The use of a quantifiable exposure method to assess the cytotoxic response of bronchial epithelial cells to whole smoke from a reduced toxicant prototype cigarette

David Azzopardi, Linsey E Haswell, Geoff Foss-Smith, Katherine Hewitt, Sarah Corke and Gary Phillips
British American Tobacco, Group Research and Development Centre, Southampton, SO15 8TL, UK

Corresponding email: David_Azzopardi@bat.com

INTRODUCTION
Prolonged cigarette smoke exposure is known to be a causative factor in the development of cardiovascular disease, chronic obstructive pulmonary disease (COPD) and cancer[1]. Cigarette whole smoke (WS) is a complex aerosol, comprising more than 6000 identified constituents distributed in the particulate and vapour phases, with the latter constituting ~95% by mass[2]. The role of each component of cigarette smoke in the development of smoking related diseases is largely unknown, however, exposure to both phases and the retention of smoke particulate has been implicated to contribute to smoking related injury and disease[1,3].

To address the role of cigarette smoke in disease development we are developing a suite of in vitro models to assist in the evaluation of reduced toxicant prototypes and the next generation of tobacco and nicotine related products. Here we describe in-house studies conducted using an in vitro smoking-related disease model of COPD (bronchial epithelial cell cytotoxicity) to evaluate the effects of reduced toxicant prototype (RTP2)™ cigarette WS on cell cytotoxicity compared to WS of reference (3R4F) and commercial control (CC) cigarettes.

Using Quartz Crystal Microbalance (QCM) technology it is possible to quantify WS particle mass. In this study we used QCMs fitted within our exposure chambers to quantify WS particle mass enabling direct comparisons of biological effects according to particle mass as opposed to smoke dilution.

METHODS
Cell culture and WS exposure
* Bronchial epithelial cells (NCI-H292; ECACC, Salisbury, UK) at passage 89 and a seeding density of 3 x 10⁴ cells/ml were grown in submerged culture on Transwell® inserts until confluent.
* The surface culture medium was removed to transition cells to the air-liquid interface (ALI) and cells were then transferred to purpose-built Perspex™ exposure chambers (Figures 1a & 1b).

![Figure 1a. BAT's whole smoke exposure chamber.](image1)

![Figure 1b. Schematic cross section of exposure chamber.](image2)

*A Borwaldt RM20S Smoking Machine (Borwaldt KC, Hamburg, Germany) was used to generate, dilute and deliver the cigarette smoke (Figure 2),

3R4F (University of Kentucky, USA: 9.4mg ISO tar), CC (7mg ISO tar) and RTP2 (7mg ISO tar) cigarettes were used.

Cigarettes were smoked to the ISO smoking regime (35 ml puff volume drawn over 2 seconds once every minute: ISO 3308:2000).

Air (AC) and cigarette WS were then delivered to the cells within the exposure chambers maintained at 37°C at the ALI.

![Figure 2. Borwaldt RM20S Smoking Machine. (A) Cigarette smoke generator. (B) Original 4 syringe system. (Bii) 4 syringe extension. (C) Air flow controller. (D) Cell culture media maintained at 37°C feeding exposure chambers with fresh cell culture medium (E) BAT exposure chamber housed at 37°C, attached to the smoke diluter and culture media.](image3)

* Cells were exposed at the ALI to a range of WS dilutions (1:2.5 to 1:4000 smoke:air, v:v) from a total of 4 cigarettes (~30 minute exposures).

* Following a 24 hour recovery period under culture medium cell viability was measured using the neutral red uptake assay.

Quantification of deposited particle mass using QCMs
* 4 BAT chambers each with a QCM (Vitrocell® Systems, Germany) fitted (Figure 3a) were connected to randomly selected smoke lines of the Borwaldt RM20S.

* Before smoke exposure the chambers were sealed and the QCM devices allowed to acclimatise at 37°C.

3R4F, CC and RTP2 cigarettes were smoked as per the biological exposures using a range of WS dilutions (1:2.5 to 1:1000 smoke:air, v:v).

The QCMs were read at a resolution of 10ng/cm²/second and mass recorded every 2 seconds.

Post exposure, QCMs were left to record real-time deposition until mass readings reached a plateau (Figure 3b).

![Figure 3a. QCMs fitted within the BAT exposure chamber.](image4)

![Figure 3b. An example real-time QCM trace.](image5)

STATISTICAL ANALYSIS
A linear regression was applied to the QCM data and a comparison of slopes performed. The cytotoxicity results were modelled using a sigmoidal four-parameter logistic curve. The effect of WS on the EC₅₀ (concentration of WS that kills 50% of the cells) was determined using the sum of squares F test. A p value of <0.05 was considered significant.

RESULTS
Quantification of deposited particle mass using QCMs
The slopes illustrate a regular linear relationship between smoke dilution and particle mass. There is a significant difference between the slopes of CC and 3R4F (P<0.05) and 3R4F and RTP2 (<0.0001). Although the CC and RTP2 cigarettes have identical tar deliveries the slopes are significantly different (P<0.05) (Figure 4).

![Figure 4. Deposited particle mass vs. smoke dilution. Each linear regression was used to convert the Borwaldt RM20S smoke dilutions used for the cytotoxicity assessments into deposited WS particle mass.](image6)

The effect of WS exposure from 3R4F, CC and RTP2 cigarettes on the cytotoxic response of NCI-H292 cells
There was no difference between the EC₅₀ of the CC and 3R4F, however, exposure to RTP2 resulted in a significant increase compared to CC (Figure 5).

![Figure 5. The effect of whole smoke exposure on NCI-H292 viability following exposure to 3R4F, CC and RTP2. The EC₅₀ for cells exposed to 3R4F, CC and RTP2 were 3.5, 3.6 and 19.2µg/cm² respectively.](image7)

SUMMARY
As determined using a quantifiable method, WS from RTP2 cigarettes is significantly less cytotoxic than WS derived from CC and 3R4F cigarettes.

CONCLUSION
Using a combination of QCM technology to quantify WS particle dose accurately and an in vitro ALI smoking-related disease model of COPD we can differentiate between the cytotoxic effects of different combustible products.

REFERENCES

Inhaled Particles XI Conference. 23-25 September 2013