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Alternatives to cytotoxicity assessment of eliquids using the neutral red uptake assay

Damian McHugh, Gianluca Cudazzo and Patrick Vanscheeuwijck

Philip Morris International R&D, Neuchatel, Switzerland.

Presentation Objectives

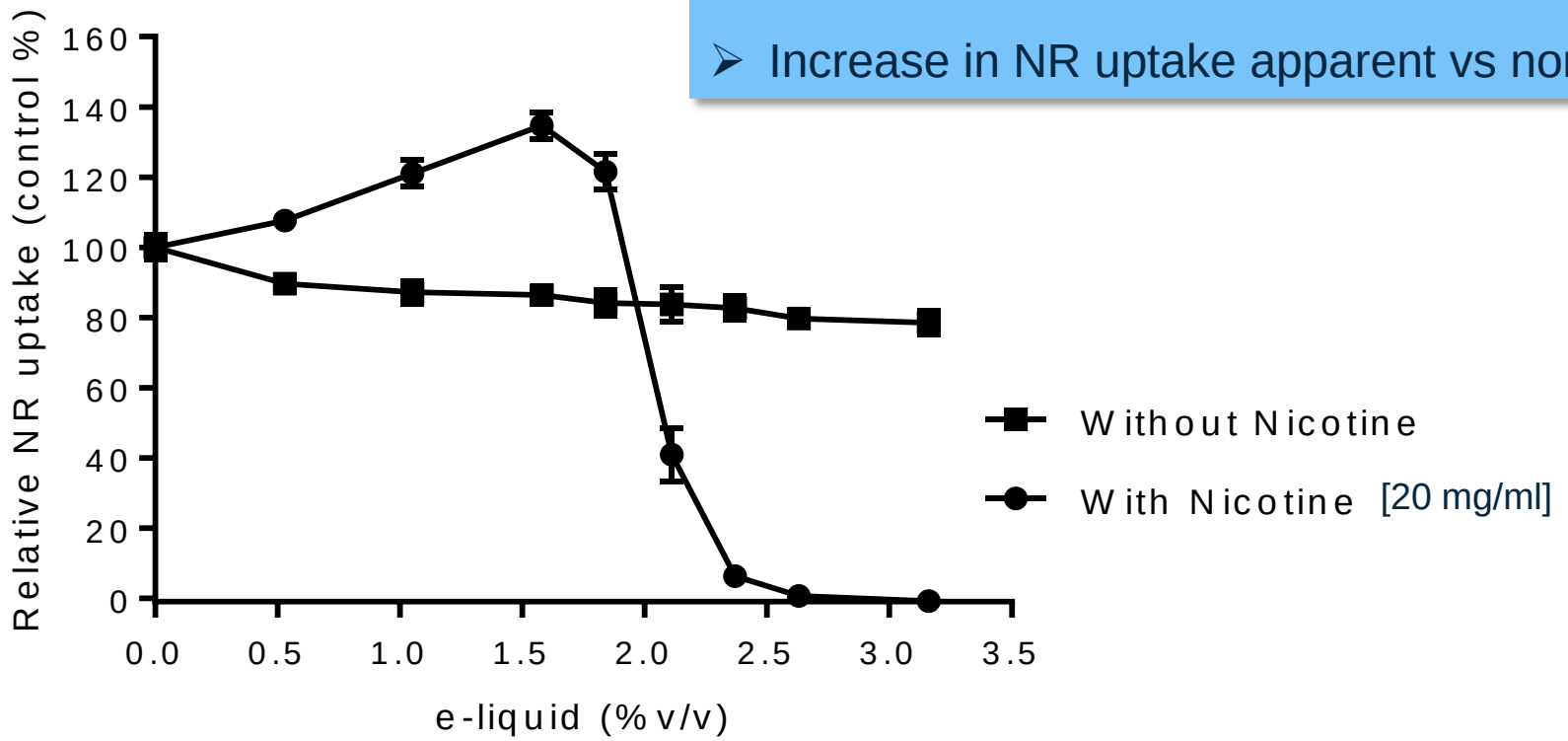
1. Details of the study protocols.
2. Neutral Red Uptake results obtained following (-)- nicotine treatment.
3. Performance of alternative approaches to assess cytotoxicity.
4. Conclusions.

Study Protocols

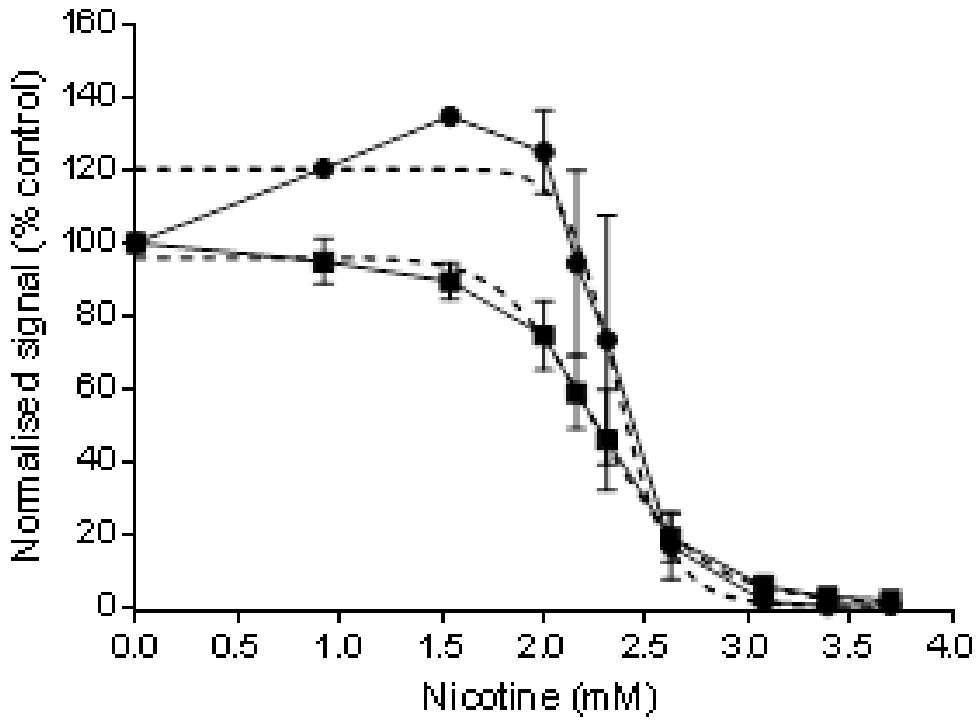
- All cells seeded at 5 x 10^3 cells ml in 96-well multiwall plates.
- Absorbance / Fluorescence read with a Tecan Safire II multi-mode plate reader operating with Magellan v7.0 software.
- Cell counts performed using a Beckman Coulter Multisizer 4 cell counter.
- Cytotoxicity scored 24-48h following start of exposure.

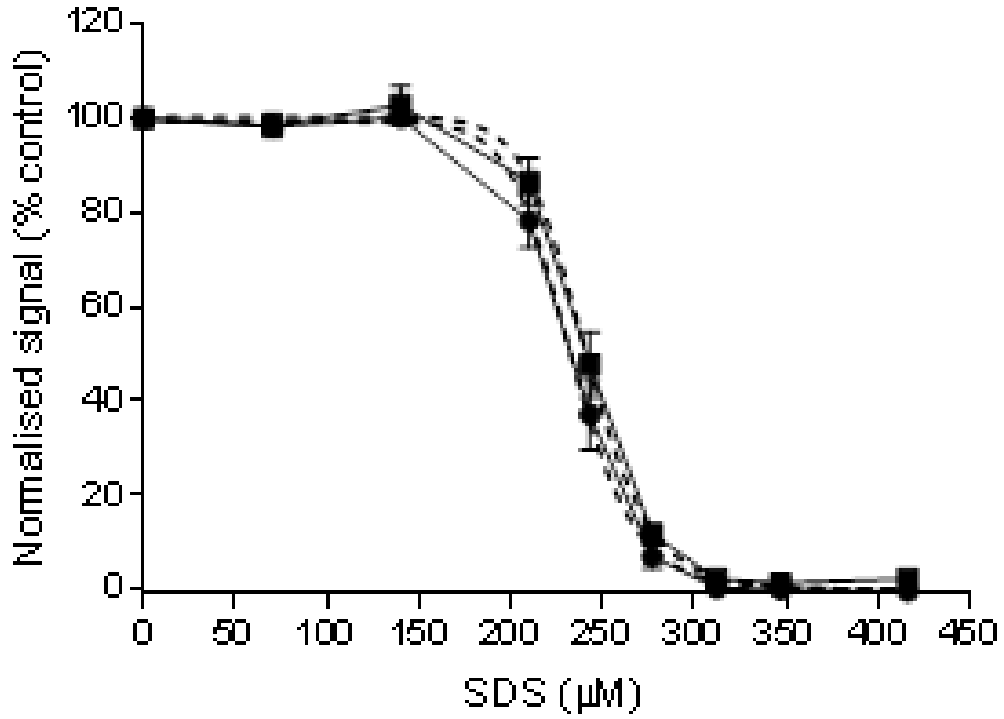
Apparent increase in neutral red uptake following exposure to eliquids containing (-)-nicotine

- Balb/c 3T3 cells treated for 24 h to eliquids ± nicotine
- Increase in NR uptake apparent vs non-nicotinated eliquid controls



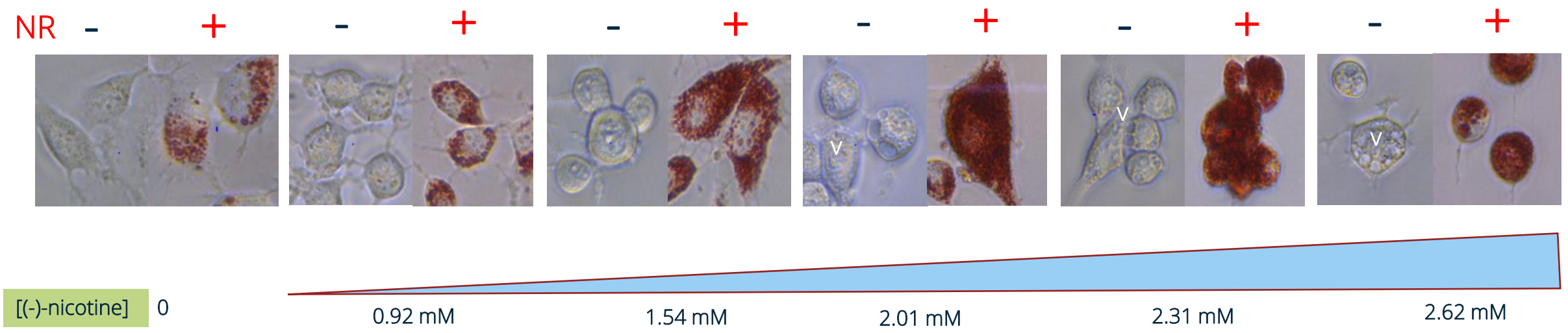
Viable cell counts not in agreement with NRU assay estimation of cell population viability following (-)-nicotine exposure





- NRU = neutral red uptake
- RCC = relative cell counts

Macroscopic changes apparent in nicotine treated cells

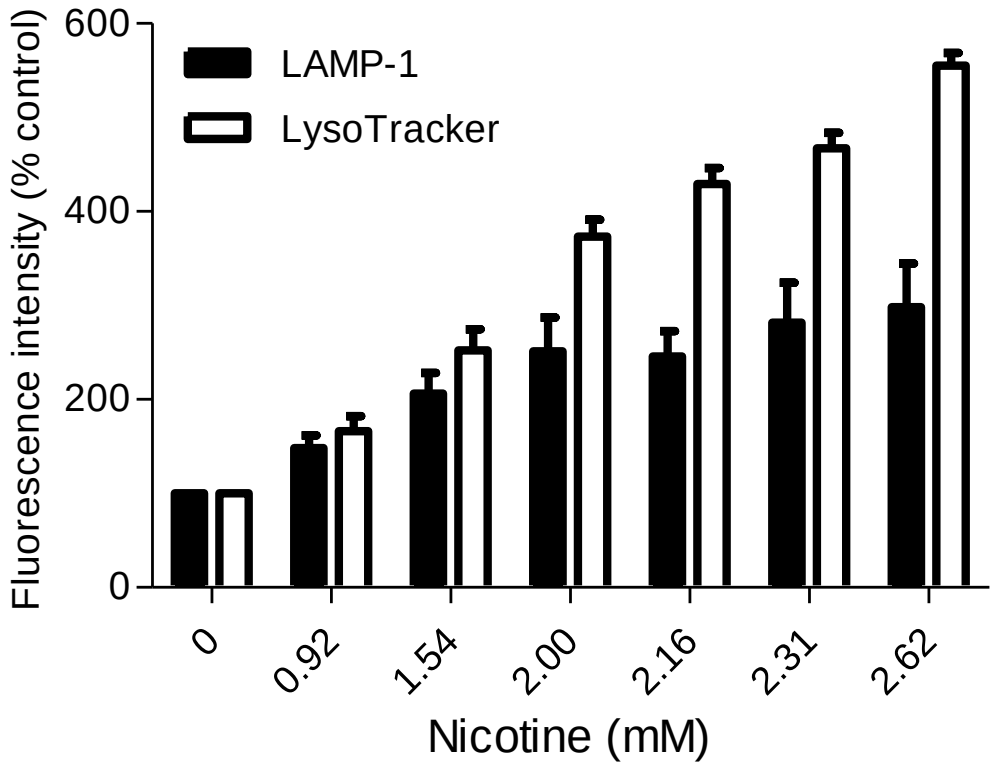


- Enhanced NR uptake coincident with macroscopic changes to cell ultrastructure

V = *vacuolisation*

Lysosome analysis via FACS confirms perturbation by (-)-nicotine

A



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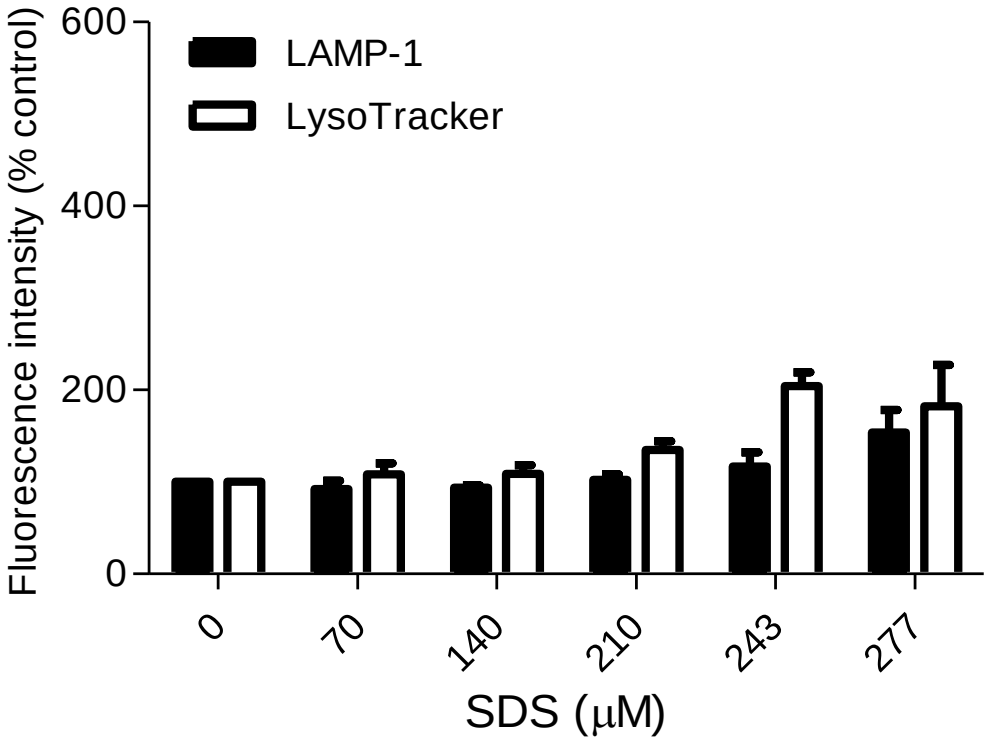

LAMP-1 staining increased in (-)-nicotine-treated Balb/c 3T3 cells

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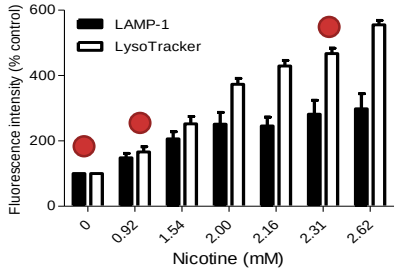

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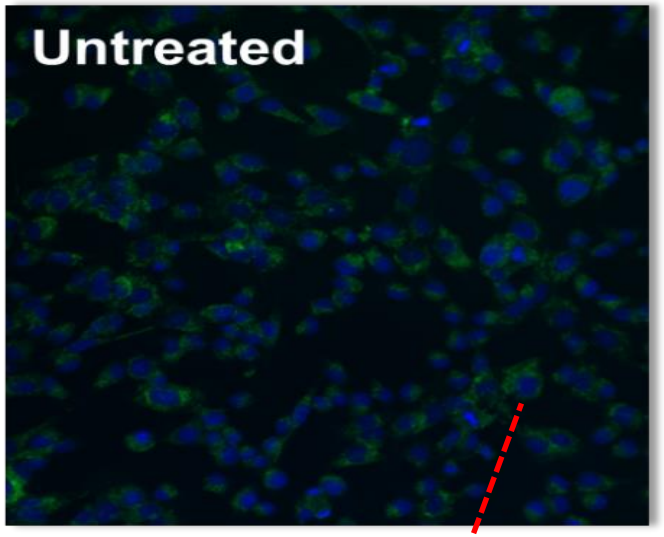
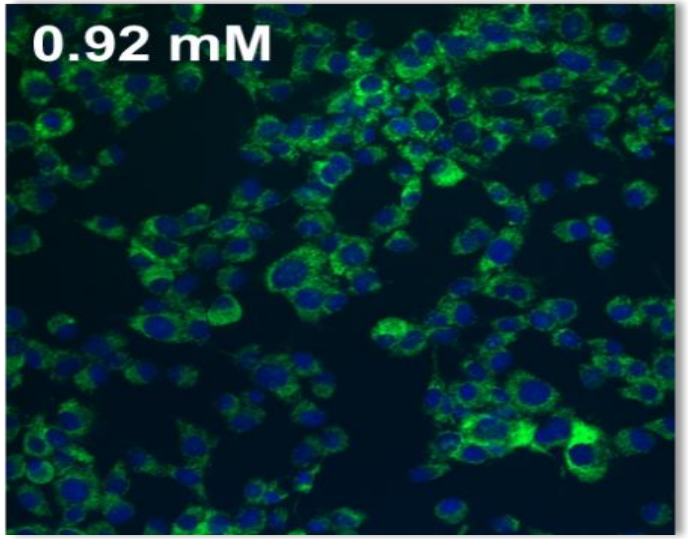
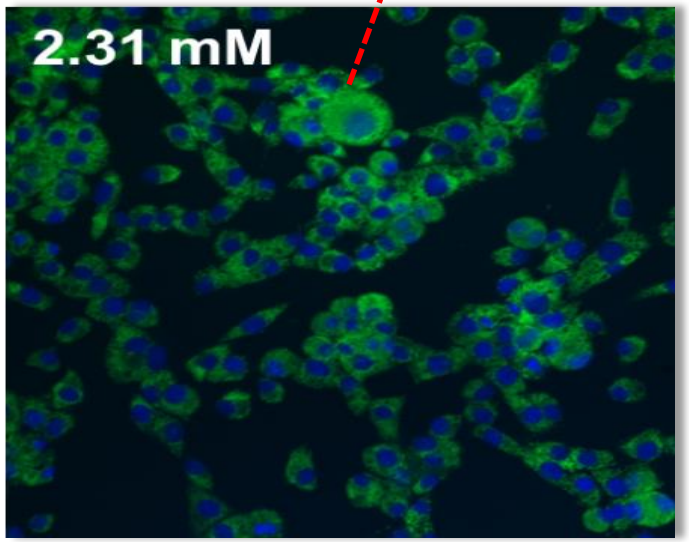




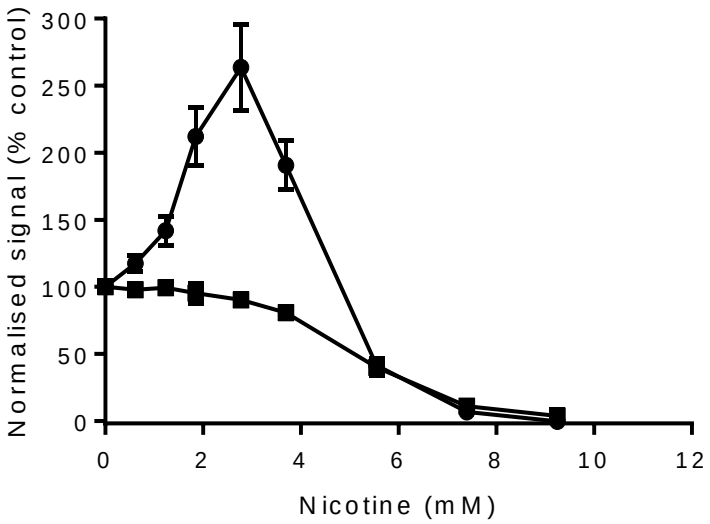


LAMP-1

nucleus

Sensitivity to nicotine-induced effects on NRU conserved across multiple human cell lines

SH-SY5Y



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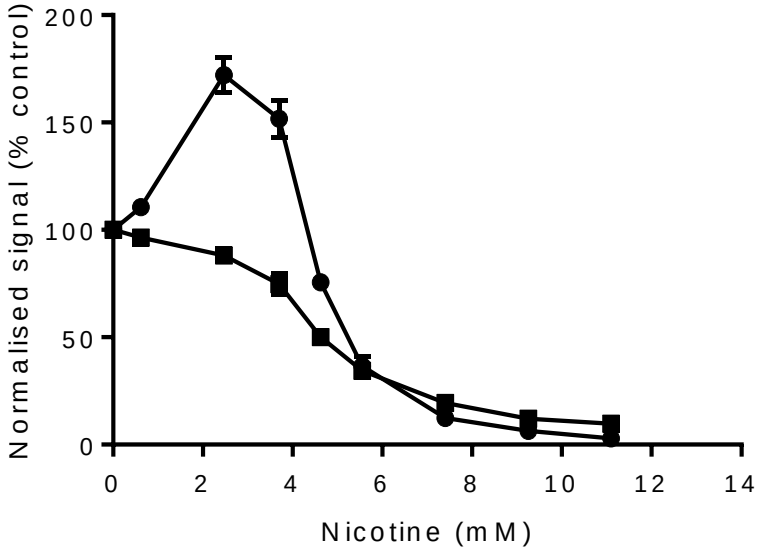

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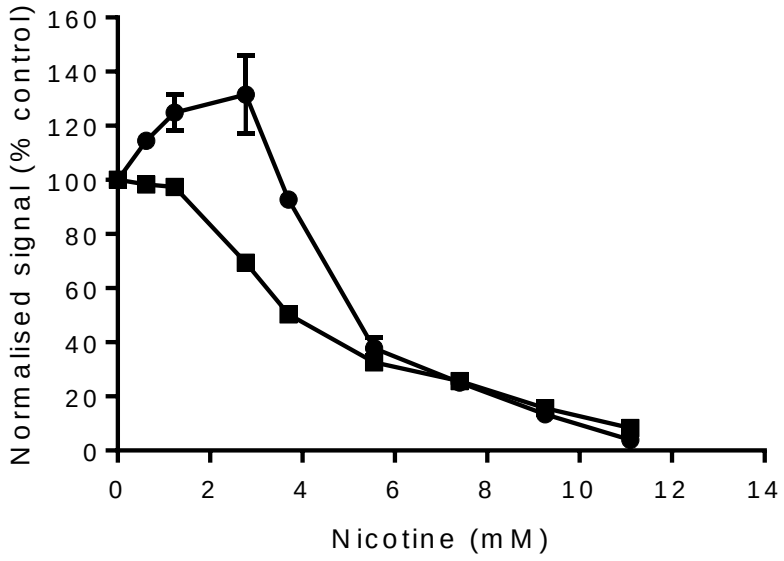
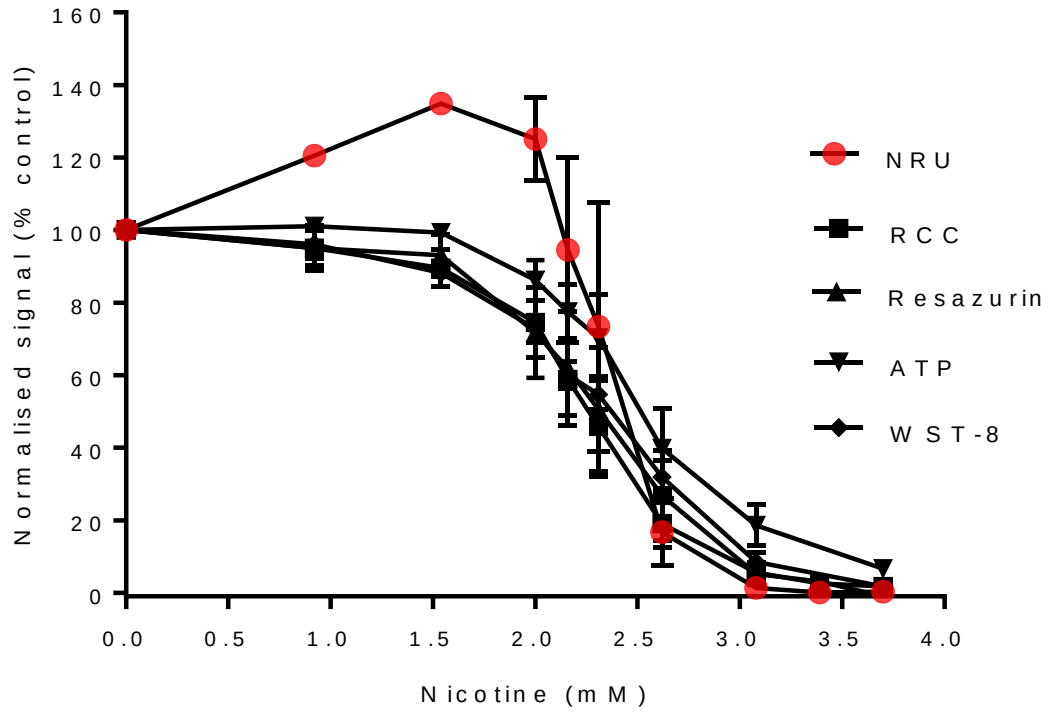


NRU = neutral red uptake
RCC = relative cell counts

High-throughput compatible approaches to determine cytotoxicity in Balb/c 3T3 cells

EC_{50} range = 2.28 -2.52 mM



- **Nicotine cytotoxicity was successfully evaluated with all assays using metabolic measures of viability**
- **No nicotine-induced artefacts were detected**
- **Agreement between the direct (RCC) and metabolic assay processes estimation of the cytotoxicity EC_{50}**

NRU = neutral red uptake
RCC = relative cell counts

Determination of the WST-8 assay intra- and inter-day variability

Repeatability			Reproducibility		
	EC_{50}			EC_{50}	
	Nicotine (mM)	SDS (µM)		Nicotine (mM)	SDS (µM)
Operator 1	2.33	248.5	Test 1	3.09	246.5
Operator 2	2.06	249.9	Test 2	2.32	250.7
Operator 3	2.36	253.5	Test 3	2.42	280.7
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Mean	2.3	250.6	Mean	2.6	259.3
SD	0.2	2.6	SD	0.4	18.7
RSD (%)	7.3	1	RSD (%)	16.0	7.2

Conclusions

- eliquids containing nicotine perturb lysosome functioning and introduce artefacts when characterising the cytotoxicity of such mixtures with the neutral red uptake assay.
- Alternatives to assess cytotoxicity with these types of mixtures include performing cell counts or using biochemical indicators of cell viability.
- Reduction of WST-8 was selected as the assay to validate and implement at PMI due to its consistency plus the possibility to assess additional endpoints in the same treated cells.
- The assay is suitable to assess cytotoxicity of nicotine-containing eliquids in multi-well plates without any changes to the upstream assay processes.

Acknowledgements

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