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Mode-of-action analysis of genotoxicity detected by the *in vitro* micronucleus assay: two industry case studies

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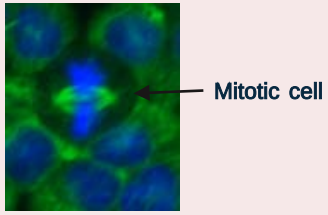
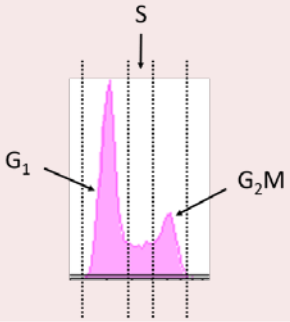
Outline

Case study one	Case study two
▪ (-)-Nicotine-induced genotoxicity	▪ Non-flavoured e-liquid-induced genotoxicity
Key concepts	
▪ Aneugenicity ▪ Advanced endpoints ▪ Lysosomotropism	▪ Advanced endpoints ▪ Cell cycle
▪ Final remarks	
▪ Info: case study data will also be presented at the poster session on 3 May 2018 (#38 and #39)	



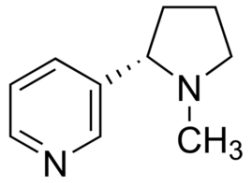


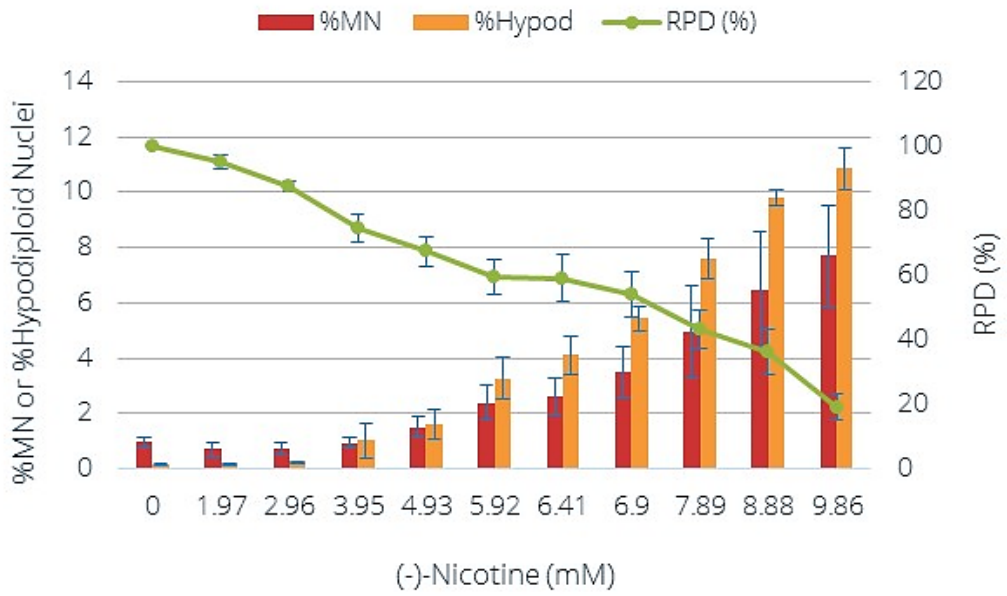
Case study one: (-)-nicotine - induced genotoxicity

Background

- **(-)-Nicotine** was evaluated in the standard battery of Good Laboratory Practice genetic toxicology assays (using Organisation for Economic Co-operation and Development (OECD) Test Guidelines) as part of the safety assessment of e-cigarette chemical components .
- **Mammalian genotoxicity:** flow cytometry -based *in vitro* micronucleus (**MN**) assay (MicroFlow®, Litron Laboratories, USA)
- **Test system:** Chinese hamster ovary-Wolff Bloom Litton (**CHO-WBL**) cell line (provenance: Merck Research Laboratories, USA)
- **Results:** concentrations up to **3.95 mM** had **no effect** on background levels of MN after 24 hours (h) treatment, but ⩾**4.93mM**, tandem increases in **MN** and **hypodiploid nuclei** were observed: evidence of **aneugenicity**.

(-)-Nicotine:





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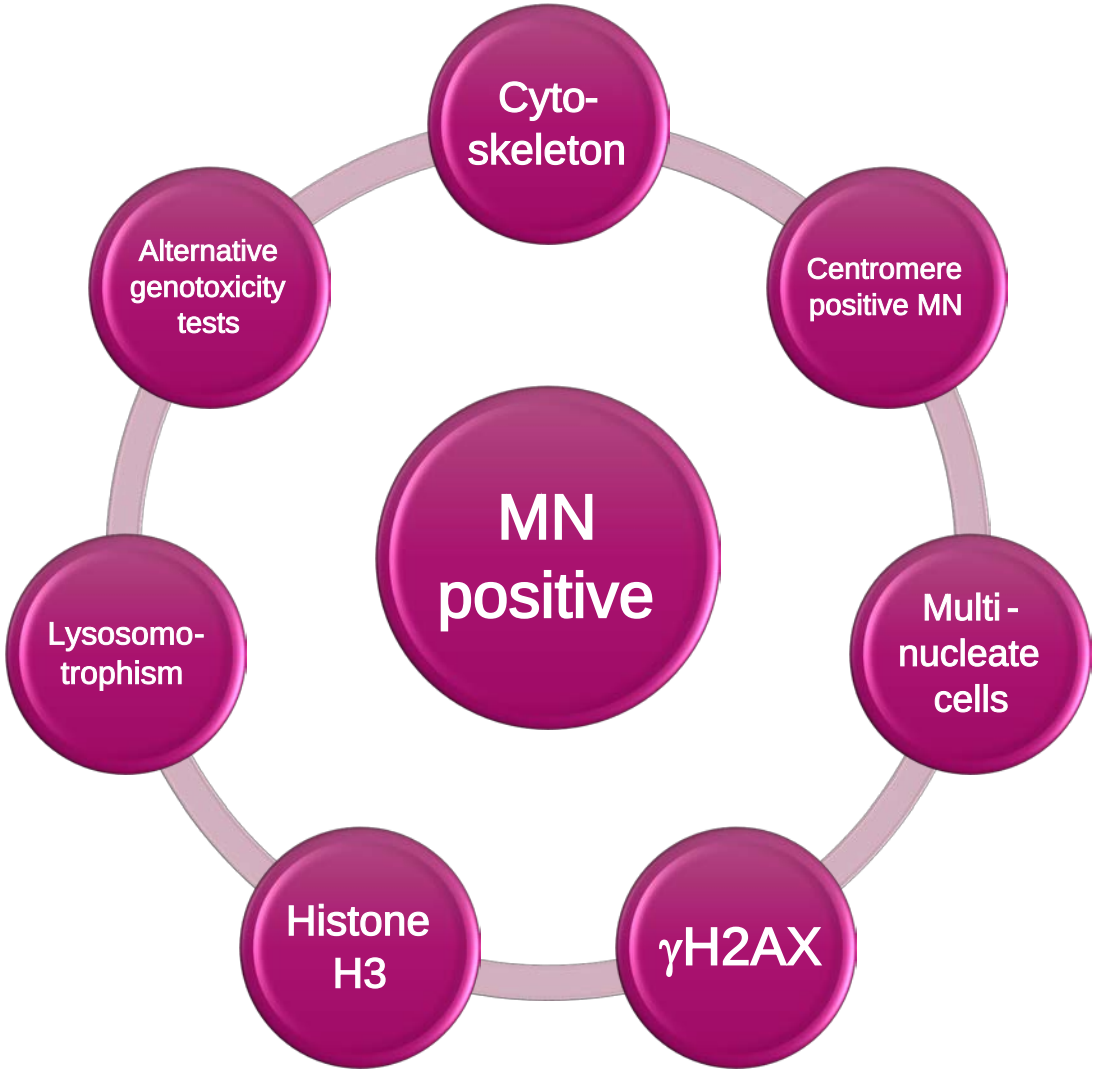
Mode-of-action analysis



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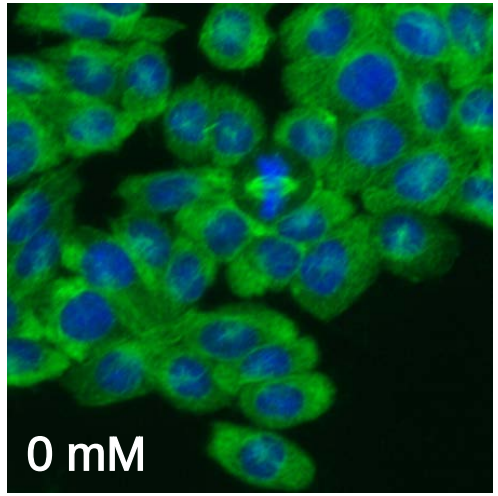
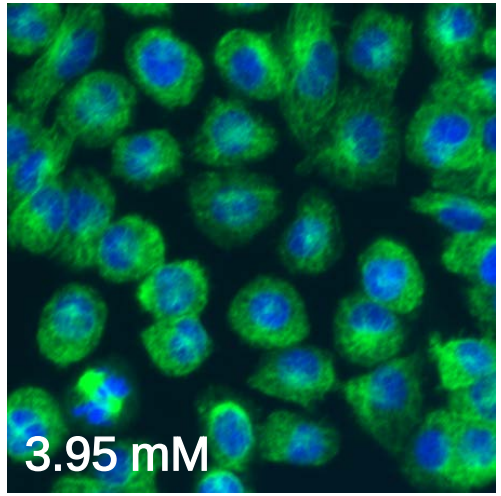
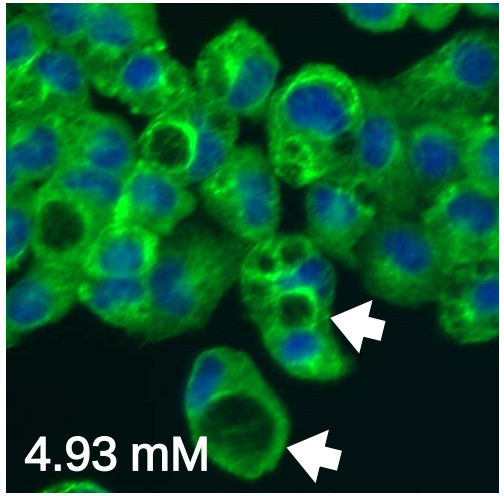
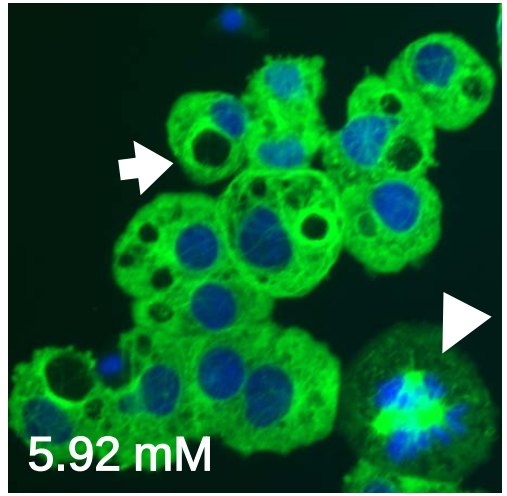
Perturbation of microtubule structure by (-)-nicotine

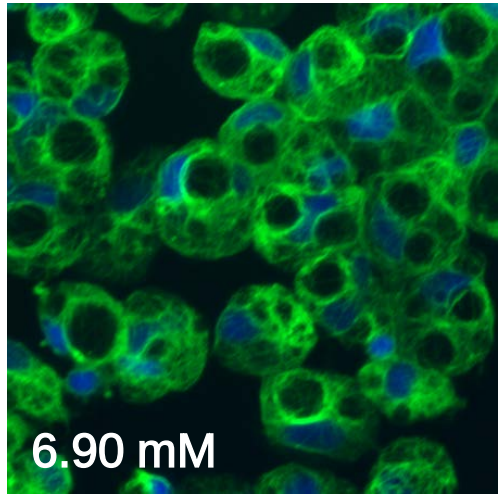
- **α-Tubulin** = Green; **Nuclear DNA** = Blue. Examples of **vacuolisation** (△) and a **tripolar spindle** (△)

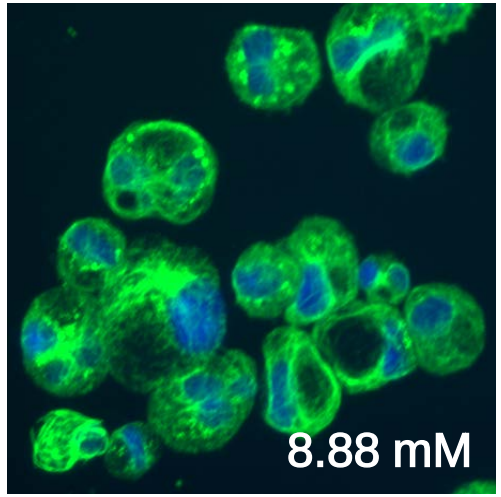


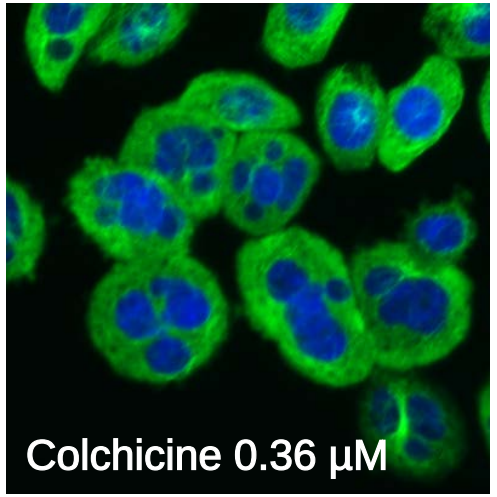


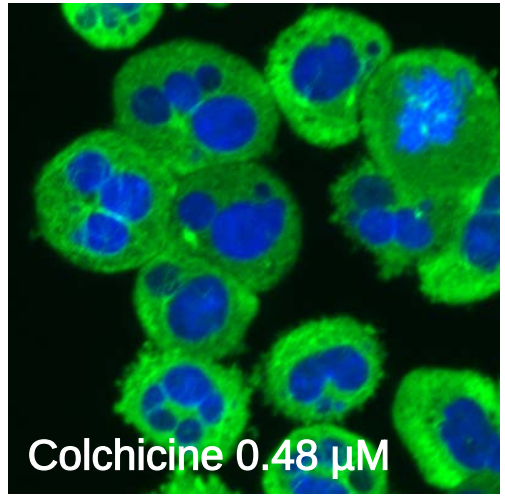








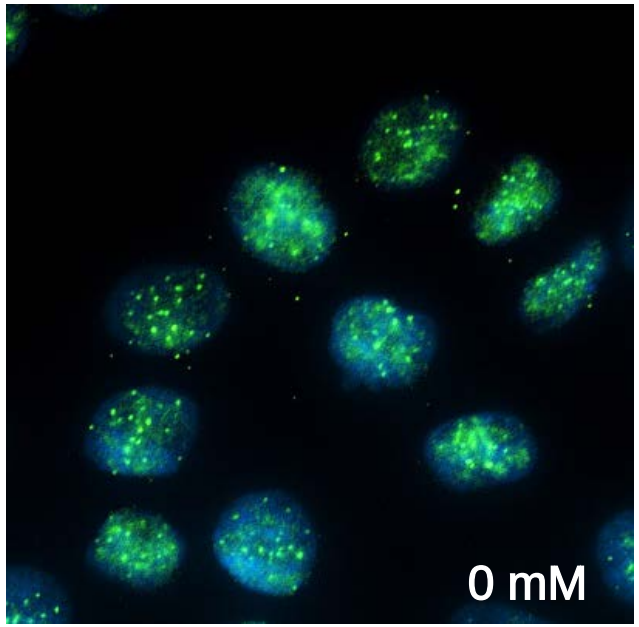


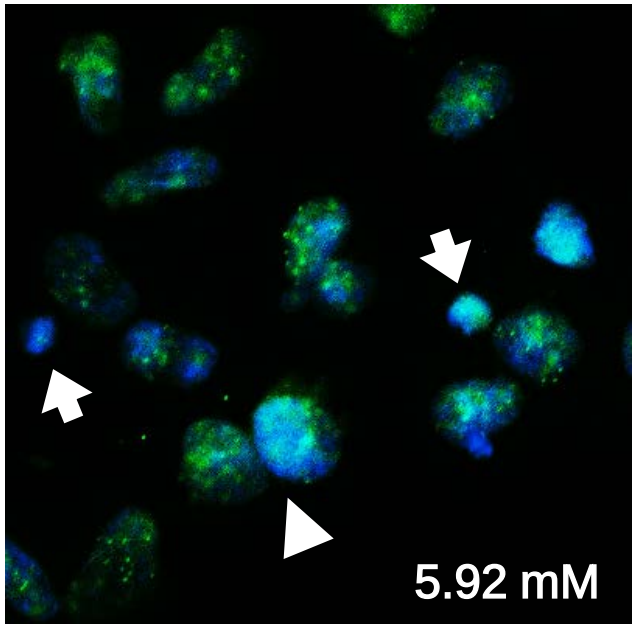


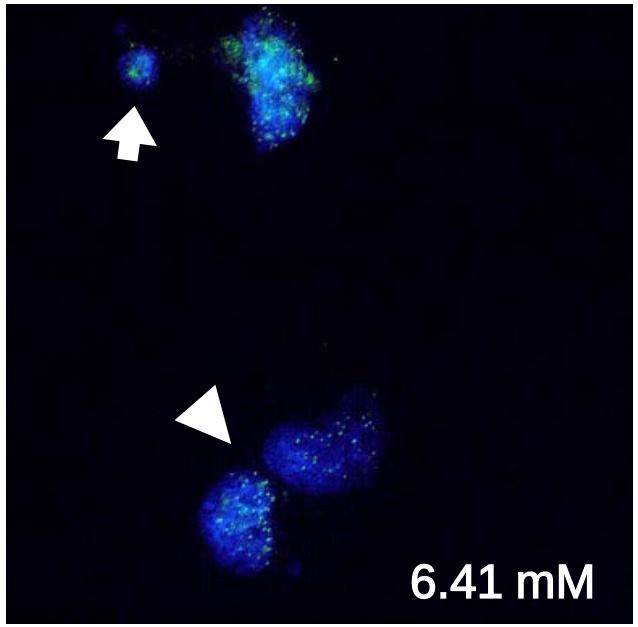
(-)-Nicotine -induced centromere -positive MN & multinuclear cells

- **Centromeres** = Green; **Nuclear DNA** = Blue

Presence of centromere-positive MN (▲) and multinucleate cells (▲) following 24 hour (-)-nicotine treatment (≥ 4.93 mM): further evidence of aneugenicity



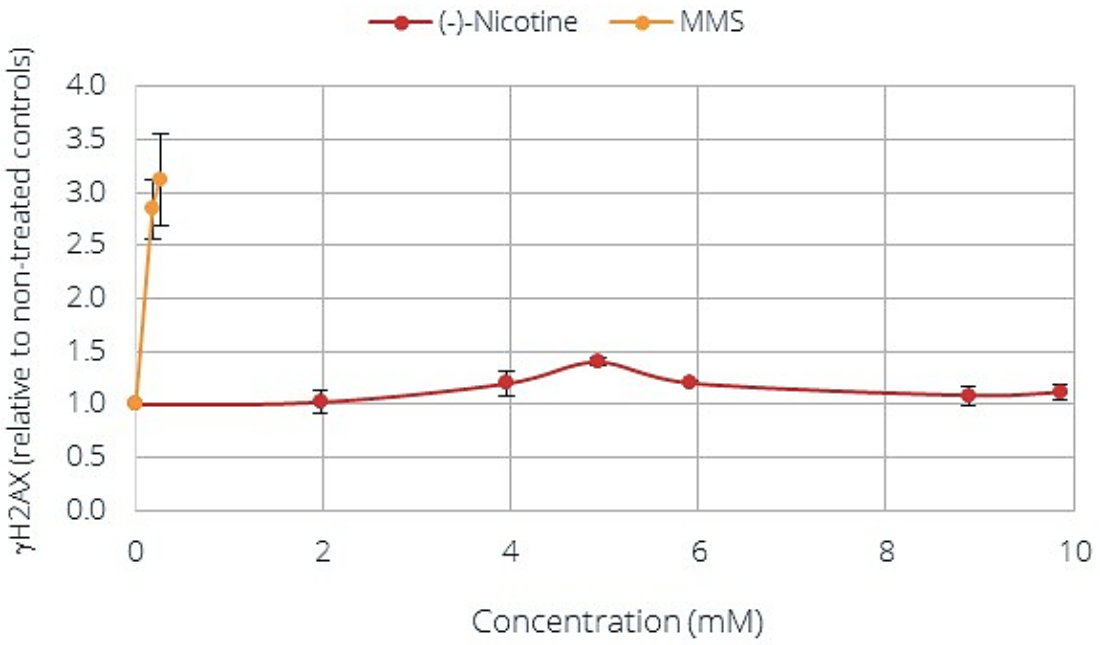




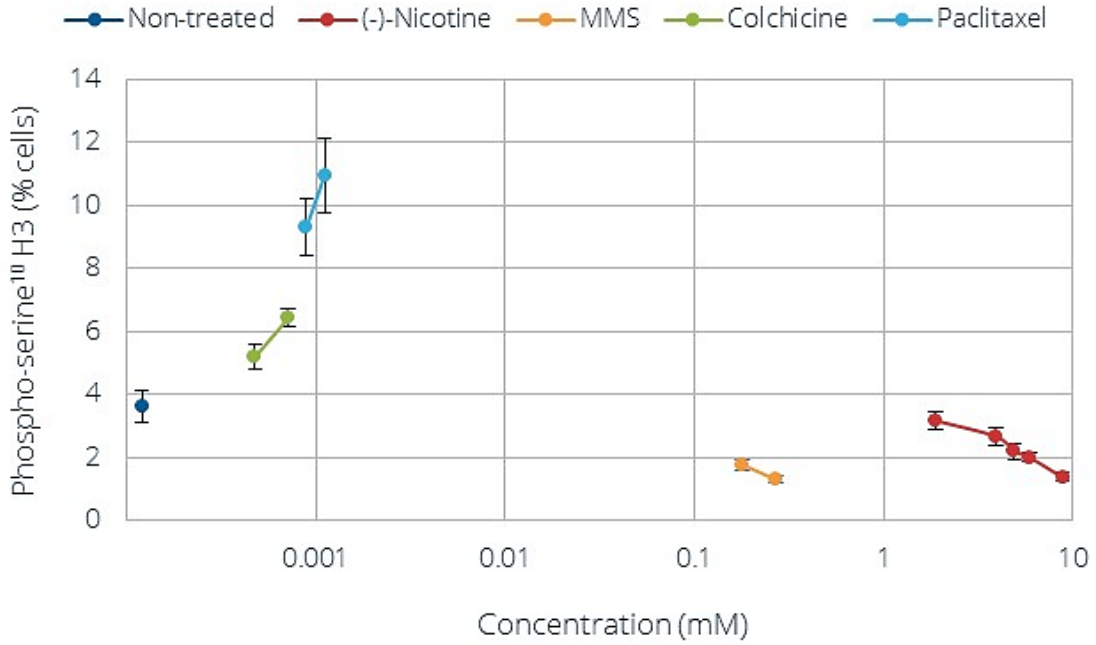
Lack of histone phosphorylation in response to (-)-nicotine

- **γH2AX:** occurs in response to DNA double-strand breaks → marker of **clastogenicity** (measured at 24 h)
- **Phospho-serine^{10} H3:** occurs in M-phase cells → marker of **aneugenicity** (measured at 4 h)

Negligible effects on γH2AX in response to nicotine treatment



Concentration-dependent decrease in phospho-H3 in response to nicotine treatment

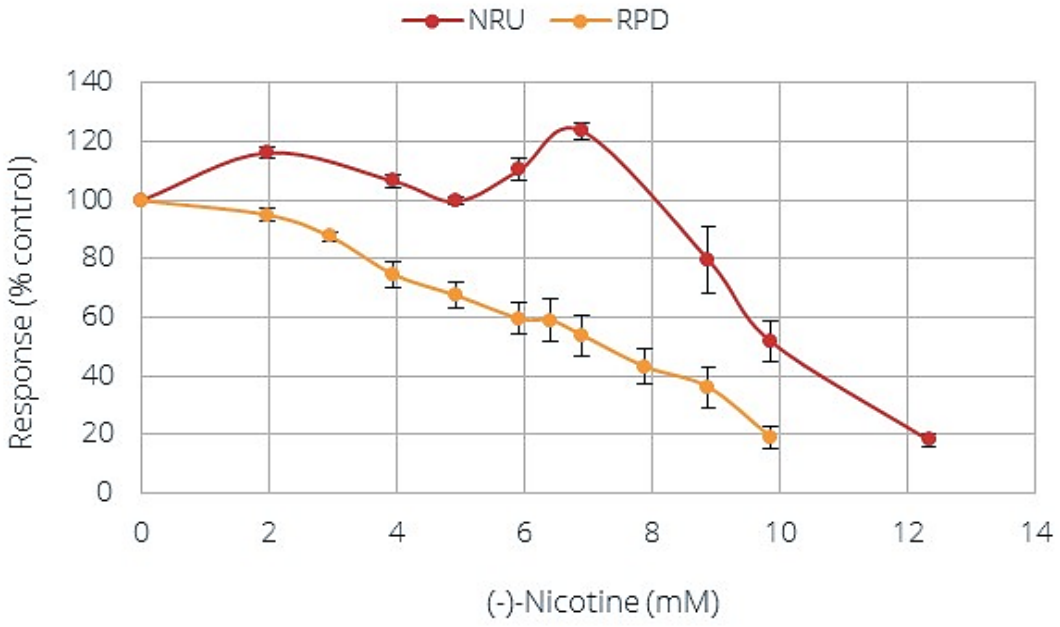


(-)-Nicotine's lysosomotropic properties drive genotoxicity

- **Neutral red uptake (NRU)** cytotoxicity assay: NRU in **acidic compartments** of living cells but not in dead/dying cells → used to assess and compare cytotoxic potency of chemicals..and now to reveal their **lysosomotropic** properties.

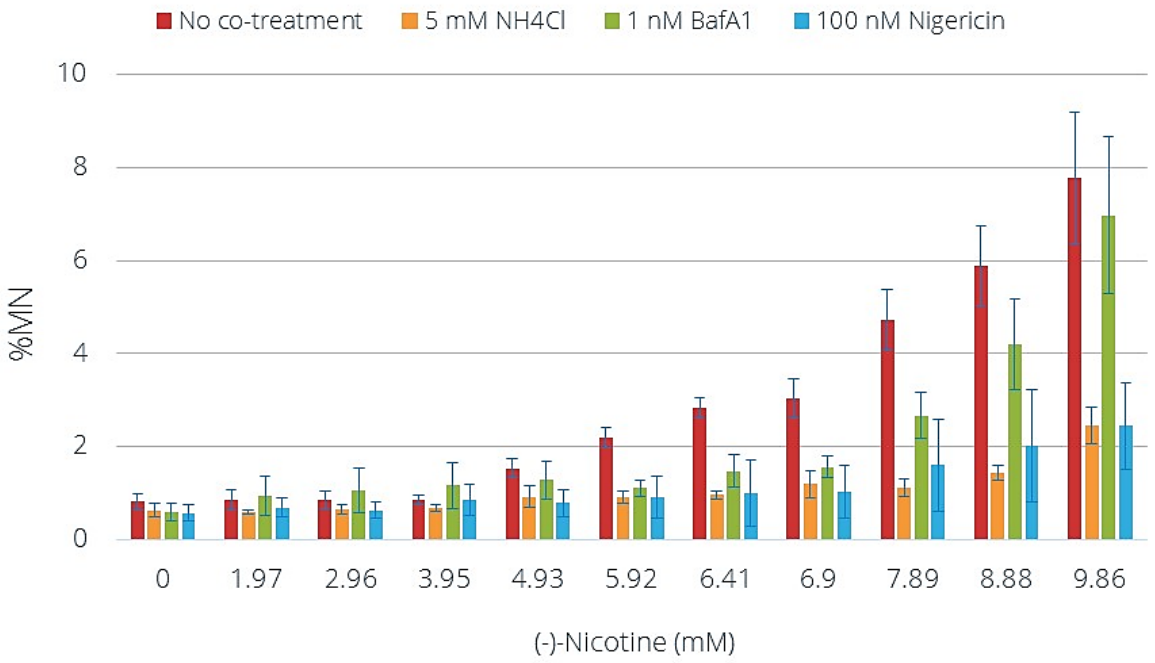
- **Lysosomotropism** : process by which chemicals become **trapped** in acidic compartments of cells, e.g. lysosomes.

Lysosomotropism → organelle swelling and/or coalescence → enhanced NRU capacity



[Cudazzo et al., *manuscript in preparation*]

Nicotine genotoxicity can be modulated by increasing pH of acidic compartments chemically



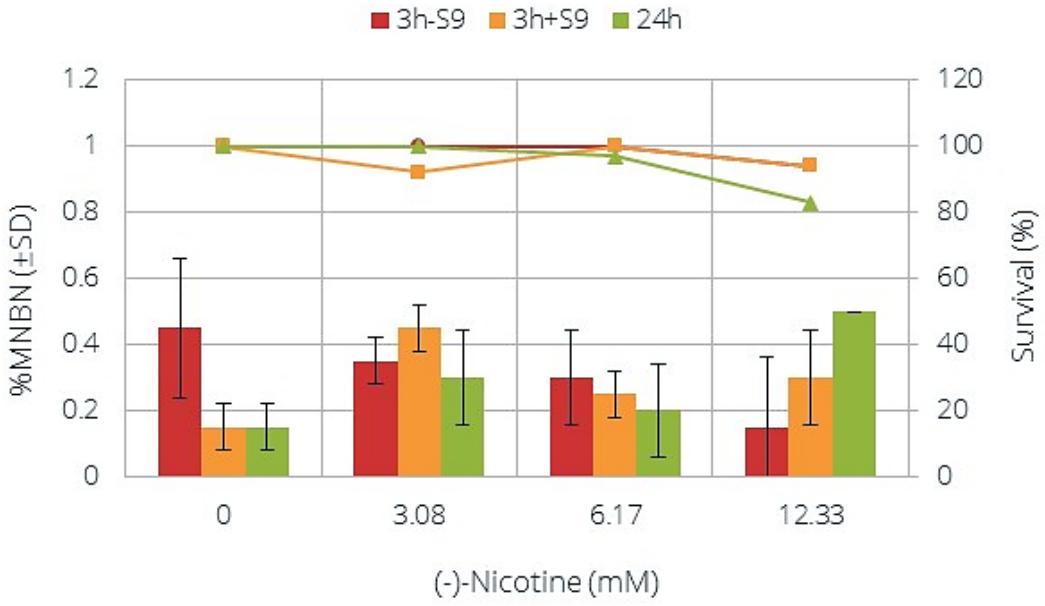
*NH_4Cl: Ammonium chloride; BafA1: Bafilomycin A1 (H^+-ATPase inhibitor); Nigericin (K^+ ionophore)

Non-genotoxic effects in alternative tests

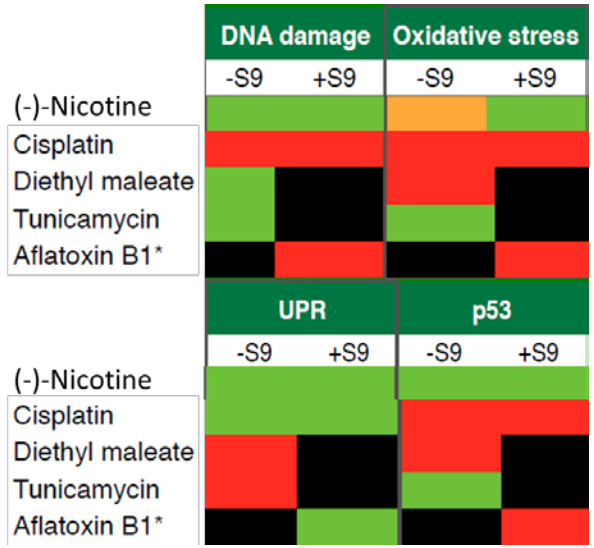
- **Human lymphocyte -based MNvit assay**: female donors; PHA-stimulated; Cyto-B arrested; 21/24 hours recovery; replication index to measure cytotoxicity

- **ToxTracker®**: state-of-the-art mouse stem cell-based reporter assay that provides mechanistic insights into genotoxic properties of chemicals → **DNA and protein (UPR) damage+ cellular (p53) and oxidative stress** endpoints vis-à-vis cytotoxicity

Non-genotoxic effects in all treatment conditions up to 12.33 mM



DNA/protein damage and cellular stress endpoints negative up to 10 mM but "weakly" positive oxidative stress response at 10 mM (24 h)



Red = positive; Green = negative; Amber = weakly positive; Black = not tested

Case study one: summary & conclusion

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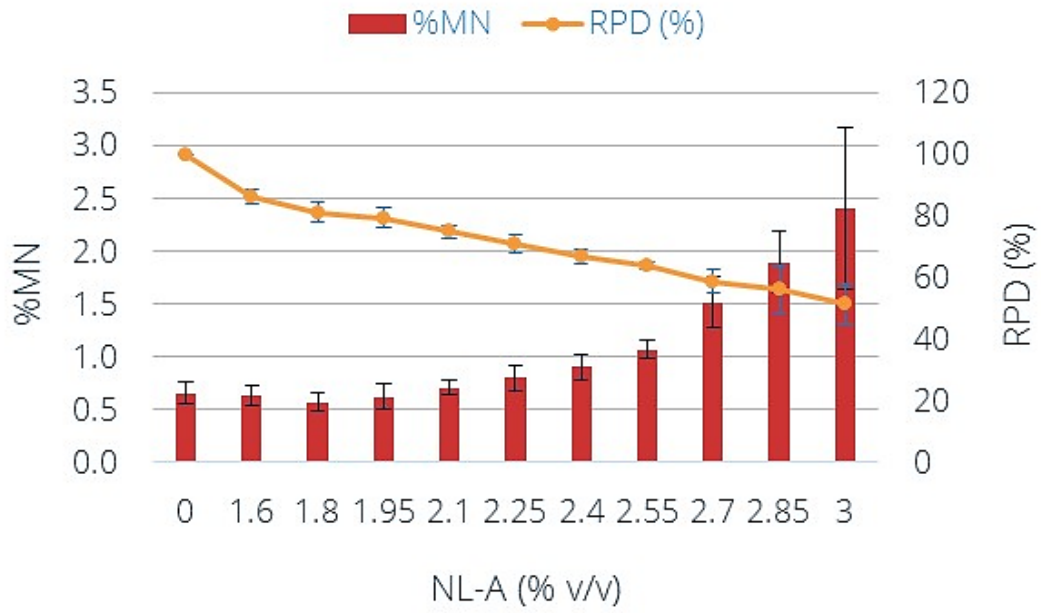
Case study two: non -flavoured e-liquid -induced genotoxicity

Background

- **Non-flavoured e-liquids (NL)** are routinely screened for ***in vitro*** **genotoxic potential** in mammalian cells alongside their novel, flavoured e-liquid counterparts.
- A NL (**NL-A**) composed of **70% propylene glycol (PG) 20% vegetable glycerin (VG)** and **20 mg/ml (-)-nicotine** was used for a particular series of novel, flavoured e-liquids.
- Mammalian genotoxicity screen: flow cytometry-based MNvit assay in CHO-WBL cells (24 h treatment)
- Results: **concentration -dependent increases** in **MN** up to **genotoxic levels** were observed **consistently** over **multiple independent** studies.



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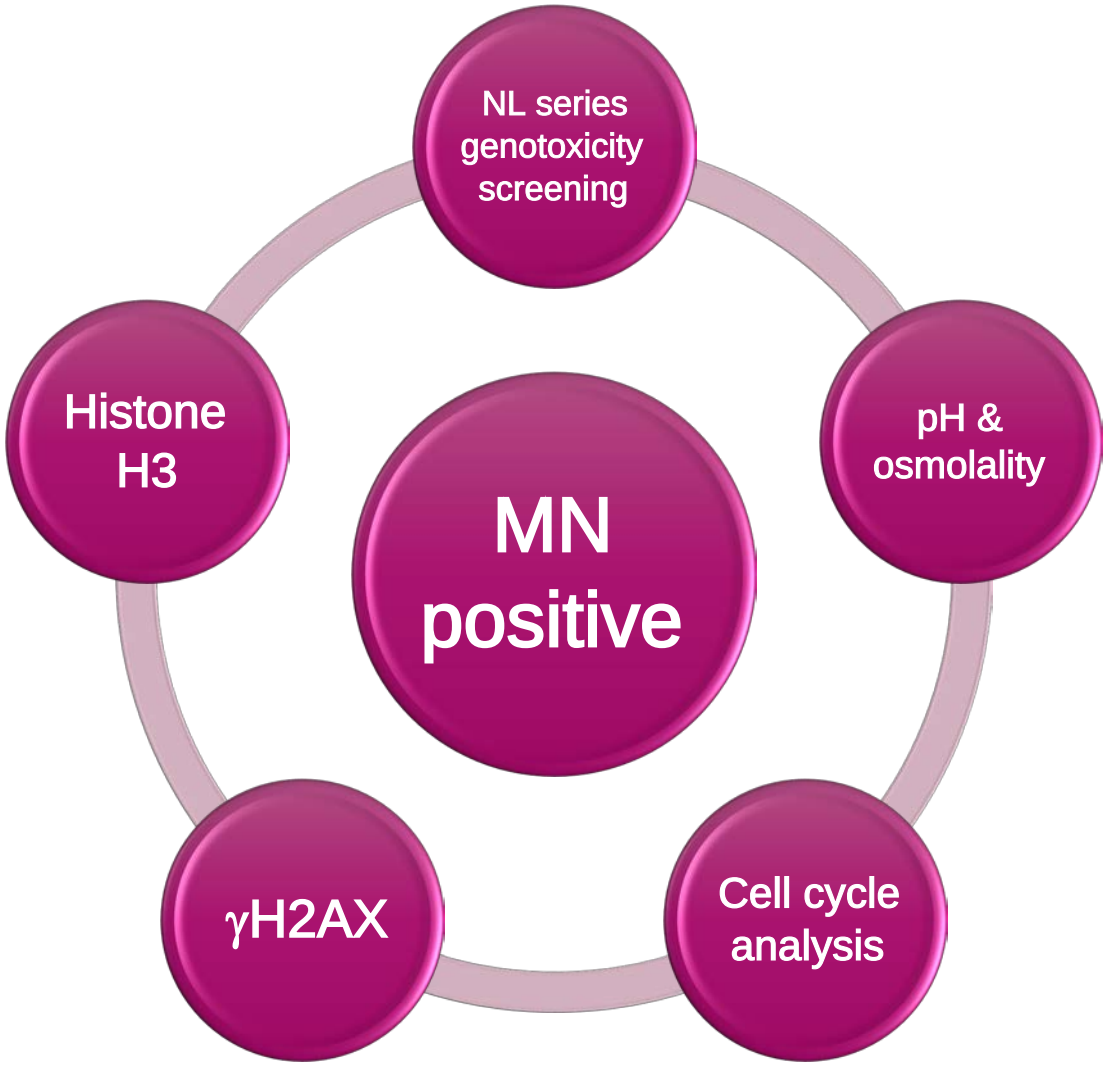
Mode-of-action analysis



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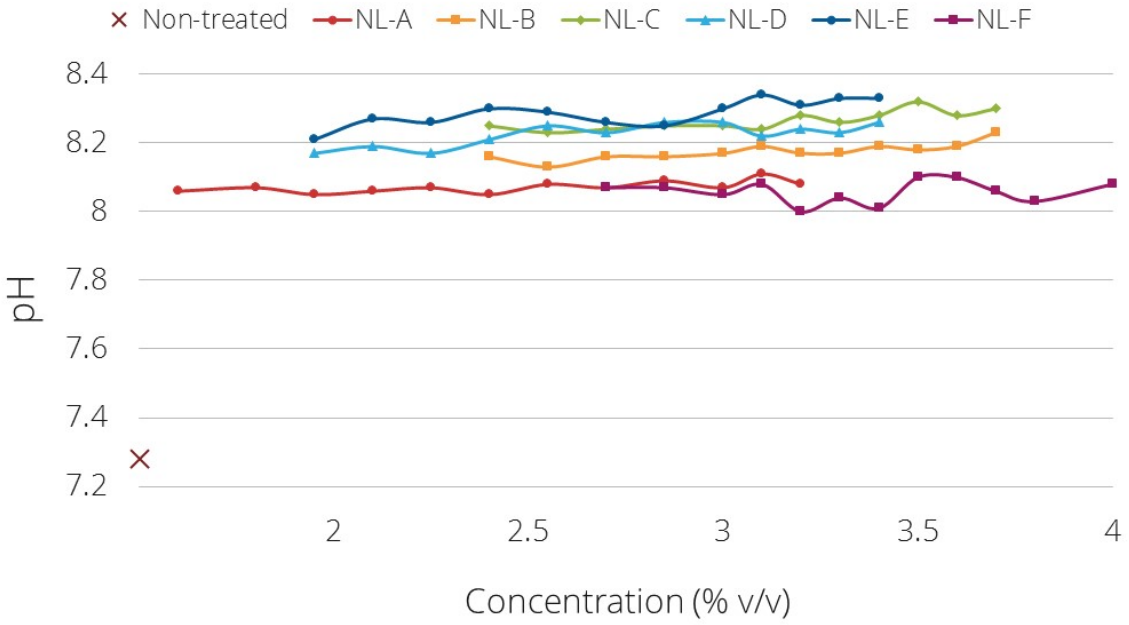
NL series

NL	PG content (%)	VG content (%)	(-)-Nicotine (20 mg/ml)
NL-A	70	20	✓
NL-B	40	40	✓
NL-C	20	73	✓
NL-D	100	0	✓
NL-E	0	100	✓
NL-F	70	20	✗

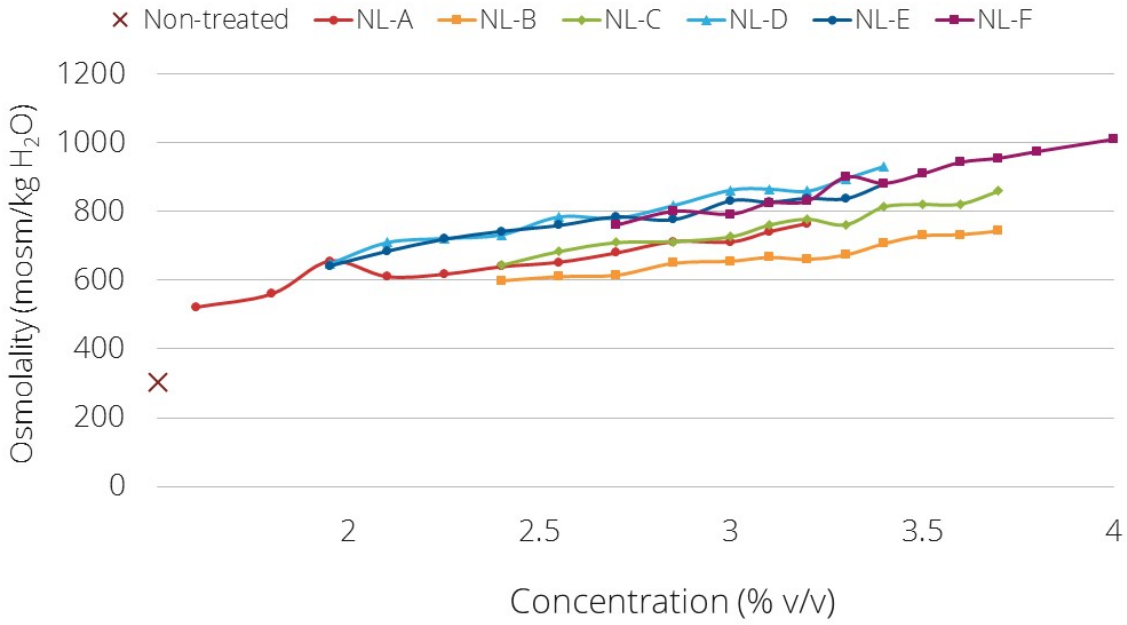
All NLs induced “extreme cell culture conditions” *in vitro*

- Changes in the pH and osmolality of the cell culture medium were measured **immediately post-NL exposure**.

In general, pH was increased by approximately 1 unit. NL-F had the least impact on pH



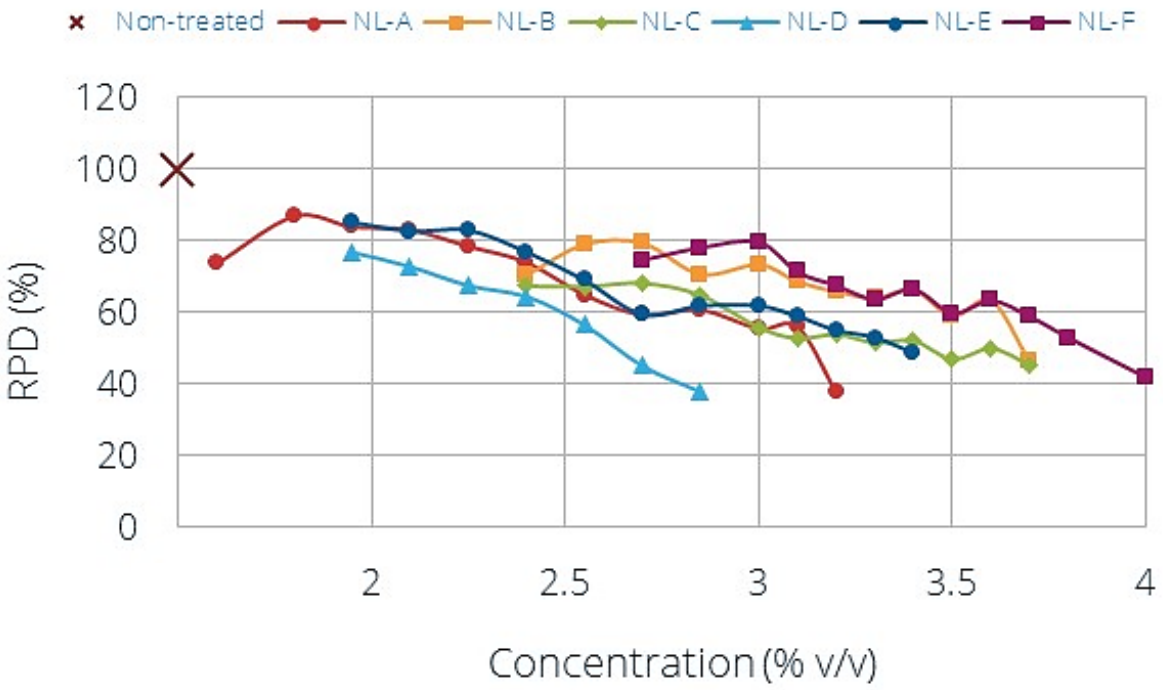
Osmolality increased in a concentration dependent manner to similar levels for all NLs. NL-B had the least impact on osmolality



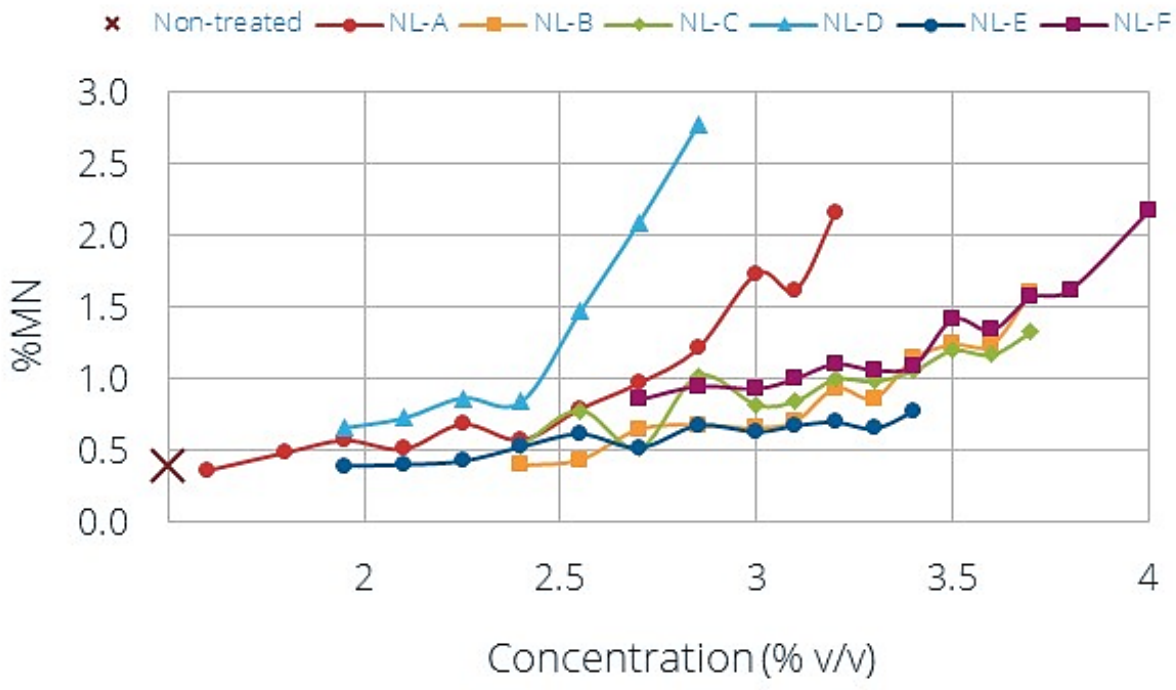
Majority PG -based NLs were more potently genotoxic *in vitro*

- Concentration ranges were established that produced cytotoxicity **at** or **just beyond the limit of the assay (relative population doubling (RPD) 40%)**

Concentration-dependent increase in cytotoxicity for all NLs



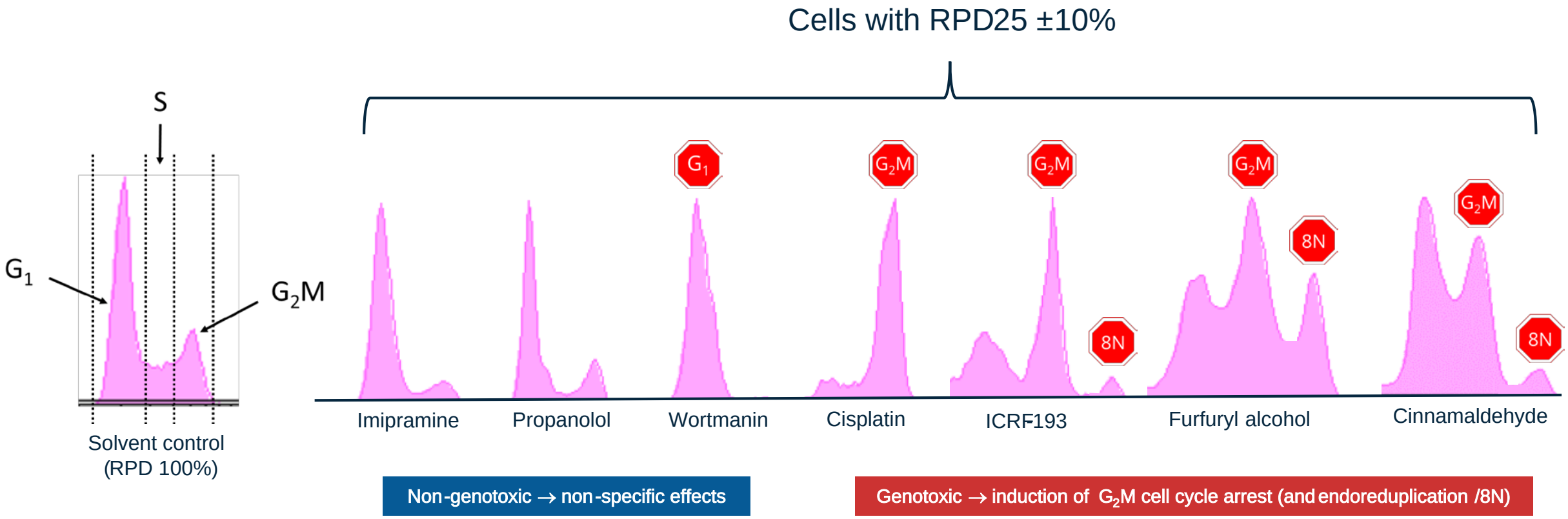
Genotoxic potency rank order (BMD @ BMR 100 RPD):
NL-D > NL-F, NL-A > NL-B
(NL-C and NL-E were defined as non-genotoxic)



*BMD: Benchmark dose; BMR: Benchmark response
→ analysis performed in PROAST (data not shown).

Unmasking cytotoxicity via the cell cycle is critical for the interpretation of genotoxicity data

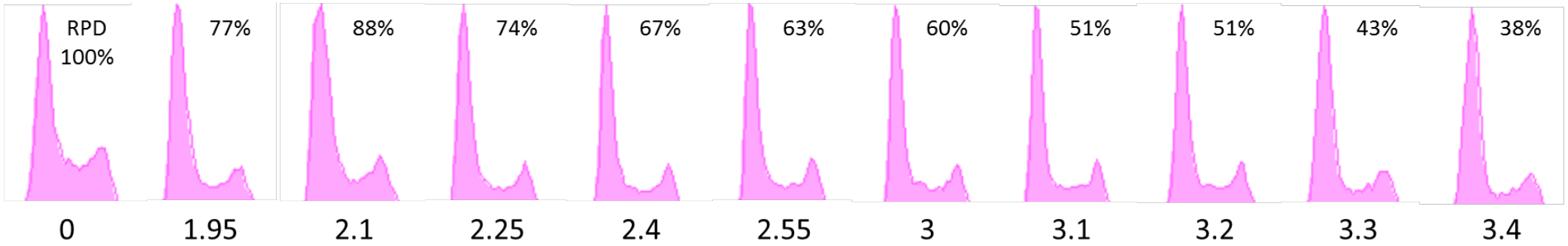
- **Cytotoxicity** is manifested from a **combination** of **cell death** and **cell cycle arrest**
- E.g., representative **cell cycle histograms** of cell populations that have undergone chemical -induced **cytotoxicity (75%)**



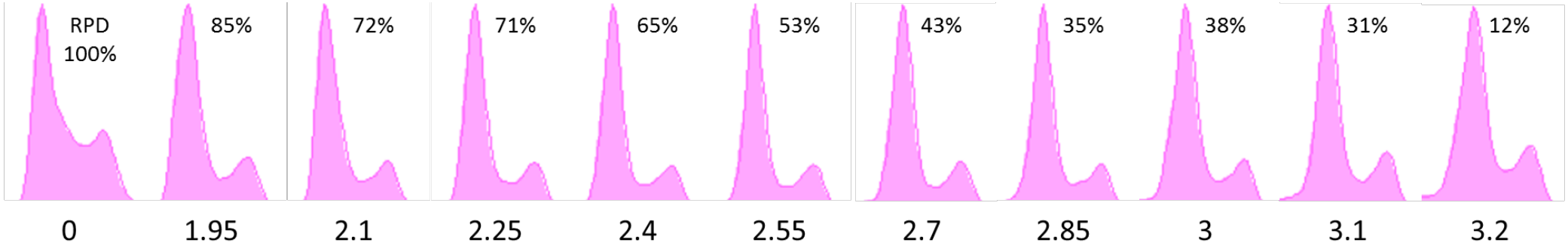
No discernible differences in cell cycle profiles of NL -exposed cells

- Representative **cell cycle histograms** of cells exposed to **NL-E and D** for 24 h.

NL-E: Non-genotoxic in MNvit



NL-D: Most potently genotoxic in MNvit

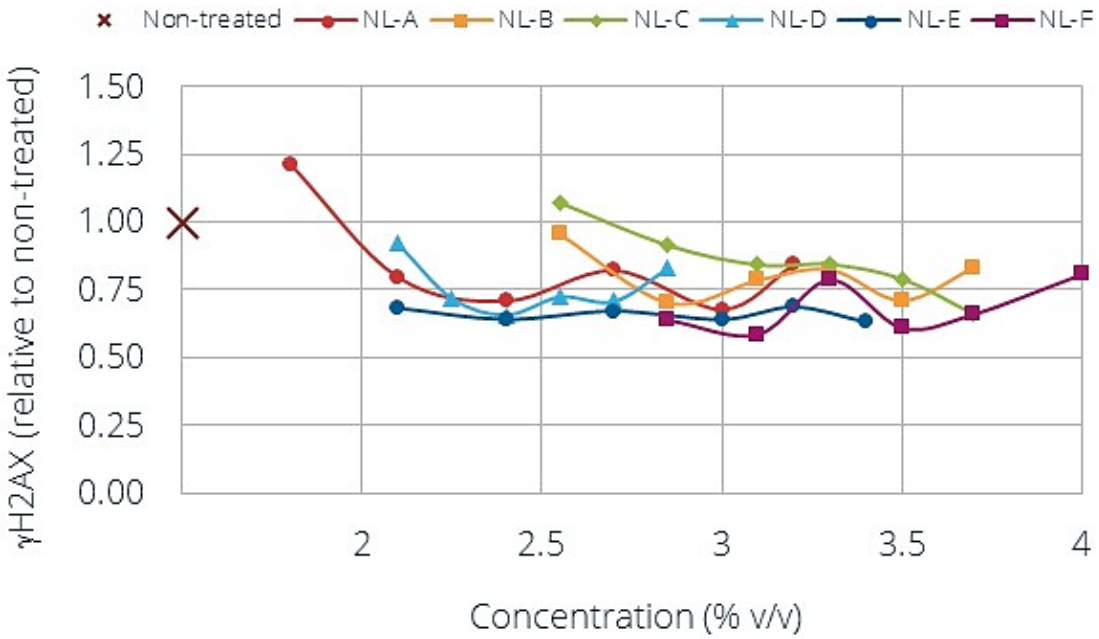


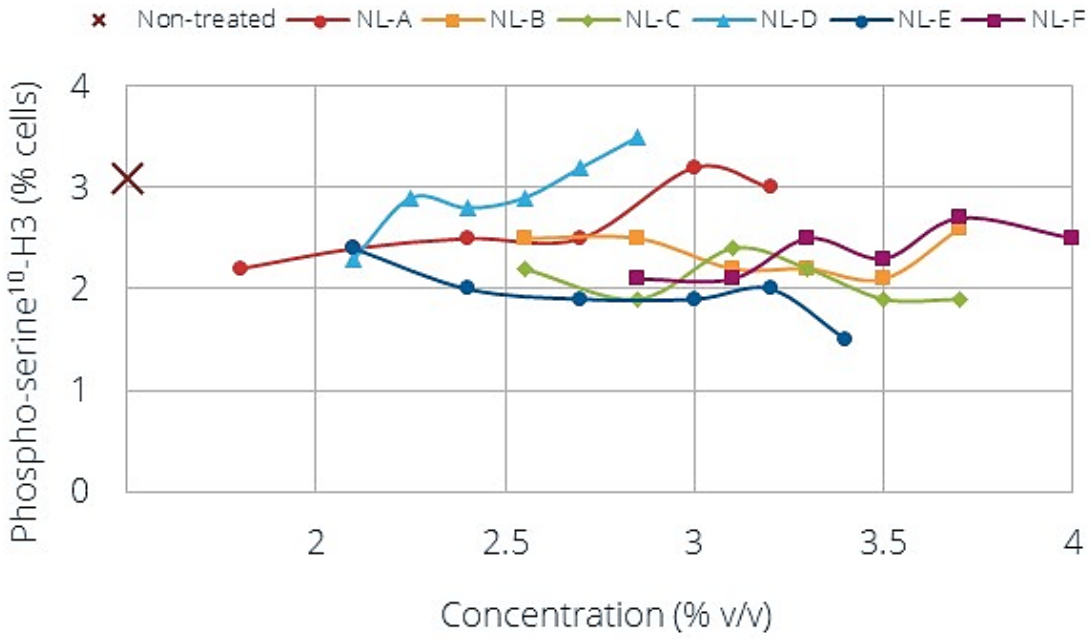
Lack of histone phosphorylation in response to NLs

- Effects on γH2AX and phospho-serine[10] H3 endpoints were used to appraise the related MN findings

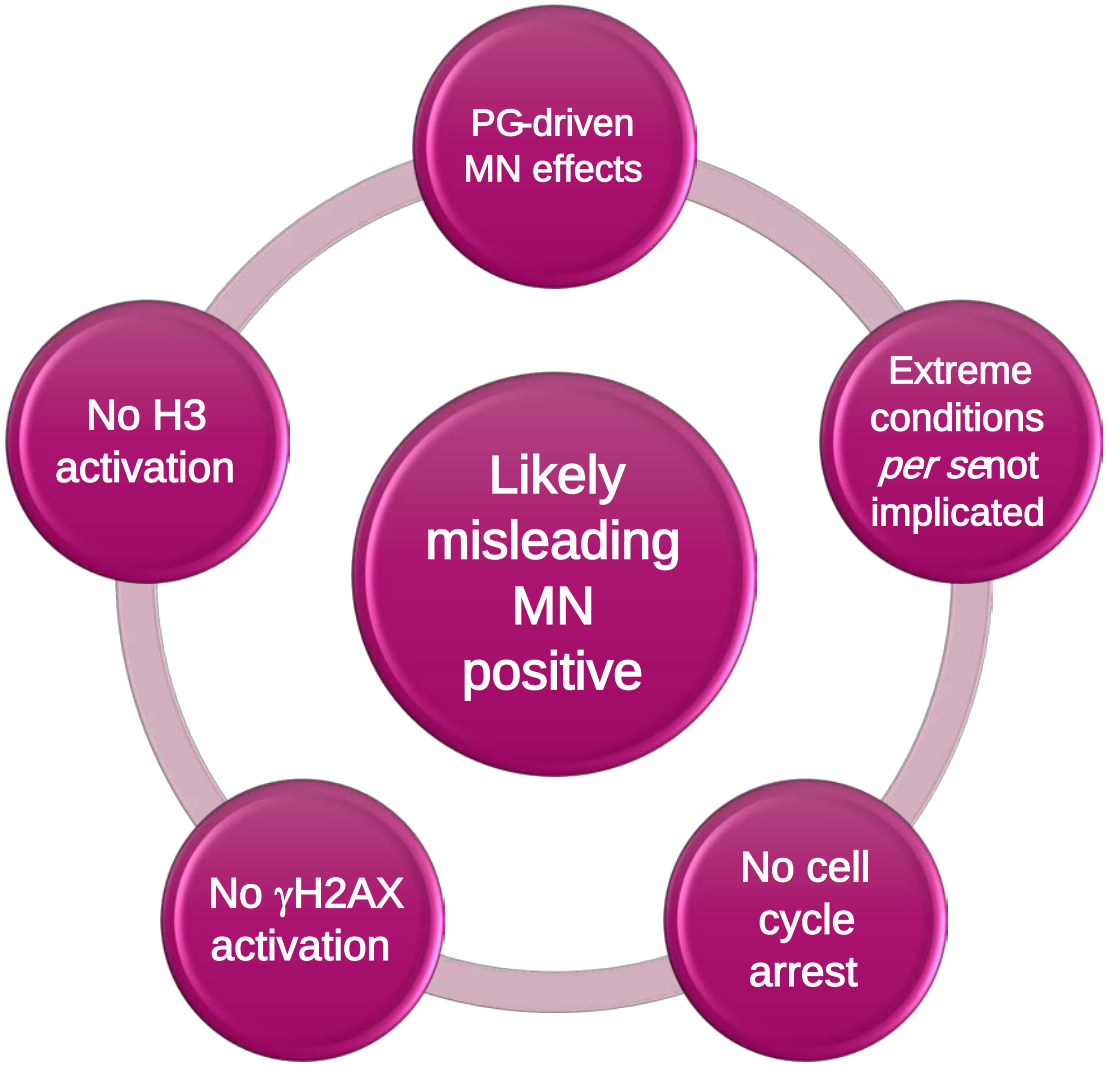
Negligible effects on γH2AX in response to NLs relative to controls

Negligible effects on phospho-serine[10] H3 in response to NLs relative to controls





Case study two: summary & conclusion



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Final remarks

- Assessing potentially-modified risk tobacco product-related chemicals and mixtures to **OECD Guideline limits** in **standard genetic toxicology assays** can result in **unexpected** genotoxicity findings.
- **Mode-of-action**-type follow-up experiments are necessary to **facilitate** the understanding of such data.
- Analysis of **advanced genotoxicity endpoints** , e.g., histone phosphorylation and cell cycle profiles, and **emerging technologies,** such as ToxTracker®, may warrant inclusion into early phases of product development.

Acknowledgements

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