Using the *in vitro* MN test for product comparisons: Comparisons within a product category and some proposed best practices

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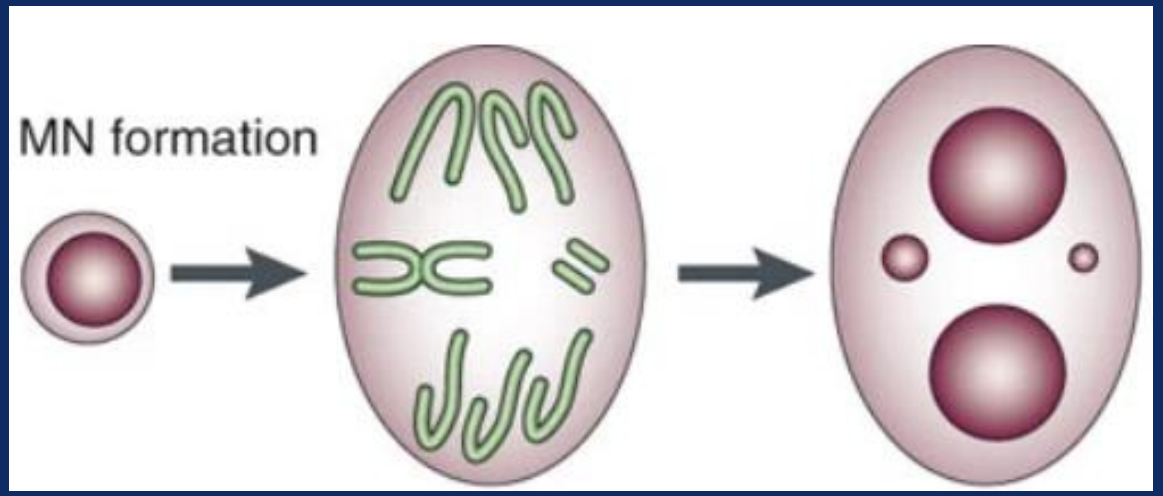
Micronucleus test overview

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

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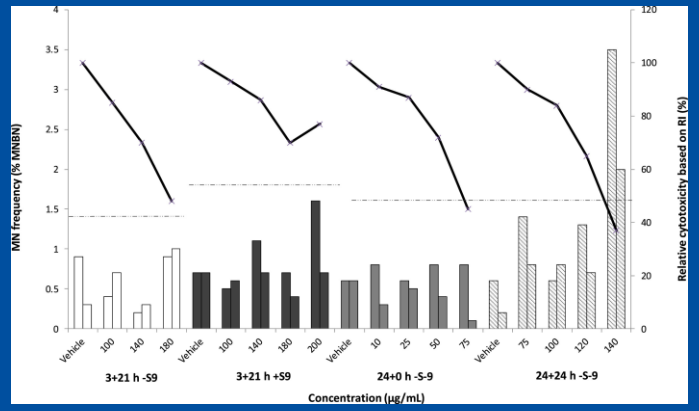
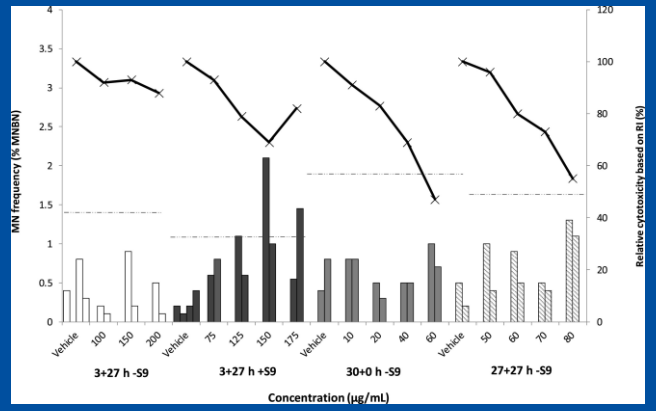
Choice of cell line

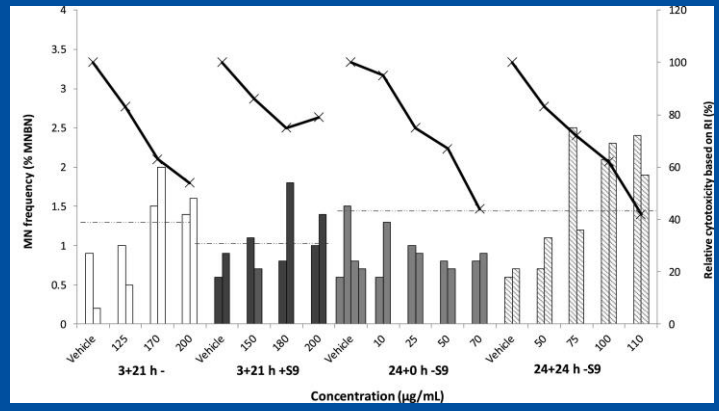
- The choice of cell line for the *in vitro* micronucleus is an important consideration
- TPM from 1R6F was generated and tested with TK6, CHO and V79 cells



CHO



TK6



V79

Condition	3+21h (-S9)[b] 3+27h (-S9)[c]	3+21h (+S9)[b] 3+27h (+S9)[c]	24+0h (-S9)[b] 30+0h (-S9)[c]	24+24h (-S9)[b] 27+27h (-S9)[c]
CHO	Negative[a]	Negative[a]	Negative	Positive[a]
V79	Positive	Weak positive[a]	Negative	Positive
TK6	Negative[a]	Weak positive[a]	Negative	Weak positive[a]

Thorne et al, https://doi.org/10.1016/j.fct.2019.05.054

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Case Study #1: Heated Tobacco Products

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Approach to testing

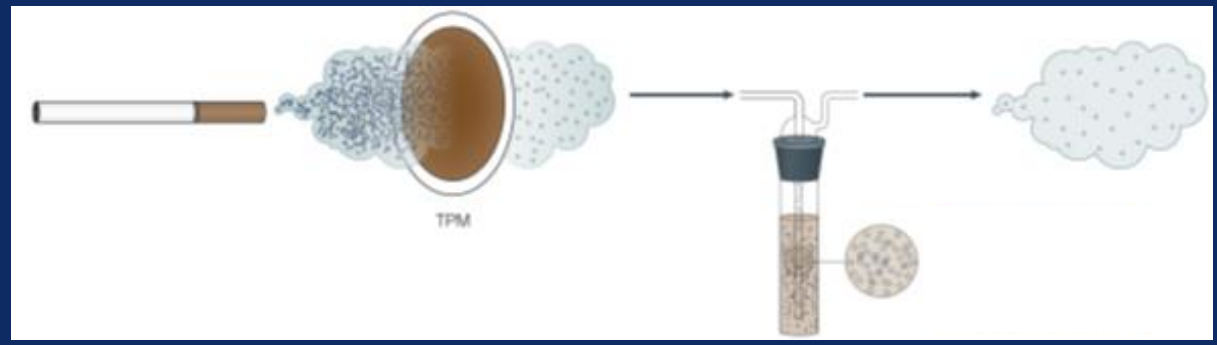
Unflavoured HTP
Reconstituted Virginia tobacco plus glycerol (14.8%)

Flavoured HTP
Reconstituted Virginia tobacco plus glycerol (14.8%), and:

Flavouring	% Inclusion
Cinnamic acid, Guaiacol, Hexanoic acid, Lime oil, Methyl butyraldehyde (3-), Methyl cyclopentonolone, Phenyl-2-propen-1-ol (3-)	0.001-0.01%
Anethole (trans), Cinnamaldehyde, Cinnamyl cinnamate, Citral, Dodecalactone (delta-), Ethyl maltol, Eugenol, Limonene, Methyl benzyl acetate (alpha-), Peppermint oil, Piperonal, Spearmint oil, Vanillin, Undecalactone (gamma-)	0.01-0.1%
Mandarin oil, Orange oil, Tangerine oil	0.1-1.0%

3R4F
Kentucky reference cigarette

Puffed to the ISO 20778 (HCI) regime
No HTP vent blocking



ACM extracted with DMSO

ACM
HTPs: 150 mg/mL

Crooks, I, et al. https://doi.org/10.1016/j.fct.2018.05.058

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The *in vitro* micronucleus test

V79 cells seeded in 75 cm^3 flasks (4 replicate cultures per concentration), using the cytochalasin B cytokinesis block method

Cells treated with a range of at least 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

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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

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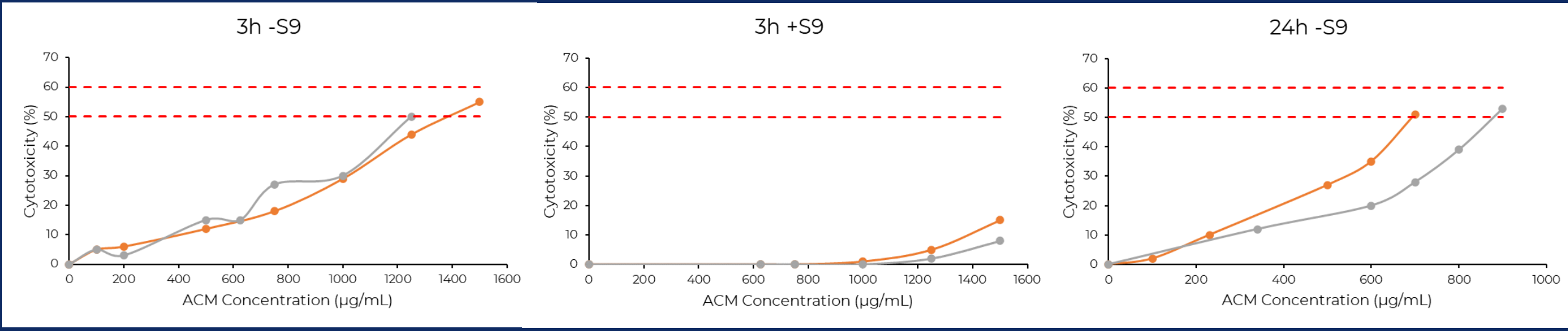
Results - cytotoxicity



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

Crooks, I, et al. https://doi.org/10.1016/j.fct.2018.05.058

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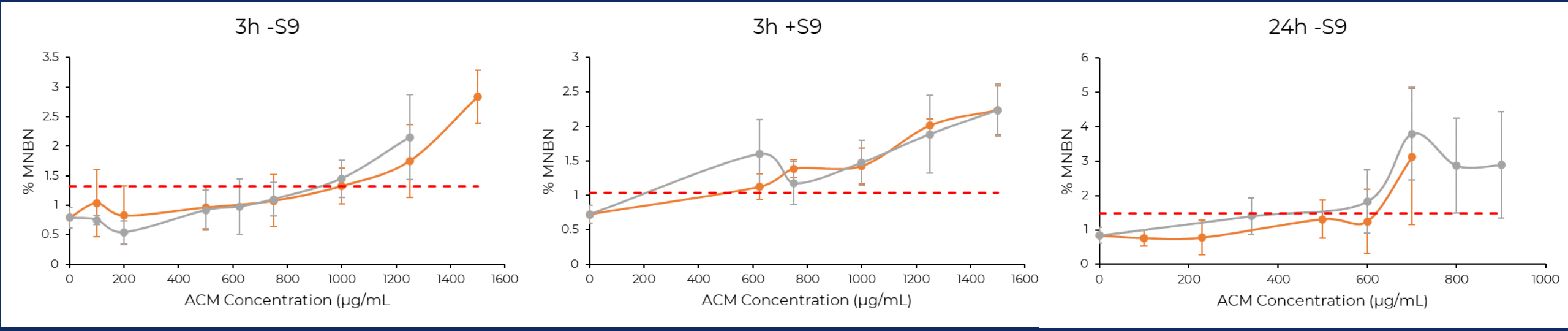
Results – micronuclei induction



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

Crooks, I, et al. https://doi.org/10.1016/j.fct.2018.05.058

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Case Study #2: Experimental Cigarettes with modified toxicants

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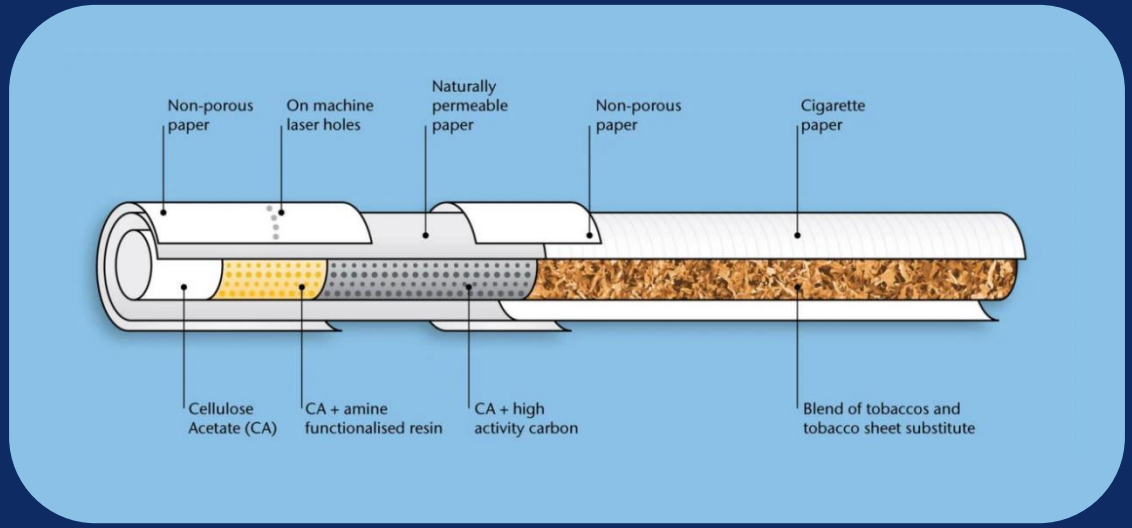
FMC technologies

Product	Blend	Filter	Format
Experimental Cigarette (EC)	50% blend treated tobacco 15% tobacco sheet substitute 35% lamina & DIET	Polymer derived activated charcoal High activity carbon	Demi-Slim
Comparator Cigarette (CC)	US-style blend	Standard cellulose acetate	King size



Analyte	CC	EC
Benzo[a]pyrene (ng/cig)	15.5 ± 1.8	13.6 ± 0.9
Formaldehyde (µg/cig)	64.4 ± 7.9	48.6 ± 4.6
Acetaldehyde (µg/cig)	1121.7 ± 39.5	576.1 ± 36.4
Crotonaldehyde (µg/cig)	46.9 ± 1.9	3.9 ± 1.4
HCN (µg/cig)	316.3 ± 12.3	109.0 ± 2.7
NNK (ng/cig)	79.9 ± 2.9	28.3 ± 2.8
Benzene (µg/cig)	68.5 ± 3.9	9.7 ± 1.1
Naphthalene (ng/cig)	1048.5 ± 38.1	142.0 ± 9.2

Dittrich, D. J., et al. https://doi.org/10.1186/2193-1801-3-374

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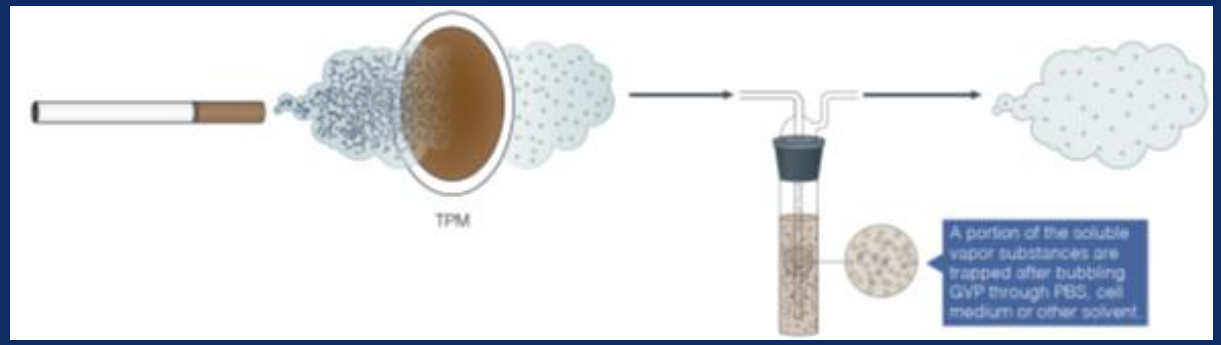
Experimental design

Puffed to the ISO 20778 (HCI) regime

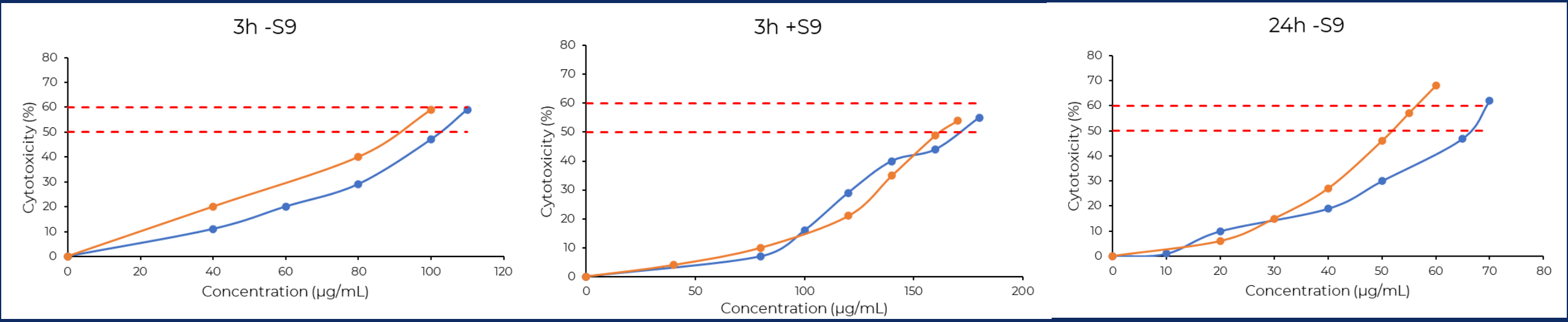


TPM extracted with DMSO

TPM
HTPs: 24 mg/mL

Crooks, I, et al. https://doi.org/10.1016/j.yrtph.2015.01.001

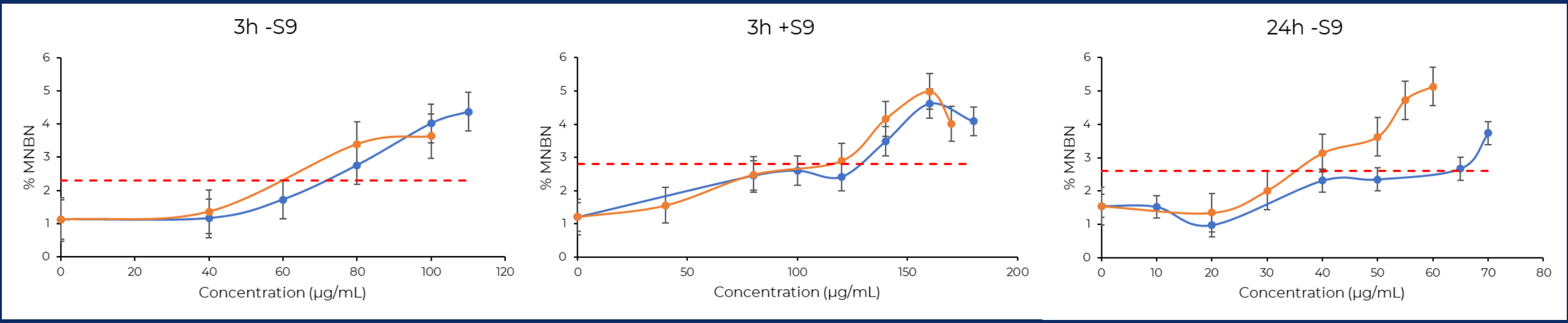
Results - cytotoxicity



Clear differences in the range of concentrations used in each treatment
Both products reached 50-60% cytotoxicity in all treatments
Indication that the CC was slightly more cytotoxic than the EC

Crooks, I, et al. https://doi.org/10.1016/j.yrtph.2015.01.001

Results – micronuclei induction



The EC and CC induced dose related increases in MN
In both 3-hour treatments, no biologically relevant differences observed
In the 24h treatment the EC was less genotoxic than the CC

Crooks, I, et al. https://doi.org/10.1016/j.yrtph.2015.01.001

Summary

HTP

Clear differences in the cytotoxicity ranges for each treatment condition

The HTPs showed weak activity in the *in vitro* micronucleus assay

No difference in the response between the unflavoured and flavoured HTP

Cigarettes

Clear differences in the cytotoxicity ranges for each treatment condition

Both cigarettes were genotoxic in each treatment condition

A reduced response was seen in the 24h treatment with the EC relative to the CC.
The EC is a non-commercial experimental prototype not shown to offer reduced risk.

Best practices

The choice of cell line is critical

Range finders for cytotoxicity should always be conducted (i.e. before performing the main endpoint) for each treatment condition. The potential for variability should also be factored in

The OECD Test Guideline should be followed and the lab should have demonstrated proficiency with the assay

The cytotoxic limits of the assay should not be exceeded and the cytotoxic limit may not need to be achieved if the test article is genotoxic below the limits of cytotoxicity

ACM/TPM/GVP extraction methodology may need to be modified for products with reduced toxicants