In House Development of In Vitro Micronucleus Assay Screening Next Generation Tobacco Products Using Microflow

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Introduction

In vitro micronucleus assay (IVMN) is a genotoxicity test for the detection of micronuclei in the cytoplasm of interphase cells. It is widely accepted by regulatory bodies as an in vitro method to investigate chromosomal damage potential after exposure to toxicants such as those found in tobacco and tobacco smoke condensates.

Studies suggest heated products (HP) emissions are less biologically active compared to cigarette smoke. The aim of this study was to establish the IVMN assay to screen HP emissions and assess the potential genotoxicity of HP aerosol compared to cigarette smoke using Litron MicroFlow® kits (Bryce SM *et al.*, 2010). This was done by comparing TPM (total particulate matter) from reference cigarette 1R6F smoke with HP aerosol collected mass (ACM) gathered from internal reference stick eHTPB2 and a commercially available HP (HP comparator).

Methodology

1 Test Articles

1R6F reference cigarette, HP reference stick eHTPB2 and a HP comparator were assessed in this study. The process of capturing cigarette smoke TPM and HP aerosol ACM is shown in Figure 1, stock concentrations of TPM and ACMs are listed in Table 1.

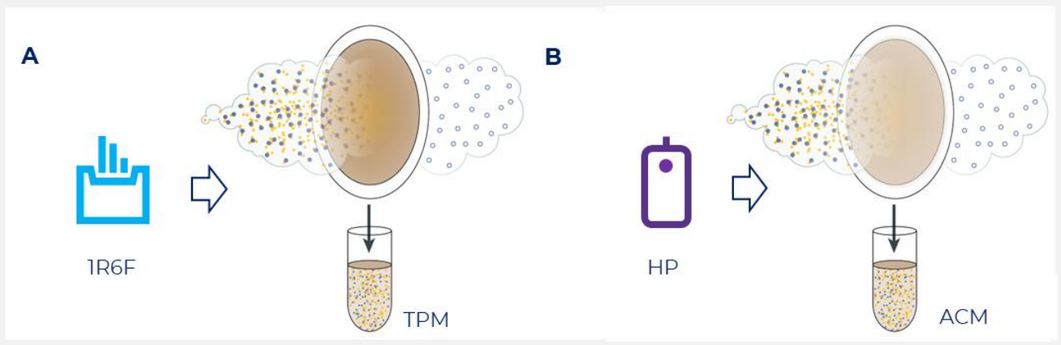

Figure 1. Process of capturing 1R6F TPM (**A**) and HP product ACM (**B**). Aerosol was generated following HCIm regime using Körber LM4E vaping machine. Appropriate amount DMSO was added to dissolve and extract TPM and ACM. TPM= total particulate matter, ACM = aerosol collected mass.

Table 1. Stock TPM and ACM concentrations.

Test Articles	Stock concentration
1R6F TPM	24 mg/mL
eHTPB2 ACM	150 mg/mL
Comparator HP product ACM	150 mg/mL

2 *In vitro* Micronucleus Assay

- Chinese hamster lung cells (V79) were seeded at 0.25 x 10^5 per well in 24-well plates.
- TPM or ACM was diluted in DMEM at maximum 1% of DMSO in stock concentration. Treatment was carried out under 3-hour –S9 and 24-hour –S9 conditions.
- Cells were treated with Litron MicroFlow® kit according to manufacture instructions.
- 300 µL of samples were transferred to 96-well plates before analysed with BD Canto II FACS machine.
- Vehicle control and untreated controls were tested, mitomycin C (MMC) was used as positive control in both testing conditions.

Results: MMC As Positive Control

Table 1. Stock TPM and ACM concentrations.

	MN (%)	Cytotoxicity (%)
MMC 2 µg/mL (3h)	17.25	59.54 ± 5.09
MMC 0.2 µg/mL (24h)	19.10	55 ± 0.97

MMC induced positive response in micronuclei induction at both 3-hour and 24-hour treatment conditions.

Results: Micronucleus And Cytotoxicity After 3-hour Treatment

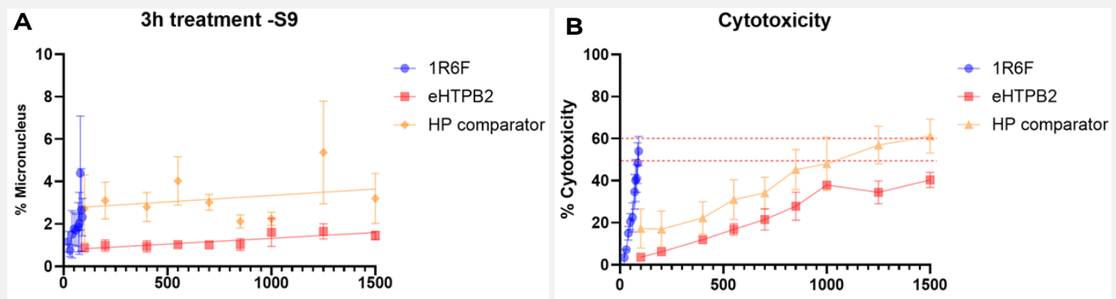

Figure 3. Micronucleus percentage (**A**) and cytotoxicity (**B**) of 1R6F TPM, eHTPB2 and HP product ACM after 3-hour treatment without presence of S9. Optimum cytotoxicity of 50-60% was highlighted in red dotted line. N=3 . Data are presented as mean ± SD.

- 1R6F, eHTPB2 and HP comparator all showed dose-related increase in micronucleus induction and cytotoxicity.
- 3-fold increase in micronuclei formation was observed for 1R6F TPM at 90 µg/mL, higher than eHTPB2 and HP comparator at 1500 µg/mL.
- 1R6F TPM reached optimum cytotoxicity range (50-60%) at 90 µg/mL, HP comparator reached this range at maximum testing concentration of 1500 µg/mL.

Results: Micronucleus And Cytotoxicity After 24-hour Treatment

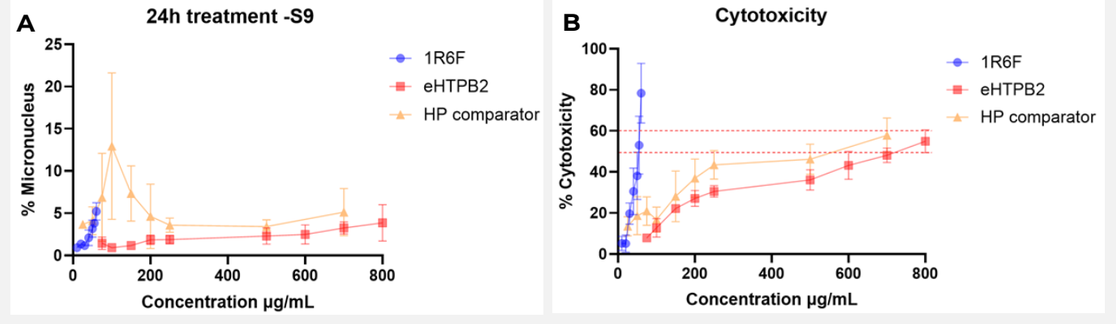

Figure 3. Micronucleus percentage (**A**) and cytotoxicity (**B**) of 1R6F TPM, eHTPB2 and HP product ACM after 24-hour treatment without presence of S9. Optimum cytotoxicity of 50-60% was highlighted in red dotted line. N=3 . Data are presented as mean ± SD.

- 1R6F, eHTPB2 and HP comparator all showed dose-related increases in micronucleus induction and cytotoxicity.
- 8-fold increase in micronuclei formation was observed in 1R6F TPM at 60 µg/mL, higher than observed for eHTPB2 and HP comparator at maximum testing concentrations.
- 1R6F TPM reached optimum cytotoxicity range (50-60%) at 60 µg/mL, HP comparator reached at maximum testing concentration of 800 µg/mL.

Conclusion

This study demonstrated that IVMN assay may be a viable tool to assess the potential genotoxicity of traditional cigarette and HPs using the method presented.

1R6F cigarette TPM and HP ACM showed dose-related responses in micronuclei induction under both 3-hour and 24-hour treatment conditions.

Both HPs we tested showed less genotoxicity and cytotoxicity compared to 1R6F cigarette, considering less micronuclei generated, and less cytotoxicity observed at the same testing concentration.

This study will be further supported with +S9 data.

Reference

Bryce SM, Shi J, Nicolette J, Diehl M, Sonders P, Avlasevich S, Raja S, Bemis JC, Dertinger SD. High content flow cytometric micronucleus scoring method is applicable to attachment cell lines. Environ Mol Mutagen. 2010 Apr;51(3):260-6.

Acknowledgements

The authors are grateful to everyone who took part in the project in pre-clinical team for the support. A special thank you goes to Steven Bryce from Litron for all the suggestions made.

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