In vitro toxicological assessment of total particulate matter of tobacco smoke and a pMRTP aerosol in human lung epithelial and coronary endothelial cells.

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Introduction

Cigarette smoke is a complex mixture of chemicals including volatile compounds contained in the gas-vapor portion (GVP) of the smoke aerosol and suspended liquid particles, referred to as total particulate matter (TPM). It is estimated that there are more than 6100 chemical constituents in tobacco smoke which lead to the development of serious diseases including cancer, chronic obstructive pulmonary disease and addiction to tobacco products [1].

In recent years, toxicological assessment of environmental agents has witnessed a strategic shift. Critical toxicity pathways perturbed upon exposure to such agents is now being identified and quantified using state-of-the-art tools and technologies including medium and high-throughput in vitro screening assays, computational toxicology, systems biology and pharmacokinetic modeling. This new strategy responds to the need of finding sound alternatives to animal testing. Some of these new approaches are based on in vitro testing using human cell lines, which better represent human biology.

Normal human bronchial epithelial cells (NHBE) and human coronary artery endothelial cells (HCAEC), two cell types relevant to respiratory and vascular diseases, respectively, were used to investigate the impact of TPM from a reference cigarette (3R4F) generated according to the Health Canada Intense (HCI) smoking protocol, and a prototype Modified Risk Tobacco Product (pMRTP).

Initial dose-range finding experiments were conducted in order to define the most appropriate smoke fraction doses to be further tested using a High Content Screening (HCS) platform. The initial evaluation of toxicity was performed using an impedance-based system, which is a real-time cellular analysis platform based on a multi-electrode array technology. The system uses tissue culture plates with sensor micro-electrodes covering approximately 70% of the area of each well bottom. The presence of the cells on top of the electrodes affects the local ionic environment at the electrode/solution interface, leading to an increase in the electrode impedance. The larger the number of cells attached to the electrodes, the larger the increases in electrode impedance. In addition, impedance readings are dependent on the quality of the cell interaction with the electrodes. For example, increased cell adhesion or spreading will lead to a larger change in electrode impedance. Thus, electrode's impedance can be used to monitor cell viability and cell number [2]. A further analysis of cell behavior was performed using a HCS platform with an automated fluorescence imaging system. A total of seven multi-parametric indicators of toxicity (Cellular count, Nuclear size, DNA structure, Mitochondrial mass, Mitochondrial membrane potential, Cytochrome C release and Cellular membrane permeability) grouped in 2 different assays were tested. This technology results from a combination of a fluorescence microscope, a high-throughput image acquisition system and a series of algorithms and software tools that allows for image processing and analysis. A schematic summary of the workflow is shown in Figure 1.

Workflow

1

Real time cell analyzer

An impedance-based measurement system is used to evaluate the toxicity of the total particulate matter fractions in NHBE and HCAEC cells to determine the appropriate doses to be further investigated in the high content screening.

High Content Screening

A total of 7 multi-parametric indicators of toxicity, grouped in 2 different assays (cytotoxicity and apoptosis/necrosis) were measured in NHBE and HCAEC cells using a high content screening (HCS) platform with automated fluorescence imaging system.

Results

- The combination of an impedance-based platform with HCS technologies provides a robust scientific approach to toxicological assessment of substance such TPM.
- This approach will be applied to a broader selection of Harmful and Potentially Harmful Constituents (HPHCs) and their mixtures to enable a systems biology-based risk assessment for pMRTPs.

Figure 1
Scheme of the workflow used for the study

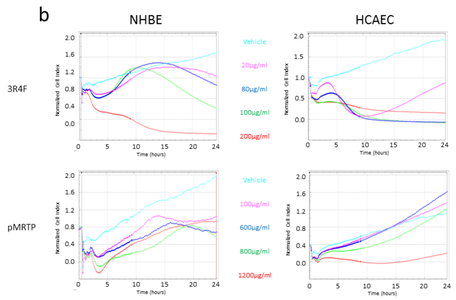
2

a

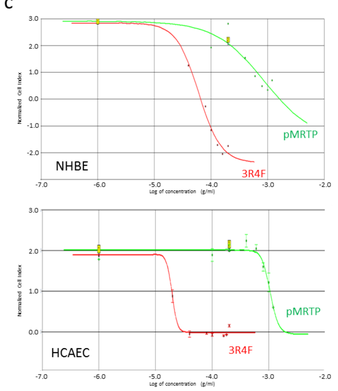
TPM concentration (µg/ml)

3R4F	pMRTP
200	1200
160	1000
100	800
80	600
50	400
40	200
20	100
0	0

b



c



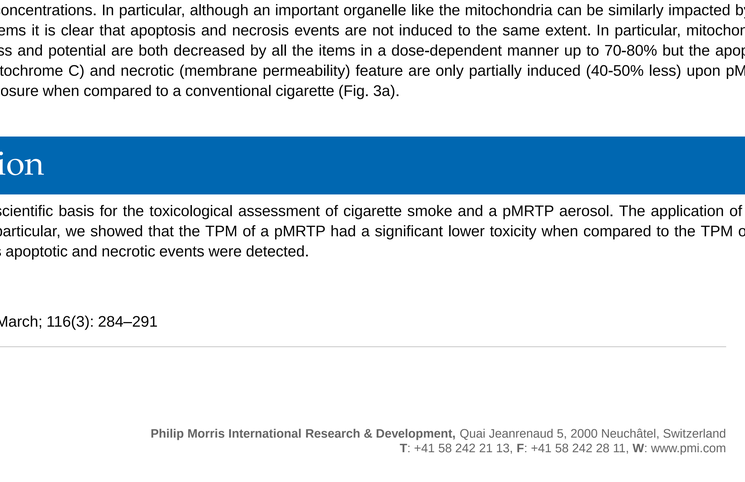
d

EC50(µg/ml) at 24h of exposure

	3R4F	pMRTP
NHBE	90	NC
HCAEC	20	1000

3

a



b

Cytotoxicity	Apoptosis/Necrosis
Cell count	Cell count
DNA structure	DNA structure
Nuclear size	Nuclear size
Mitochondrial mass	
Mitochondrial potential	
Cytochrome C release	Membrane permeability

Figure 3
(a) Scatter plot of the 7 different endpoints tested with the HCS platform. Only the NHBE data are shown.
(b) Table of HCS-based feature investigated in two different protocols.
(c) Dose response curve based on the cell index at 24 hours.
(d) EC50(µg/ml) at 24h of exposure.

Materials and Methods

Real time cell analyzer:

- NHBE cells were seeded in E-culture plates at a density of 7200 cells in 100 µl of culture media per well.
- HCAEC cells were seeded in a Collagen A coated E-culture plate at a density of 2000 cells in 100 µl of culture media per well.
- Cells were incubated for 24 h in triplicates before exposure to increasing doses of TPM.
- Appropriate positive (Carbonyl cyanide m-chlorophenyl hydrazone) and negative controls (ethanol, culture medium) were included in each experimental plate.

High Content Screening:

- NHBE cells were seeded in black, clear-bottom 96-well tissue culture plates at a density of 12000 cells in 100 µl of culture media per well.
- HCAEC cells are seeded in black, collagen-coated clear-bottom 96-well tissue culture plates at a density of 6500 cells in 100µl of culture media per well.
- Cells were incubated for 24h in culture media before exposure to increasing doses of TPM.
- Appropriate positive (Carbonyl cyanide m-chlorophenyl hydrazone) and negative controls (ethanol, culture medium) were included in each experimental plate.
- Cells were exposed in triplicates for 24h.
- Seven multi-parametric indicators of toxicity, grouped in two separate assays were measured.

Results

In this study, we examined the effects of a conventional reference cigarette (3R4F) and a pMRTP on NHBE cells and HCAEC, two cell types relevant to respiratory and vascular diseases.

Cells were exposed to TPM at different doses for 24 hours. Our initial assessment performed using the impedance-based real-time cellular analysis system showed that the effects of TMP from 3R4F on cell index, a surrogate of impedance, were dose-dependent in both cell types (Fig. 2b). Although the highest possible dose of TPM from the pMRTP was used in NHBE cells, no EC50 calculation was possible since none of the concentrations tested induced a complete cell death. EC50 values were calculated in HCAECs as the effect was 50 times higher compared to values obtained for conventional cigarettes (Fig. 2c-d). A comparison of the EC50s obtained from the two different cellular models suggests that HCAECs were more sensitive to the effects of TPM from 3R4F compared to NHBE cells; in fact, the 3R4F curve was shifted to the left and its EC50 was 4.5 times lower compared to NHBE cells (Fig. 2d).

HCS data further confirmed the lower toxicity of the pMRTP, in some of the parameters tested the highest concentrations could not match the toxic effects given by the conventional cigarette which were tested at a lower range of concentrations. In particular, although an important organelle like the mitochondria can be similarly impacted by the 2 items it is clear that apoptosis and necrosis events are not induced to the same extent. In particular, mitochondrial mass and potential are both decreased by all the items in a dose-dependent manner up to 70-80% but the apoptotic (Cytochrome C) and necrotic (membrane permeability) feature are only partially induced (40-50% less) upon pMRTP exposure when compared to a conventional cigarette (Fig. 3a).

Conclusion

The current study shows that the combination of impedance-based measurements and HCS-assessment provides a robust scientific basis for the toxicological assessment of cigarette smoke and a pMRTP aerosol. The application of both platforms allowed a better evaluation of the biological impact of TPM to compare a pMRTP with a conventional cigarette. In particular, we showed that the TPM of a pMRTP had a significant lower toxicity when compared to the TPM of the conventional cigarette 3R4F at comparable doses and although the mitochondrial mass and potential are equally impacted less apoptotic and necrotic events were detected.

References

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2. Xia, M. et al. *Compound cytotoxicity profiling using quantitative high-throughput screening.* Environ Health Perspect. 2008 March; 116(3): 284–291

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