Recent improvements in *in vitro* models need to be supported with refinements in dosimetry to enable possible future *in vivo* data extrapolation

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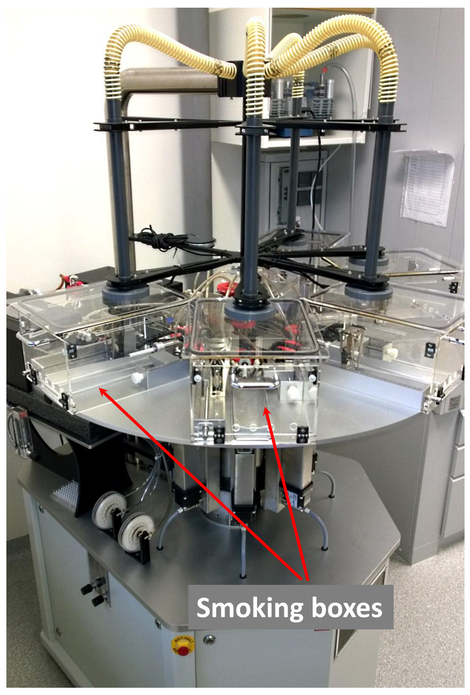
1. Introduction/Context

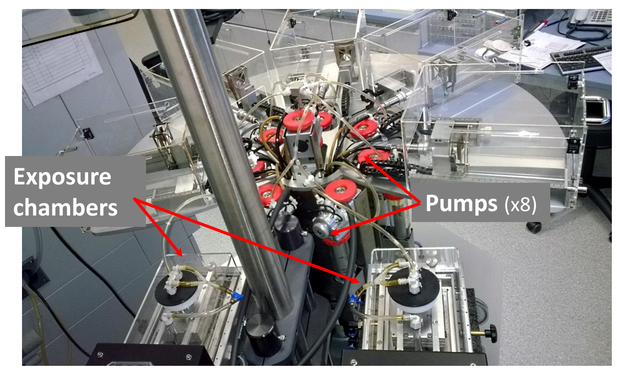
Based on the National Research Council (NRC) report "Toxicity Testing in 21st Century (2007)(1)" there is a focus on using human based *in vitro* assays as the ultimate replacement of animal studies. The use of 2D and 3D human lung cell models can be used to replicate physiologically relevant exposure to tobacco smoke and Electronic Vapour Products (EVP) in users.

For the assessment of tobacco additives, Imperial Tobacco toxicologists routinely use consumption data collected for the different product types, product specific additive transfer rates and assume 100 % deposition in the human respiratory tract. The estimated additive exposure is compared to relevant published animal data using standard uncertainty factors to calculate a Derived No Effect Level (DNEL). Estimated exposure values below the DNEL are considered tolerable. A better understanding of the dosimetry of whole smoke is required to investigate how *in vitro* assays could be potentially extrapolated to estimate human exposure levels. Future work will focus on *in vitro* exposure at the cellular level.

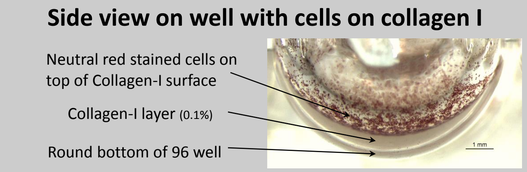
An additional consideration, is that the toxicological assessment of EVPs poses a challenge for standard assay systems. Due to the low toxicity of the vapour produced by EVPs, the sensitivity of *in vitro* test systems towards vapour components is of crucial importance. The direct exposure of human cells to the freshly generated smoke/vapour at air liquid interface (ALI) seems to be the most promising approach. Here, the cells need to be kept under optimal conditions during prolonged experiments to better replicate human exposure. Our in-house system enables accurate delivery of smoke/vapour to cells for extended periods and is key for potential incorporation of *in vitro* data into human exposure/hazard assessments.

2.Methods: Aerosol Exposure in Vitro System (AEIVS)



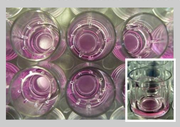


Side view on well with cells on collagen I



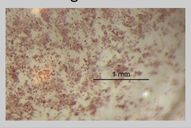
Optimal ALI exposure of cells on:

Porous membrane - Insert



or

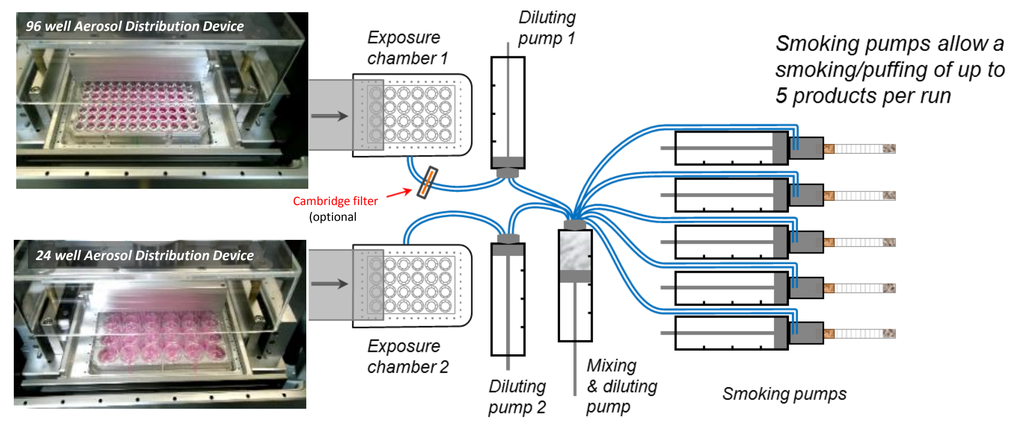
Collagen I matrix



Measurement of cytotoxicity - NRU

- Preparation of 96 MWP with Collagen I matrix
- Seeding of BEAS-2B cells
- Medium removed by cell washer and centrifugation
- Exposure time up to 3 hours
- Addition of medium and further incubation
- Exposure to neutral red and measurement at 540nm

AEIVS – Diagram of experimental set up



3. Results

Condensate evaluation

Nicotine vs. TPM extract in DMSO

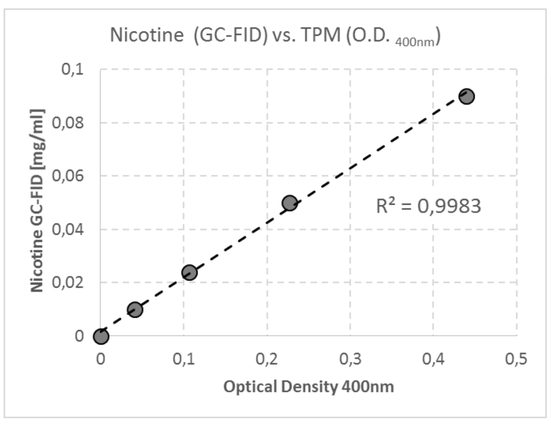


Fig.1: Nicotine trapped on Cambridge filter was measured by GC-FID and correlated to the optical density (400nm) of TPM extract in DMSO.

Smoke dosimetry in 24 well plates

- Each well of the 24 multi well plates (MWP) was filled with 300µl of DMSO
- Plates were exposed to differently diluted smoke of CM7 (undiluted - 1:10)
- Particulate matter concentrations were estimated by optical density (O.D. at 400 nm)

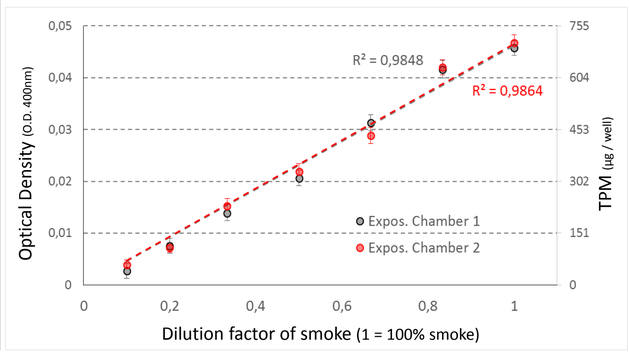


Fig.2: TPM concentration per well was calculated over calibration curve with known TPM concentration of CM7 in DMSO; R^2 confirms the linear smoke dilution function in each exposure chambers

Cytotoxicity of smoke and vapour to BEAS-2B cells in NRU assay

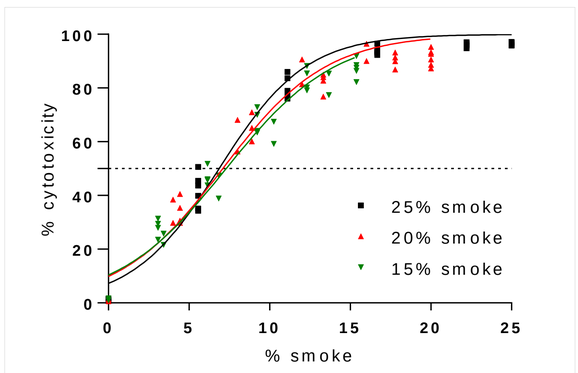


Fig.3: BEAS-2B cells were exposed to different whole smoke dilutions from the CM7 test piece (DF – Dilution Factor). EC50 calculations following the correction for the dilution result in nearly identical results showing that the dilution does not produce any artificial results.

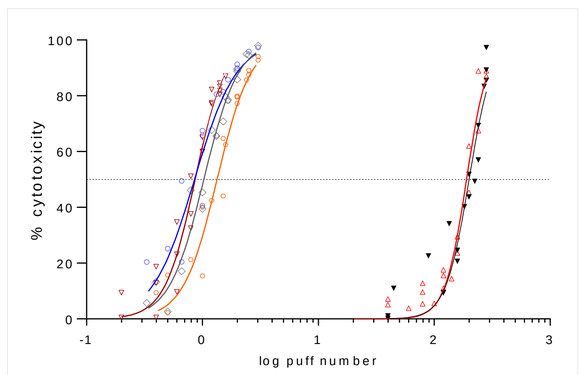


Fig.4: Cytotoxicity profiles of fresh smoke/aerosol of different product categories as determined in the AEIVS on a puff basis . From left to right the profiles of 4 market cigarettes (grey, burgundy, pink and orange) and 2 E-cigarette aerosols (black and red) are presented.

Effects of repeated smoke and vapour exposure using 3D lung model EpiAirway (MatTek)

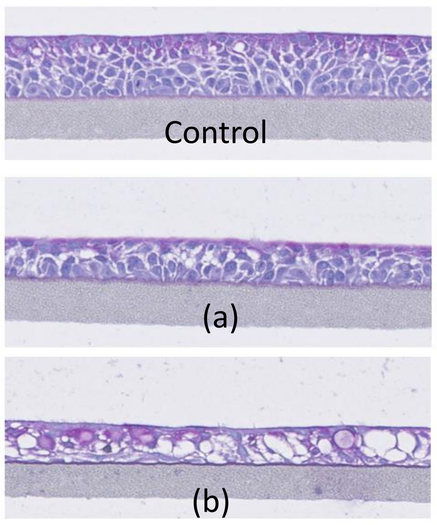


Fig.5: EpiAirway (MatTek) 3D model after 14 days incubation (untreated, 4 x exposed to e-cigarettes or 3R4F). Goblet cells are stained magenta with PAS. Clear areas represent an absence of cells.

(a) EpiAirway after exposure to 1200 puffs of an e-cigarette in total

(b) EpiAirway after exposure to 3.6 puffs of 3R4F smoke in total (36 puffs diluted 1:10)

4. 3Rs* Impact of research

- Imperial do not commission or conduct research involving animals and would not undertake such research unless formally requested to do so by a recognised regulatory authority
- Imperial Tobacco favours human *in vitro* alternatives, due to ethical considerations, physiological relevance, the speed of the assay and flexibility when compared to the use of animal models (2)
- The use of human derived cells/organ typical systems at the air-liquid interphase is considered to be representative of human exposure
- Human derived cellular systems are cost effective and provide potentially relevant insights into possible toxicity risks in humans

5. Conclusions/Future work

- Using AIEVS the linear dilution of smoke/vapour is both accurate and highly reproducible
- Achieves rapid delivery of whole smoke/vapour to the cells (within 10 seconds)
- The use of separated dilution pumps for both exposure chambers allows highly flexible and independent testing
- Prolonged exposure periods at ALI are possible (up to 3 hours)
- AEIVS enables a direct comparison of products and potential effects of product modifications at the same time

Future Research Initiatives

- Investigate AEIVS exposure using a 3D model for dosimetry, focusing on the internal exposure concentration of chemicals in cells (3)
- Explore possible extrapolation of *in vitro* data using a reverse Physiologically Based Pharmacokinetic (PBPK) model.
- Compare derived *in vivo* data to any relevant clinical trial data.
- Derive putative human no effect concentrations, using *in-vitro* data from a range of alternate assays (e.g. High Content Screening, HCS)

References

(1) National Research Council (NRC, 2007): Toxicity testing in 21st Century: A vision and a strategy https://www.nap.edu/catalog/11970/toxicity-testing-in-the-21st-century-a-vision-and-a; (2) Paur HR *et al.,* (2011) *In vitro* cell exposure studies for the assessment of nanoparticle toxicity of the lung – a dialogue between aerosol science and biology *J Aerosol Sci.* **42**; 668-692; (3) Groothius *et al.,* (2015) Dosimetric considerations in *in vitro* assays to improve quantitative *in vitro in vivo* extrapolations *Toxicol* **332**; 30-40. * Reduction , refinement and replacement of animals

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