able to detoxify furan(s) (CYP2E1)
- Human lung S9 generates reactive sulfates of hydroxymethyl-furans (e.g. 5-HMF)

Human organotypical tissue cultures exposed to whole smoke from the reference cigarette 3R4F

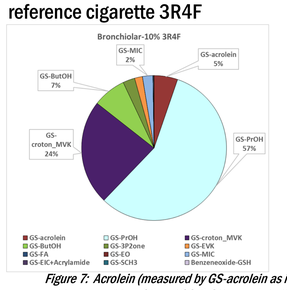


Figure 7: Acrolein (measured by GS-acrolein as initial adduct, see Figure 6B and GS-PrOH as metabolic reduction product), crotonaldehyde and MVK[1] are responsible for the majority (approx. 92-93%) of the GSH-adducts identified

[1] MVK: methylvinylketone

Migration Kinetics

Human organotypical bronchiolar tissue cultures exposed to cigarette smoke

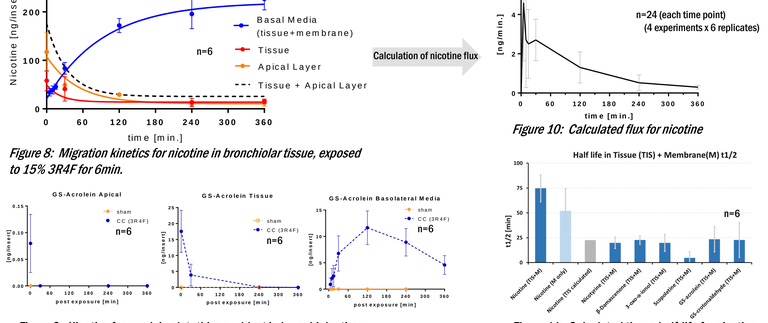


Figure 8: Migration kinetics for nicotine in bronchiolar tissue, exposed to 15% 3R4F for 6min.

Figure 9: Kinetics for acrolein glutathione adduct in bronchiolar tissue, exposed to 15% 3R4F for 6min.

Figure 10: Calculated flux for nicotine

Figure 11: Calculated tissue half-life for nicotine and additional compounds

Conclusions

- Subcellular fractions (microsomes, S9) are useful tools to study in vitro lung metabolism:
  - Metabolite profiles and reactive metabolites are relevant for toxicology & kinetics
  - Results from metabolism models using subcellular fractions can be utilized in more complex models, such as organotypical tissue cultures
- Metabolism studies will further substantiate the use of organotypical tissue culture models as a tool for in vitro respiratory toxicology testing
- Analytical methodologies were established to characterize the exposure of organotypical tissue cultures:
  - Nicotine and glutathione adducts of reactive carbonyls can be determined to describe the dose in exposed tissues
  - Glutathione serves as an additional marker for tissue functionality
- Dynamics of the exposed organotypical tissue cultures help to understand the biological system:
  - Migration kinetics (including adsorption, metabolism, excretion kinetics): => bioavailability of aerosol (compounds) by area under the curve (AUC)

References

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2) *Peterson L. A. Reactive metabolites in the biotransformation of molecules containing a furan ring. Chem. Res. Toxicol. 26:6 (2013).*
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Competing Financial Interest

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