

The genotoxicological assessment of a tobacco heating product aerosol relative to cigarette smoke



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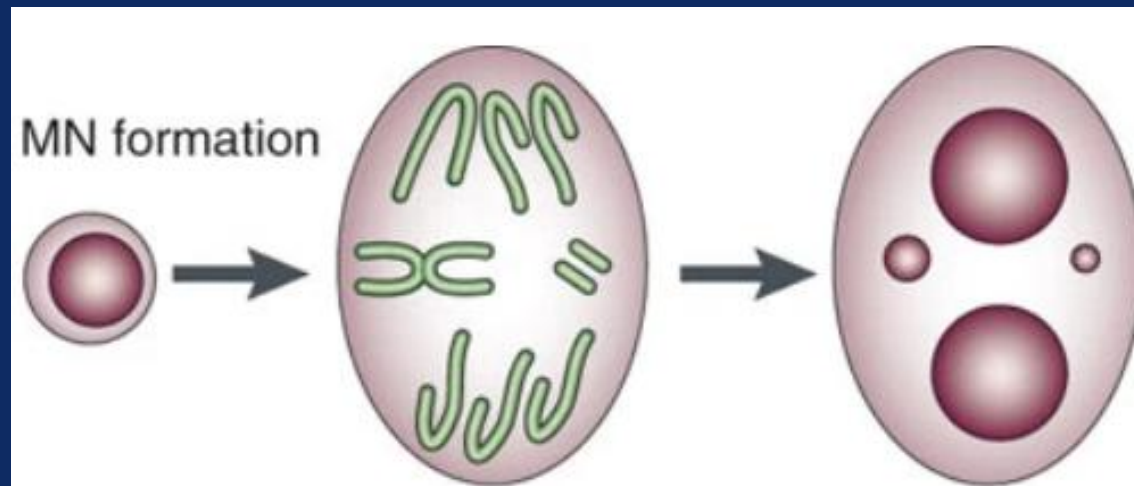
Micronucleus test overview



IVMN

Measure of
genotoxicity

The ability to
measure the
potential for genetic
damage



Following exposure to
a genotoxic substance,
damage to the genetic
material can occur

The damaged
genetic material is
left behind as
'micronuclei'

Chromosomal damage is an important stage in
carcinogenesis and other diseases

Approach to testing

Unflavoured HTP

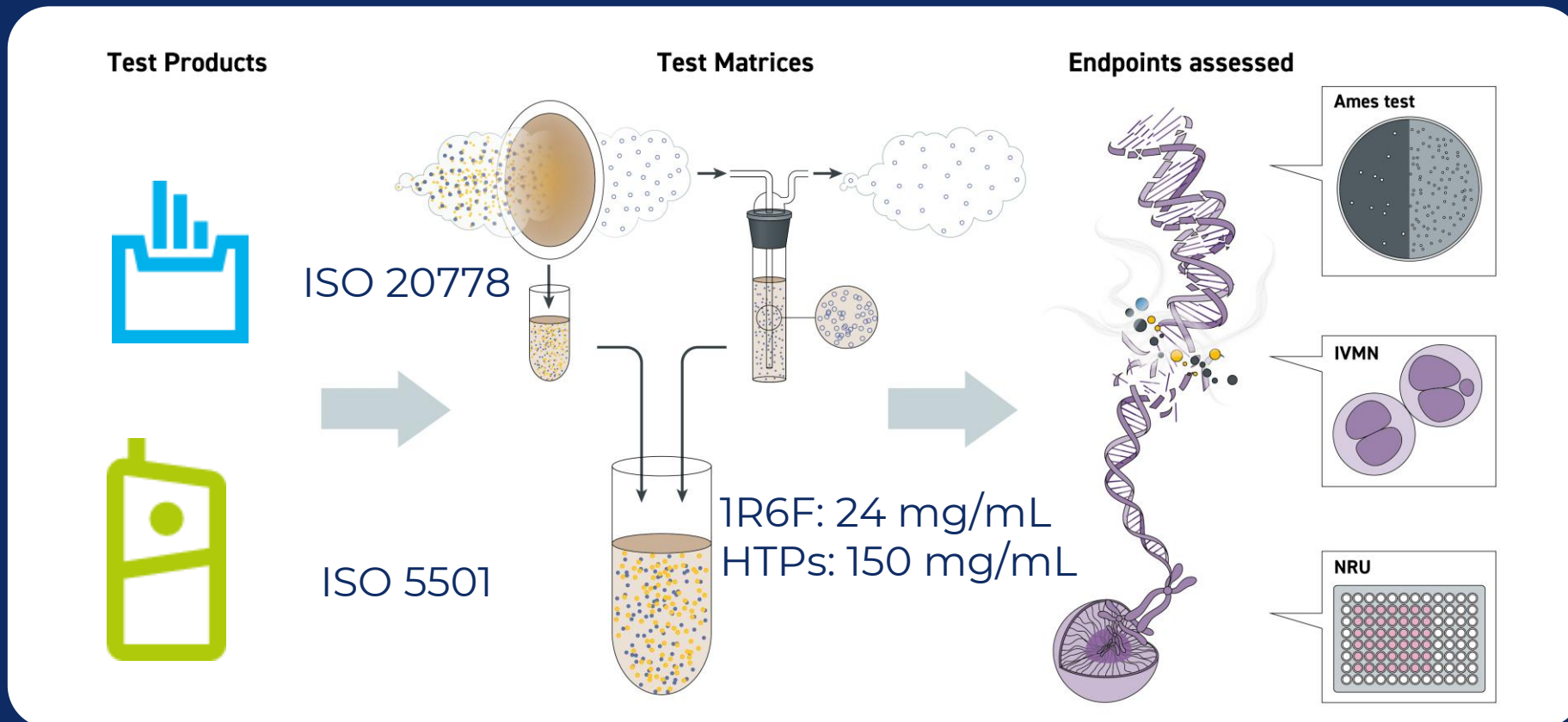
Reconstituted
Virginia tobacco
plus glycerol (14.8%)

Flavoured HTP

Reconstituted
Virginia tobacco
plus glycerol (14.8%),
and flavourings

3R4F

Kentucky reference
cigarette



The *in vitro* micronucleus test

V79 cells seeded in 75 cm³ flasks (4 replicate cultures per concentration), using the cytochalasin B cytokinesis block method

Cells treated with a range of at least 5 concentrations for 3 hours with a 21-hour recovery period in the absence and presence of S9, and for 24 hours with no recovery period in the absence of S9.

Positive controls used were cyclophosphamide in the 3h+S9 treatments, mitomycin C in the 3h –S9 treatment and vinblastine in the 24h –S9 treatment

Following the treatment/recovery period, slides were prepared with staining for manual scoring

An initial dose range finder was used to identify the appropriate concentration range to test, to avoid concentrations that exceeded 60% cytotoxicity

Acceptance & evaluation criteria

Acceptance Criteria

Vehicle controls are within the historical control ranges

Positive controls induced statistically significant increases in micronucleated binucleate cells

At least 50% of cells had gone through one cell division

Evaluation Criteria

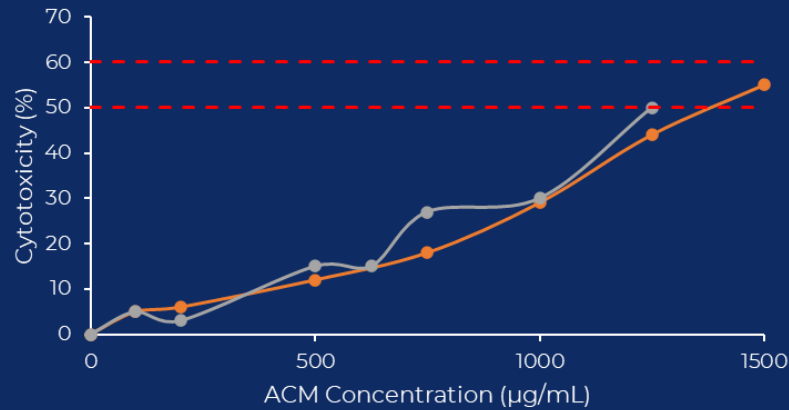
The TPM/ACM induced statistically significant increases in micronuclei

A concentration related increase was seen

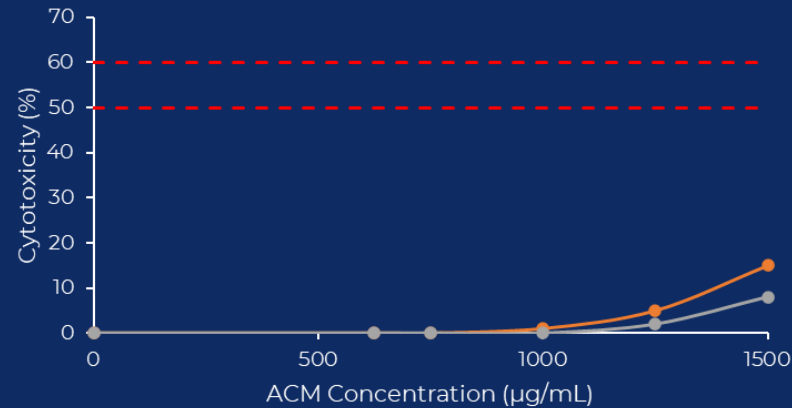
The induction of micronucleated binucleate cells exceeded the historic vehicle control ranges

Results - cytotoxicity

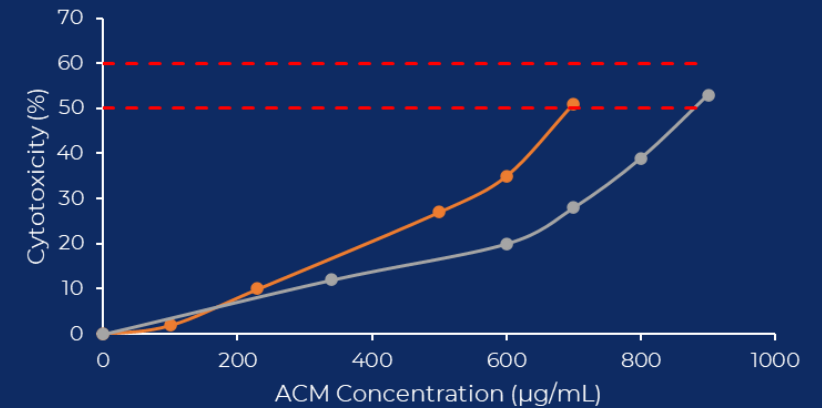
3h -S9



3h +S9

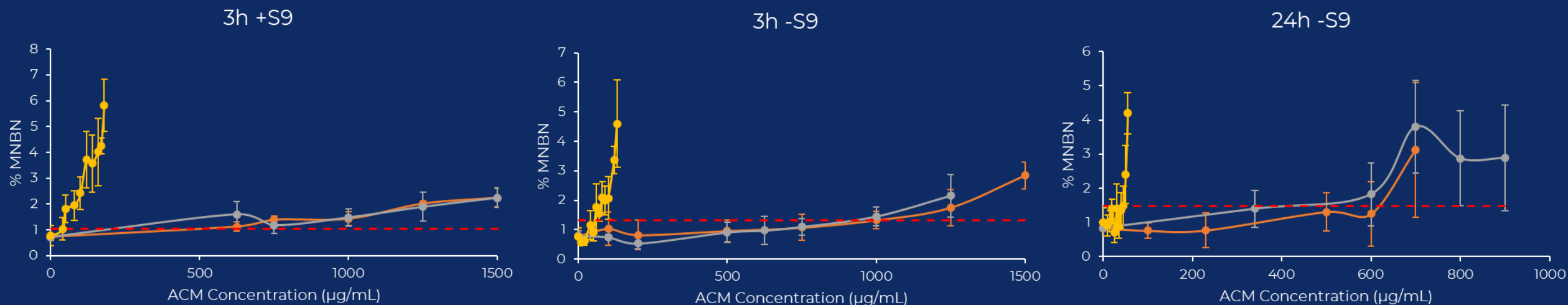


24h -S9



Clear differences in the range of concentrations used in each treatment condition
Only the 3h+S9 did not reach adequate levels of cytotoxicity, despite testing at the maximum feasible concentration

Results – micronuclei induction



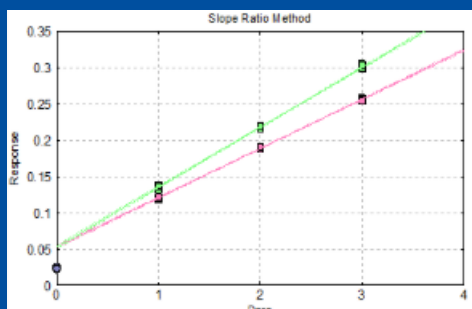
Both the unflavoured and flavoured HTP induced increases in micronuclei in all treatments
The response of the unflavoured and unflavoured HTP were similar to each other

—●— Flavoured
—●— Unflavoured
—●— 3R4F

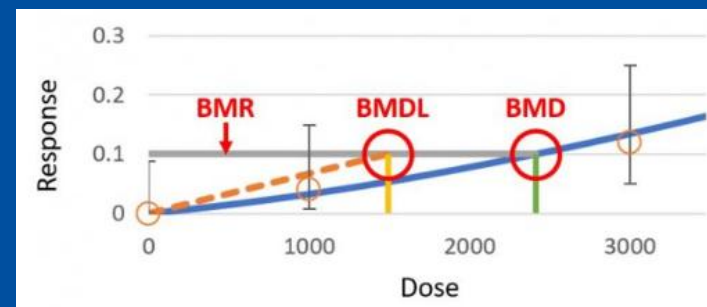
Approaches to analysis

Comparative analysis was only applied if both products to be compared provided dose related increases that were statistically significant and biologically relevant

Slope analysis



BMD analysis



BMR: Benchmark Reference
BMD: Benchmark Dose
BMDL: lower bound 95% confidence limit

<https://www.epa.gov/bmds/frequent-questions-about-bmds-modeling-tools>
<https://www.unistat.com/guide/bioassay-analysis-slope-ratio-method/>

Focus on BMD approach

TPMs were compared using a benchmark dose (BMD) approach with BMDS 2.6 software (www.epa.gov/bmds)

The application was run using default the wizard settings (BMDS Wizard 1.10)

The benchmark reference was set as the 95% upper limit of the vehicle control as the threshold and the selected model was based on the recommendation of the wizard and the lowest Akaike Information Criteria (AIC).

The BMD was defined as the concentration of the test item to induce a response that was equal to the 95% upper limit of the historic vehicle control range and the BMDL was defined as the lower bound 95% confidence limit.

Focus on BMD approach

	3h -S9				3h +S9				24h -S9			
	TPM		Nicotine		TPM		Nicotine		TPM		Nicotine	
	BMD	BMDL	BMD	BMDL	BMD	BMDL	BMD	BMDL	BMD	BMDL	BMD	BMDL
3R4F	46.5	40.3	2.22	1.92	17.7	11.7	0.84	0.56	44.4	41.6	2.12	1.99
Flavoured	946	848	21.58	19.35	463	329	10.56	7.51	572	474	13.05	10.81
Unflavoured	875	795	20.05	17.98	341	68.6	7.81	1.57	356	273	8.16	6.26

Conclusions

The IVMN is a valuable tool for evaluating tobacco and nicotine-containing products

The sensitivity of the cell line used needs to be taken into consideration, as does the highest concentration of the respective test articles, and their preparation

Several methods exist to valuate the data, including slope analysis and benchmark dosing

In all instances, the methods/software and parameters used should be documented, and the rationale for the BMD justified