Mode-of-action analysis of a positive *in vitro* micronucleus finding in CHO-WBL cells

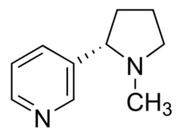
D McHugh, DJ Smart, FR Helbling, and P Vanscheeuwijck

PMI R&D, Philip Morris Products S.A., Quai Jeanrenaud 5, CH-2000 Neuchâtel, Switzerland

Introduction and Goals

- As part of the safety assessment of individual e-liquid components, (-)-nicotine (Figure 1) was evaluated for genotoxicity in the *in vitro* micronucleus (MNvit) assay.

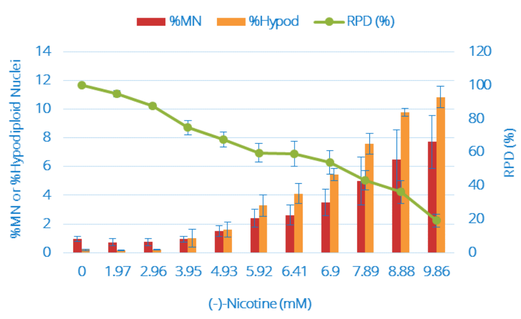
Figure 1. The chemical structure of (-)-nicotine.



- Chinese hamster ovary (CHO) cells were exposed to concentrations of (-)-nicotine up to 9.86 mM (their cytotoxic limit) for 24 hours prior to harvesting nuclei and micronuclei (MN) for enumeration by flow cytometry.

- A non-linear MN response was observed; concentrations up to 3.95 mM had no effect on background levels of MN. However, at concentrations ≥4.93 mM, tandem increases in MN and hypodiploid nuclei were evident (Figure 2).

Figure 2. The concentration-response of (-)-nicotine in the MNvit assay after 24-hour treatment.



- A series of follow-up experiments was carried out with t tion of (-)-nicotine in the CHO-based MNvit assay as well as to provid findings.

Materials and Methods

- **Cell line:** CHO-Wolff Bloom Litton (CHO-WBL; Merck Research Laboratories, USA). Cells were seeded in 96-well plates (Nunclon®, USA) at 4500 cells/well for 24 hours prior to treatment in McCoy's 5A + GlutaMAX™ medium + 10 % HI-FBS + 100 units/ml Penicillin + 0.1 mg/ml Streptomycin and incubated at 37°C with 5% CO_2 and 70% relative humidity.

- **Chemicals:** Pharmaceutical-grade (-)-nicotine was purchased from Nicobrand, UK, while all other chemicals and reagents were purchased from Sigma-Aldrich, Switzerland, unless otherwise specified.

- **MNvit 24-hour testing procedure:**

Treatment & nuclei count → **24 hours** → Nuclei count & MN evaluation

- **Genotoxicity assessment:** Nuclei and MN were prepared using the *in vitro* MicroFlow® kit (Litron Laboratories, USA) and analysed with a FACS Canto II flow cytometer with an integrated high-throughput sampler (Becton Dickinson, USA). Concomitant absolute nuclei counts were made at the start and end of treatment (24 hours) and used to calculate the relative population doubling (RPD) cytotoxicity parameter. At least 2,000 nuclei were evaluated for each concentration assessed.

- **Genotoxicity evaluation:** A response was defined as genotoxic if there were 1) a statistically significant increase in %MN relative to concurrent solvent controls, 2) a concentration-related increase in %MN relative to concurrent solvent controls, and 3) at least one concentration-induced %MN outside the laboratory's historical solvent control range at RPD ≥40%.

- **α-Tubulin and centromere analysis:** Cells were seeded at 11,000 cells/well in eight-well chamber slides (Nunc™ Lab-Tek™ II Chamber Slide™ System, Thermo Fisher Scientific) and cultivated for 24 hours. Following treatment, cells were fixed with Cytofix/Cytoperm™ solution (BD Bioscience, USA), and nuclei were stained with the mouse anti-α-tubulin monoclonal antibody (Sigma-Aldrich) or the human anti-centromere protein polyclonal antibody (Antibodies Inc., USA). ProLong® Gold antifade with DAPI (Thermo Fisher Scientific, Switzerland) was used as the coverslip mountant before analysis via fluorescence microscopy (Nikon Eclipse Ti-E and NIS-Elements v4.0 software, Japan).

- **Phosphorylated histone analysis:** Cells were seeded at 130,000 cells/well in six-well plates (Nunclon®, USA) and cultivated for 24 hours. Following treatment, cells were fixed as above, and nuclei were stained with 7-AAD and either the mouse anti-H2A.X Phospho (Ser139) FITC-conjugated antibody (Biolegend, USA) or the rabbit anti-phospho-histone H3 (Ser10) Alexa Fluor® 488-conjugated antibody (Cell Signaling Technology, USA) before analysis via flow cytometry.

- **MNvit in presence of pH-raising chemicals:** Cells were co-exposed to the same range of (-)-nicotine concentrations and a fixed concentration of either ammonium chloride (NH_4Cl; 5 mM), bafilomycin A1 (BafA1; 1 nM), or nigericin (100 nM) for 24 hours. After the treatment period, nuclei and MN were harvested and enumerated as above.

- **Neutral red uptake (NRU) cytotoxicity assay:** This assay was performed in general accordance with the INVITTOX guideline (1990). Briefly, cells were seeded in 96-well plates at 4,500 cells/well for 24 hours prior to treatment. Following treatment, cell culture medium was replaced with medium containing 50 µg/mL neutral red (NR) dye supplemented with 20 mM HEPES. Following incubation for three hours, intracellular NR dye was extracted using a destaining solution (ethanol, water, and acetic acid, mixed in a 50:49:1 ratio), and absorbance was measured at 540 nm using a plate reader (Safire 2 with Magellan Tracker v7.0 software, Tecan, Switzerland).

- **Human lymphocyte-based MNvit assay:** This assay was performed in general accordance with Organisation for Economic Co-operation and Development Guideline 487 under Good Laboratory Practice conditions by a Contract Research Organisation (CRO). Specifically, females were used as donors, and lymphocytes were stimulated to proliferate using phytohaemagglutinin and then arrested using cytochalasin B. Cell cultures were allowed to recover for 21-24 hours, and the replication index was subsequently used to measure cytotoxicity before MN were enumerated.

- **ToxTracker® analysis:** The state-of-the-art mouse stem cell-based reporter assay that provides mechanistic insights into genotoxic properties of chemicals (DNA and protein damage and cellular and oxidative stress endpoints vis-à-vis cytotoxicity) was carried out by a CRO (Hendriks et al., 2012) using a limit concentration of 10 mM.

Results

Figure 3. The endpoints evaluated to shed light on the genotoxic mode-of-action of (-)-nicotine in the CHO cell-based MNvit assay.

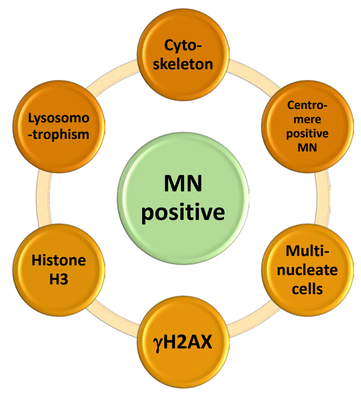


Figure 4. Perturbation of microtubule structure by (-)-nicotine (images 1-6) and the prototypical aneugen colchicine (images 7 and 8). (α-Tubulin = green; nuclear DNA = blue).

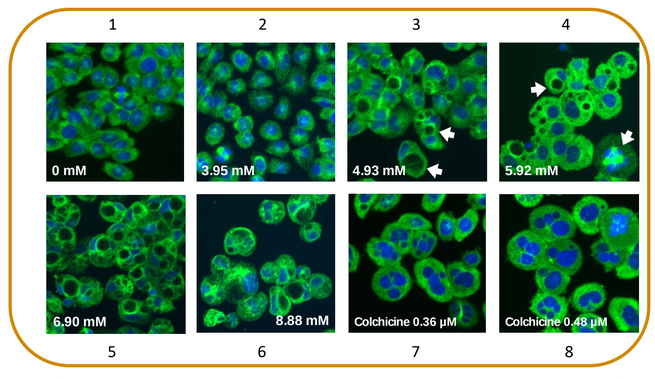


Figure 5. Presence of centromere-positive MN and multinucleate cells following 24-hour nicotine treatment (≥ 4.93 mM) (Centromeres = green; nuclear DNA = blue).

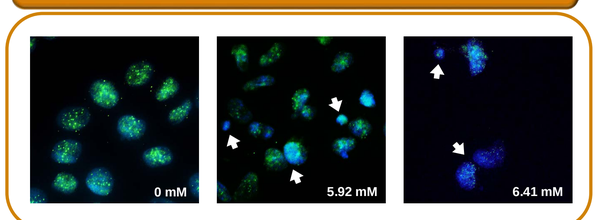


Figure 6. Lack of histone phosphorylation in response to (-)-nicotine but expected effects following treatment with the positive control chemicals: MMS, colchicine, and paclitaxel. **A.** γH2AX. **B.** Phospho-H3.

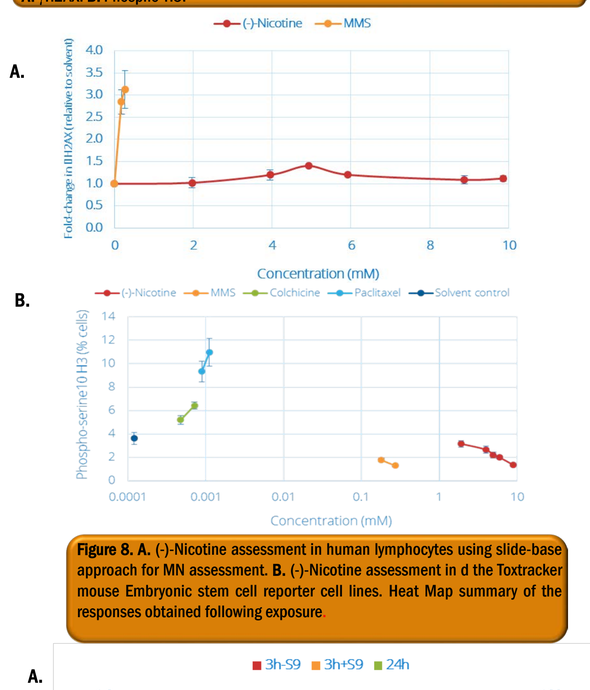


Figure 7. (-)-Nicotine's lysosomotropic properties drive genotoxicity. **A.** Lysosomotropism leads to organelle swelling/coalescence and enhanced NRU capacity. **B.** Genotoxicity can be modulated by increasing pH of acidic compartments chemically.

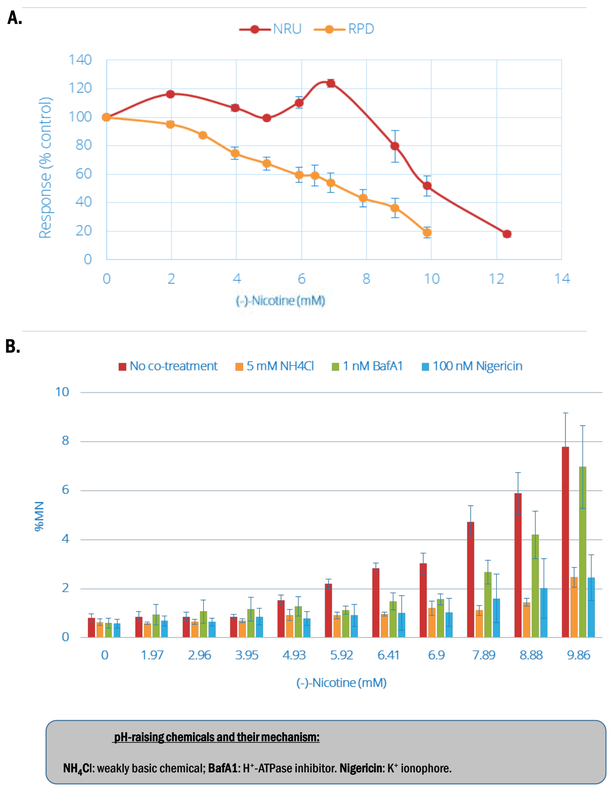
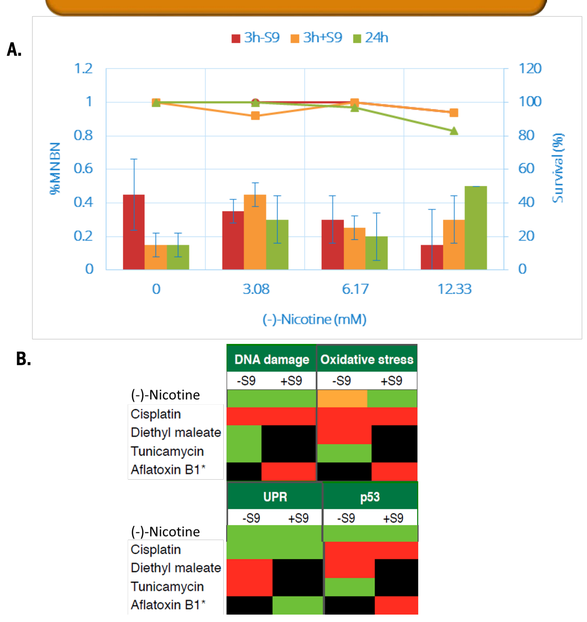


Figure 8. A. (-)-Nicotine assessment in human lymphocytes using slide-base approach for MN assessment. **B.** (-)-Nicotine assessment in d the Toxtracker mouse Embryonic stem cell reporter cell lines. Heat Map summary of the responses obtained following exposure.



Discussion and Conclusions

- Concentrations of (-)-nicotine approaching 5-10 mM can induce apparent aneugenic effects in CHO-WBL cells:
  - Non-linear MN and hypodiploid nuclei responses as well as cytoskeleton perturbations and presence of multinuclear cells were observed.
- The genotoxicity is likely driven by a novel lysosomotropic mode-of-action because:
  - Cellular vacuolization and enhanced NRU are co-associated.
  - Modulated by compounds that elevate the pH of intracellular acidic compartments.
  - Does not involve activation of histones H2AX and H3.
- However, this response is unlikely to be of physiological relevance, as:
  - High, sustained concentrations (i.e., 5-10 mM) required to elicit the response in CHO-WBL cells orders of magnitude higher than maximal plasma concentrations achieved in heavy nicotine consumers (i.e., 0.1-0.6 µM) (Ginzkey et al., 2014).
  - MN and ToxTracker® studies in more relevant/robust cell lines reported non-genotoxic findings up to and beyond 10 mM.

References

- Ginzkey *et al.,* 2014 *Toxicol. Lett.* 229: 303–310; Hendriks G *et al.,* 2012, *Toxicol. Sci.* 125, 285-298; INVITTOX (1990). Protocol 3; OECD Test Guideline 487 (2016).

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Competing Financial Interest

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