Multiplexed High Content Screening Analysis Reveals Reduced Toxicity of a Prototypic Modified risk Tobacco Product (pMRTP) as Compared to a Conventional Cigarette

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 # 537

Introduction

Cigarette smoke (CS) is a complex mixture with more than 6100 chemicals [1]. Exposure to CS causes lung toxicity and increases the risk of developing chronic obstructive pulmonary disease and cancer. Gaining insight into the cellular processes impacted upon exposure would be very useful to understand CS toxicity and aid the development of Modified Risk Tobacco Products (MRTP) products.

In this study, normal human bronchial epithelial (NHBE) cells were used to investigate the biological impact of total particulate matter (TPM) from the University of Kentucky reference research cigarette 3R4F and a prototype Modified Risk Tobacco Product (pMRTP). TPM samples were generated under a Health Canada smoking regimen. Cell viability was measured using a real-time cellular analysis system based on a multi-electrode array technology. In addition, eleven multi-parametric toxicity endpoints were measured using a High Content Screening (HCS) platform. The different endpoints were measured over a range of TPM concentrations and at two different exposure time points (4h and 24h).

Materials and Methods

Real-time cell analyzer

High Content Screening

Real time cell analyzer:

NHBE cells (purchased from Lonza, Basel, Switzerland) were seeded in 96-well tissue culture plates and incubated in medium for 24h. Cells were then exposed in triplicate to increasing doses of TPM from 3R4F and pMRTP for additional 24h. Impedance values were recorded in each well every 15 minutes as a readout of cell viability. Ethanol was used as vehicle. Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) was used as a positive control.

High Content Screening:

NHBE cells were seeded in 96-well tissue culture plates and incubated in medium for 24h. Cells were then exposed in triplicate to increasing doses TPM from 3R4F and pMRTP for additional 4h or 24h. Ethanol was used as vehicle. Appropriate positive controls were used for each endpoint. Eleven multi-parametric toxicity endpoints, grouped into different assays were measured.

- **Cell count**
- **Nuclear size**
- **DNA structure**
- **P-H2AX (DNA damage)**
- **P-cJun (Stress kinase)**
- **P-H3 (Mitosis)**
- **Mitochondrial potential**
- **Mitochondrial mass**
- **GSH content**
- **Cell membrane permeability**
- **Cytochrome C release**

Conclusions

❖ Our study provides mechanistic insight into the toxicity pathways activated by CS TPM on lung epithelial cells, and how, by lessening the biological impact on key cellular processes, exposure to a pMRTP reduces overall toxicity when compared to a conventional product, the reference cigarette 3R4F.

❖ Furthermore, the ability to detect biological perturbations at sub-toxic doses strongly supports the use of a combination of impedance-based measurements and a HCS-based approaches for the toxicological assessment of pMRTPs.

❖ This study will be used to support the approach to develop a systems biology-based risk assessment for MRTPs

References

1. Rodgman, A., and Perfetti, T.A. *The Chemical Components of Tobacco and Tobacco Smoke.* 2013.
2. GraphPad Prism Version 5.00 for Windows. GraphPad Software, San Diego, California, USA

RESULTS

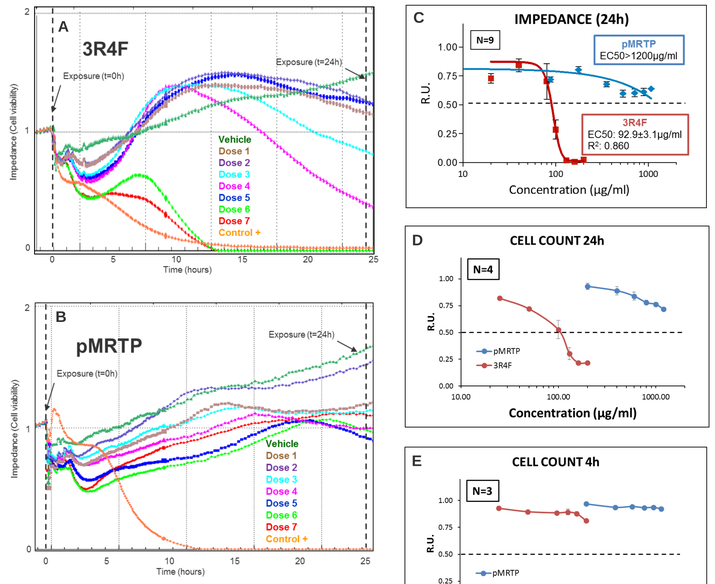

Figure 1. Cell Viability.

A) & **B)** Representative real-time cell analysis experiment showing the effect of TPM from 3R4F and pMRTP on NHBE cell viability. Impedance is used as a read-Out of cell viability. Data was acquired every 15 minutes and represents the average of three replicate wells. Vertical dotted lines delimitate the exposure period (0-24h). CCCP was used as positive control (control +) **C)** Normalized impedance values after 24 h of exposure. Data represents average ± SEM of 9 independent experiments and was fitted using GraphPad Prism® 5.0 [2]. **D) & E)** Average number of cells per well, measured using a HCS platform after exposure to 3R4F and the pMRTP for 4h or 24h respectively. Values represent average ± SEM of 3-4 independent experiments. Horizontal dotted line indicates 50% cytotoxicity. R.U. means relative units.

Longer exposures (24h) to 3R4F TPM further increased DNA damage and stress kinase activation, compared to the 4h time-point. In addition, we observed the presence of oxidative stress (measured as a decrease in GSH content) and the presence of apoptosis (measured as cytochrome C release) and necrosis (measured as cellular membrane permeability). By contrast, only moderate responses in DNA damage, GSH content and cellular membrane permeability were observed in cells exposed to TPM from pMRTP, and only at the highest doses tested.

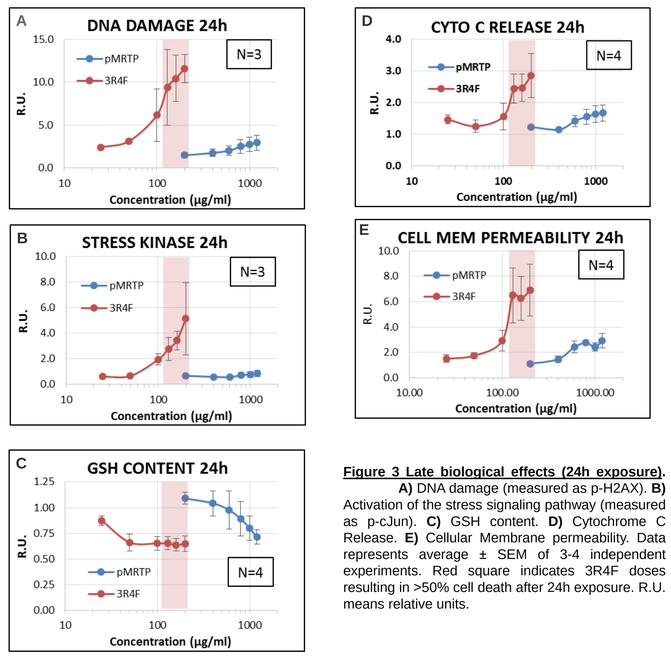

Figure 3 Late biological effects (24h exposure).

A) DNA damage (measured as p-H2AX). **B)** Activation of the stress signaling pathway (measured as p-cJun). **C)** GSH content. **D)** Cytochrome C Release. **E)** Cellular Membrane permeability. Data represents average ± SEM of 3-4 independent experiments. Red square indicates 3R4F doses resulting in >50% cell death after 24h exposure. R.U. means relative units.

Short exposures (4h) to TPM from either 3R4F or pMRTP caused no apparent cellular death (**Figure 1D**). Exposure to 3R4F TPM resulted in increased DNA damage, a dose-dependent activation of the stress kinase pathway and increased cellular membrane permeability. Moreover 3R4F TPM caused a decrease in mitochondrial membrane potential without changes in mitochondrial mass (data not shown), suggesting the induction of mitochondrial toxicity. By contrast, only mild responses were observed upon exposure to TPM from pMRTP at any given endpoint, and only at the highest doses tested.

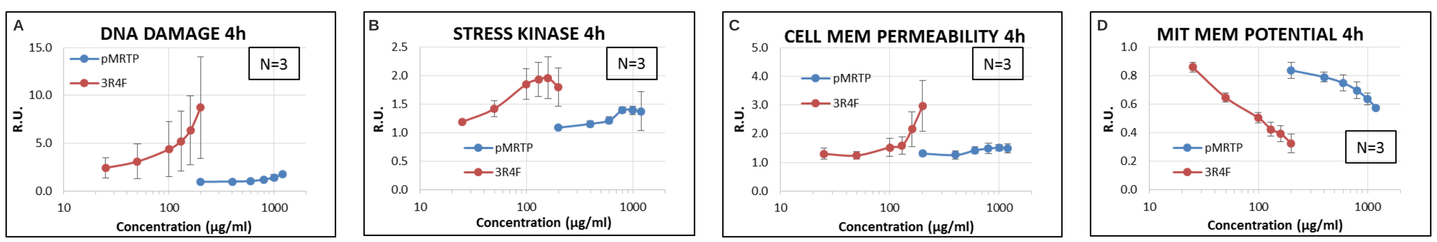

Figure 2 Early biological effects (4h exposure). A) DNA damage (measured as p-H2AX). **B)** Activation of the stress signaling pathway (measured as p-cJun). **C)** Cellular membrane permeability. The dose-response effect observed in 3R4F-exposed cells occurred in the absence of apparent cell death (**Figure 2D**). **D)** Mitochondrial membrane potential. No changes in mitochondrial mass were observed for either 3R4F or pMRTP (Data not shown). Data represents average ± SEM of 3 independent experiments. R.U. means relative units.

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