An *in vitro* genotoxicity assessment for next generation nicotine delivery products

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Presentation outline

- An introduction to next generation products (NGPs)
- *In vitro* product assessment framework overview
- *In vitro* exposure methods with cigarettes/ NGPs
- Case study: Application of (experimental) electronic vapour product/ cigarette-derived samples in the ToxTracker assay

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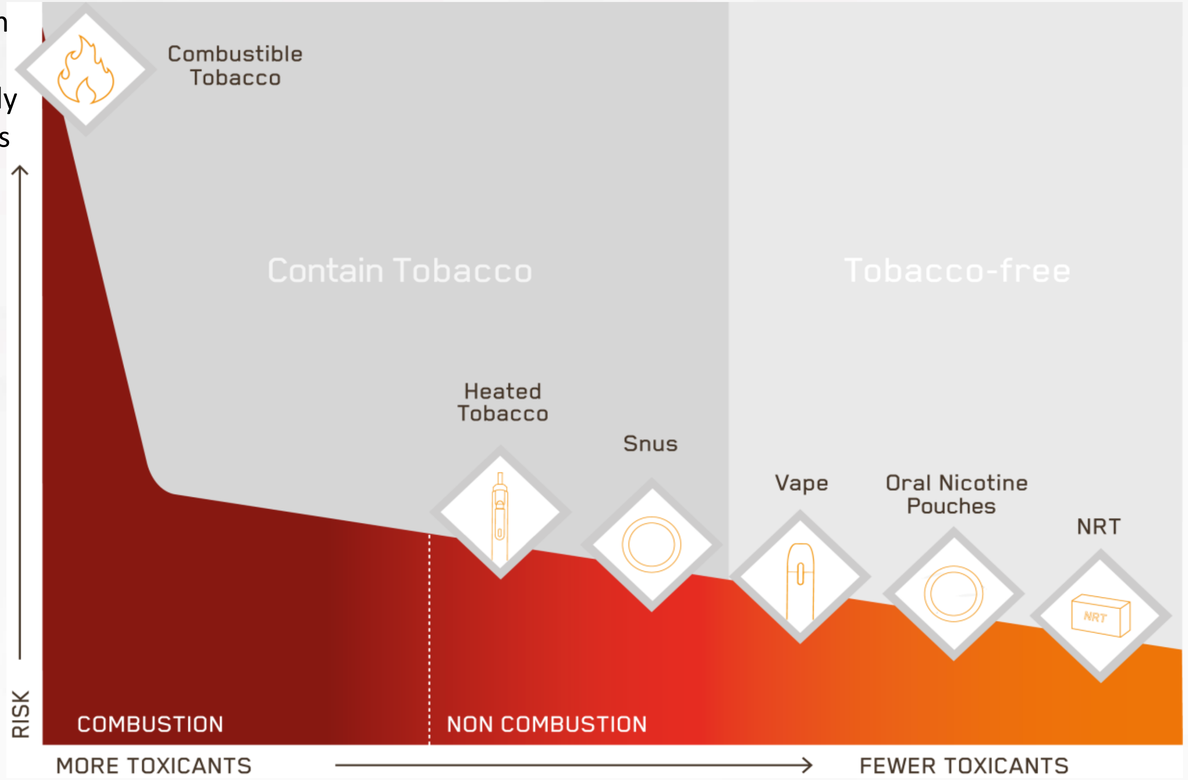
Next generation products

- Next generation products (NGPs) offer a means of potentially reduced harm nicotine delivery to adult smokers
- Categories include electronic vapour devices (EVPs), heated tobacco products (HTPs) and oral nicotine pouches

>7000 chemicals in cigarette smoke, around 100 of which are classified as harmful or potentially harmful constituents (HPHCs)



- HTP: reconstituted tobacco stick heated (but not burned) to produce nicotine-containing aerosol
- Snus: oral resting (between gum and top lip) products containing tobacco
- EVP: (flavoured) e-liquid (base constituents propylene glycol (PG) + vegetable glycerine (VG)) heated to produce vapour (containing nicotine or nicotine free)
- Oral nicotine pouches: tobacco-free oral resting pouches
- NRT: nicotine replacement therapies (e.g., lozenges)
- Test samples generated from these are complex chemical mixtures

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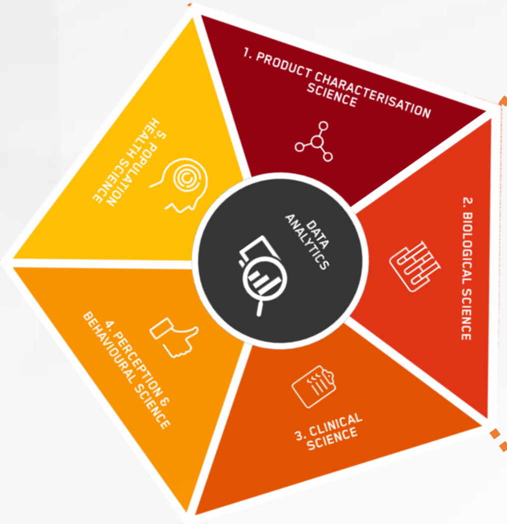
Background: *in vitro* toxicity testing framework

- The *in vitro* assays used avoid the need, and act as a good surrogate, for animal testing
- The assays also utilise human-derived cells wherever possible
- The *in vitro* toxicity testing framework is a combination of established regulatory toxicity assays and newer methodologies



Regulatory assays	Cardiovascular	Carcinogenesis	COPD	Reproductive toxicity	*In silico* methods	Clinical samples
Cytotoxicity (neutral red uptake)	*Scratch wound assay*	*Cellular transformation assay*	*3D lung models*	*devTOX quickPredict*	*AOP development*	*Analysis of clinical samples*
Micronucleus assay	*Cardio quickPredict*	*ToxTracker*			*Regional lung deposition*	*Application of clinical samples in vitro*
Ames bacterial reverse mutation test					*QVIVE*	
	High content screening					
	'Omics technologies					

Available tools but not yet routinely used

Routinely used

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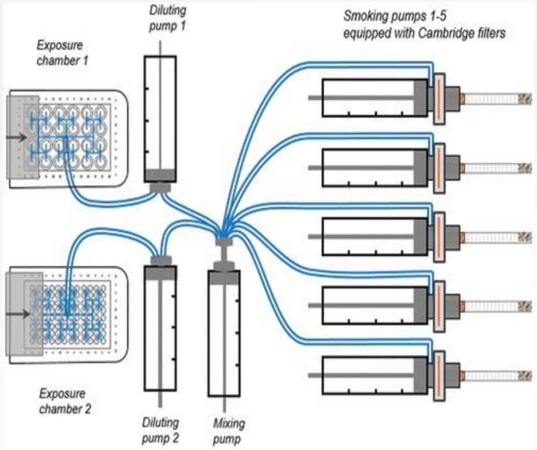
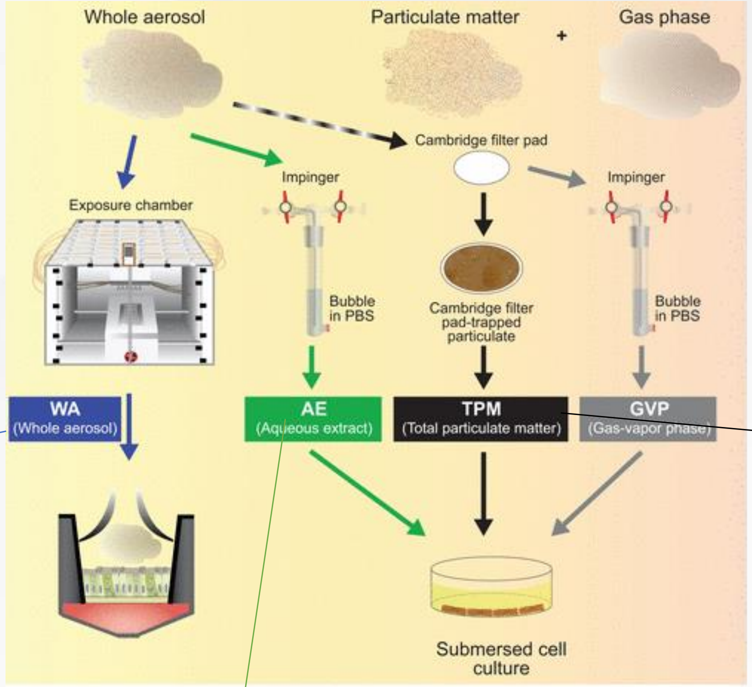
Methods for smoke/ aerosol exposure *in vitro*

Smoke/ Aerosol Exposure *In Vitro* System (SAEIVS)

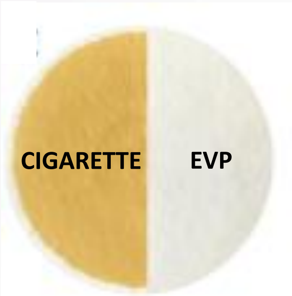
- Achieves exposure to **fresh** smoke/ aerosol at the air-liquid interface
- Most human relevant *in vitro* smoke/ aerosol exposure scenario
- Not practical for submerged samples



Rudd *et al.*, 2020

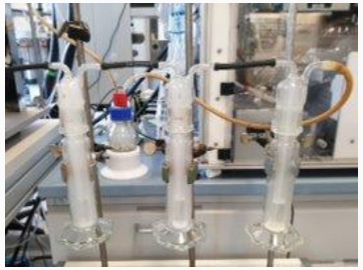


Boué *et al.*, 2020



Smoke/ aerosol trapping on Cambridge filter pads

- Includes **lipophilic fraction**
- Requires suitable solvent (e.g., DMSO) for addition to aqueous *in vitro* systems



Aqueous bubbling

- Can bubble **fresh** smoke/ aerosol through bacterial cultures
- Can bubble through PBS or medium and add to aqueous *in vitro* systems (can be stored frozen, used for several tests and shipped to between laboratories) – **aqueous soluble fraction**

E-liquids can also be added directly to aqueous medium

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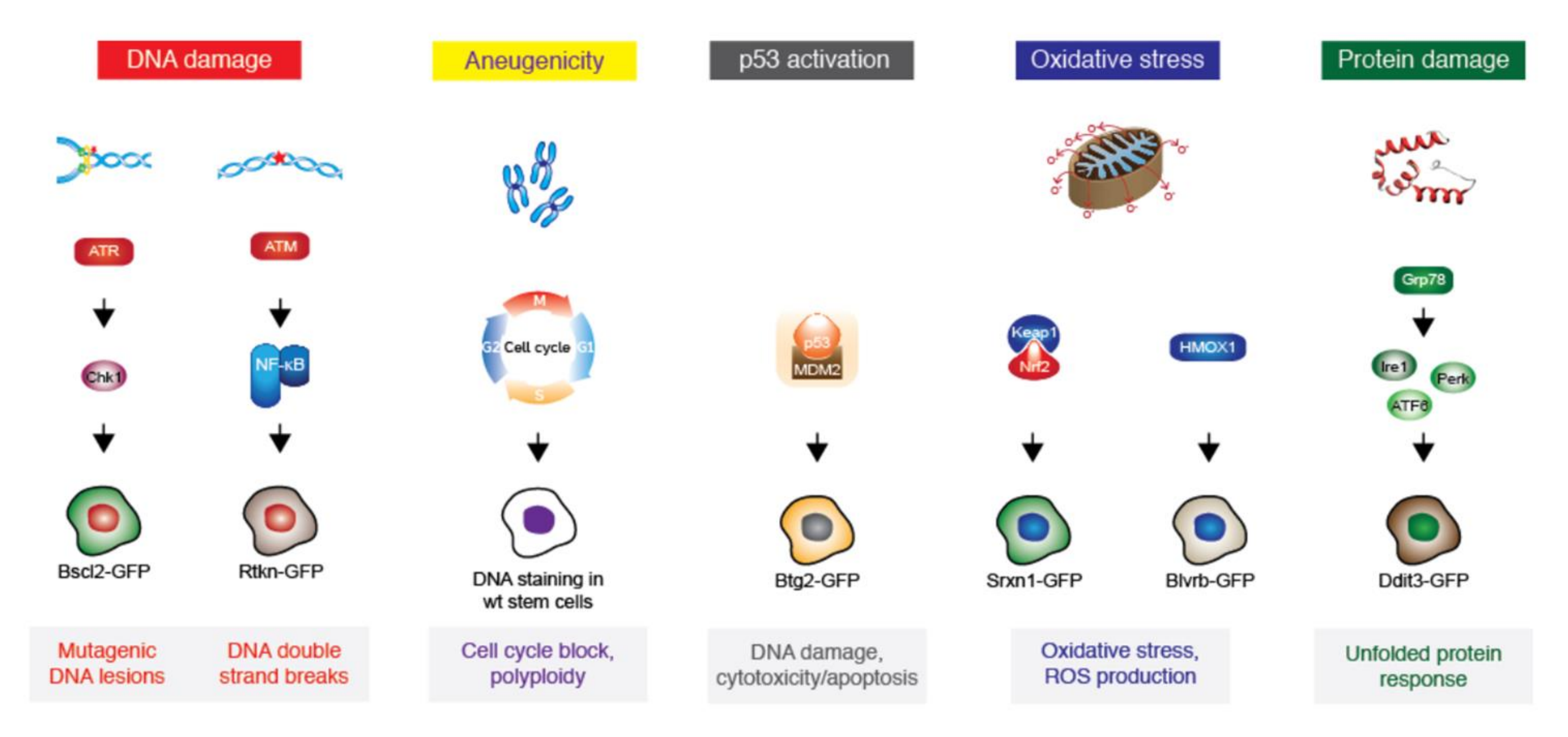
Application of EVP/ cigarette-derived samples in the ToxTracker assay

- Aim: to study the effects of EVP and 1R6F reference cigarette smoke-derived samples in the ToxTracker assay

- ToxTracker assay: mouse embryonic stem cells modified with bacterial artificial chromosome recombineering, producing 6 green-fluorescent protein (GFP) reporter cell lines

- Cells in submerged culture
    - Cells exposed to experimental e-liquids (neat or aerosol bPBS) and 1R6F smoke (TPM or smoke bPBS) (24h; +/-S9)



Toxys, accessed online, 11-2020

Test articles

	Test article	Description	Max. tested concentration (%)
Neat	EVP-neat-NS-TF	Neat e-liquid base containing 1.6% nicotine salt and tobacco flavouring	1
Neat	EVP-neat-FB-TF	Neat e-liquid base containing 1.6% freebase nicotine and tobacco flavouring	1
Neat	EVP-neat-TF	Neat e-liquid base containing 0% nicotine and tobacco flavouring	1
Neat	EVP-neat-1:1 PG:VG	Neat e-liquid base, 1:1 PG and VG only	1
Aqueous	EVP-bPBS-NS-TF	PBS-bubbled aerosol of e-liquid base containing 1.6% nicotine salt and tobacco flavouring	10
Aqueous	EVP-bPBS-FB-TF	PBS-bubbled aerosol of e-liquid base containing 1.6% freebase nicotine and tobacco flavouring	10
Aqueous	EVP-bPBS-TF	PBS-bubbled aerosol of e-liquid base containing 0% nicotine and tobacco flavouring	10
Aqueous	EVP-bPBS-1:1 PG:VG	PBS-bubbled aerosol of e-liquid base containing 1:1 PG and VG only	5
Aqueous	1R6F-bPBS	PBS bubbled smoke of the 1R6F reference cigarette	10
TPM	1R6F-TPM	DMSO extract of cigarette smoke trapped in a filter pad	1

- 24h exposures
- + or – S9 (metabolism)

Neat = 100% e-liquid stock

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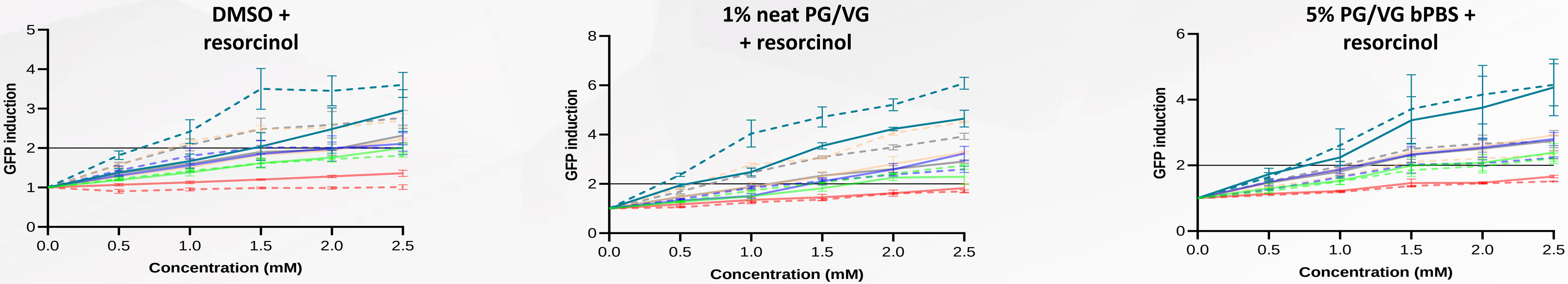
Does PG/VG base liquid interfere with responses to additional ingredients?



- Positive control compounds (resorcinol, vinblastine, B[*a*]P) spiked into system to test for the effects of combination with PG/VG base e-liquid components
- Positive compounds added to cells
  - + DMSO
  - + 1% 1:1 neat PG:VG
  - + 5% 1:1 PG:VG aerosol bPBS
- No significant differences in the responses observed in each of the three vehicles

Resorcinol:
- DNA damage
- Oxidative stress
- p53 related response

Endpoint	*Marker*
DNA damage	Bscl2 -S9 Bscl2 +S9 Rtkn -S9 Rtkn +S9
p53 activation	Btg2 -S9 Btg2 +S9
Oxidative stress	Srxn1 -S9 Srxn1 +S9 Blvrb -S9 Blvrb +S9
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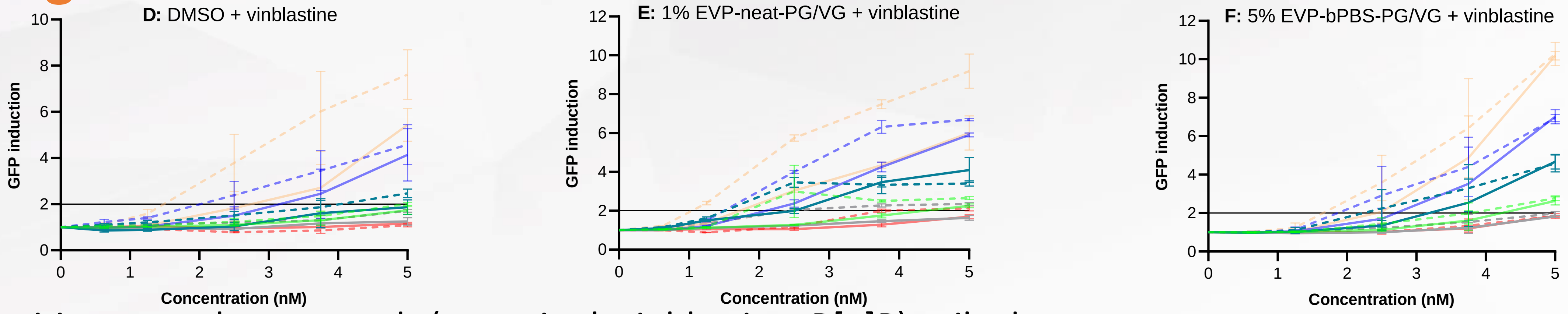

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- Similar case with B[*a*]P and vinblastine

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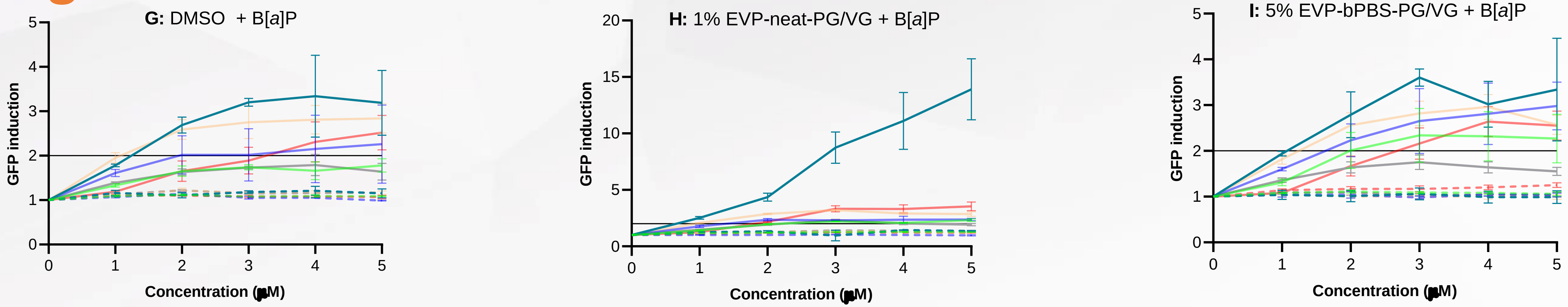

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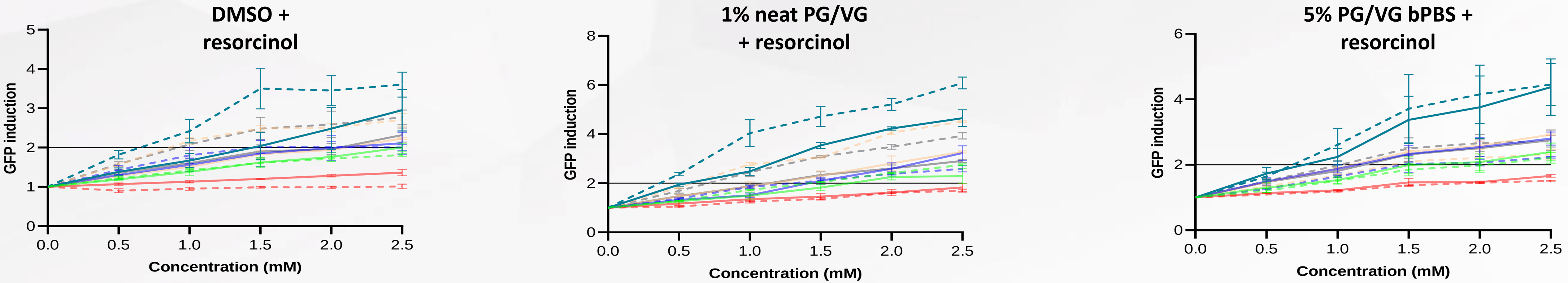

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- Positive compounds added to cells
  - + DMSO
  - + 1% 1:1 neat PG:VG
  - + 5% 1:1 PG:VG aerosol bPBS
- No significant differences in the responses observed in each of the three vehicles
- Conclusion: The system still had sensitivity to positive control compounds in the presence of PG/VG samples → additional flavourings/ nicotine can be tested

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DNA damage	Bscl2 -S9 Bscl2 +S9 Rtkn -S9 Rtkn +S9
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Protein damage	Ddit3 -S9 Ddit3 +S9
Positive induction	

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How does the system respond when screening neat e-liquids?

Oxidative stress response at higher concentrations of (tobacco) flavoured e-liquids tested

Endpoint	Marker
DNA damage	Bscl2 -S9 Bscl2 +S9 Rtkn -S9 Rtkn +S9
p53 activation	Btg2 -S9 Btg2 +S9
Oxidative stress	Srxn1 -S9 Srxn1 +S9 Blvrb -S9 Blvrb +S9
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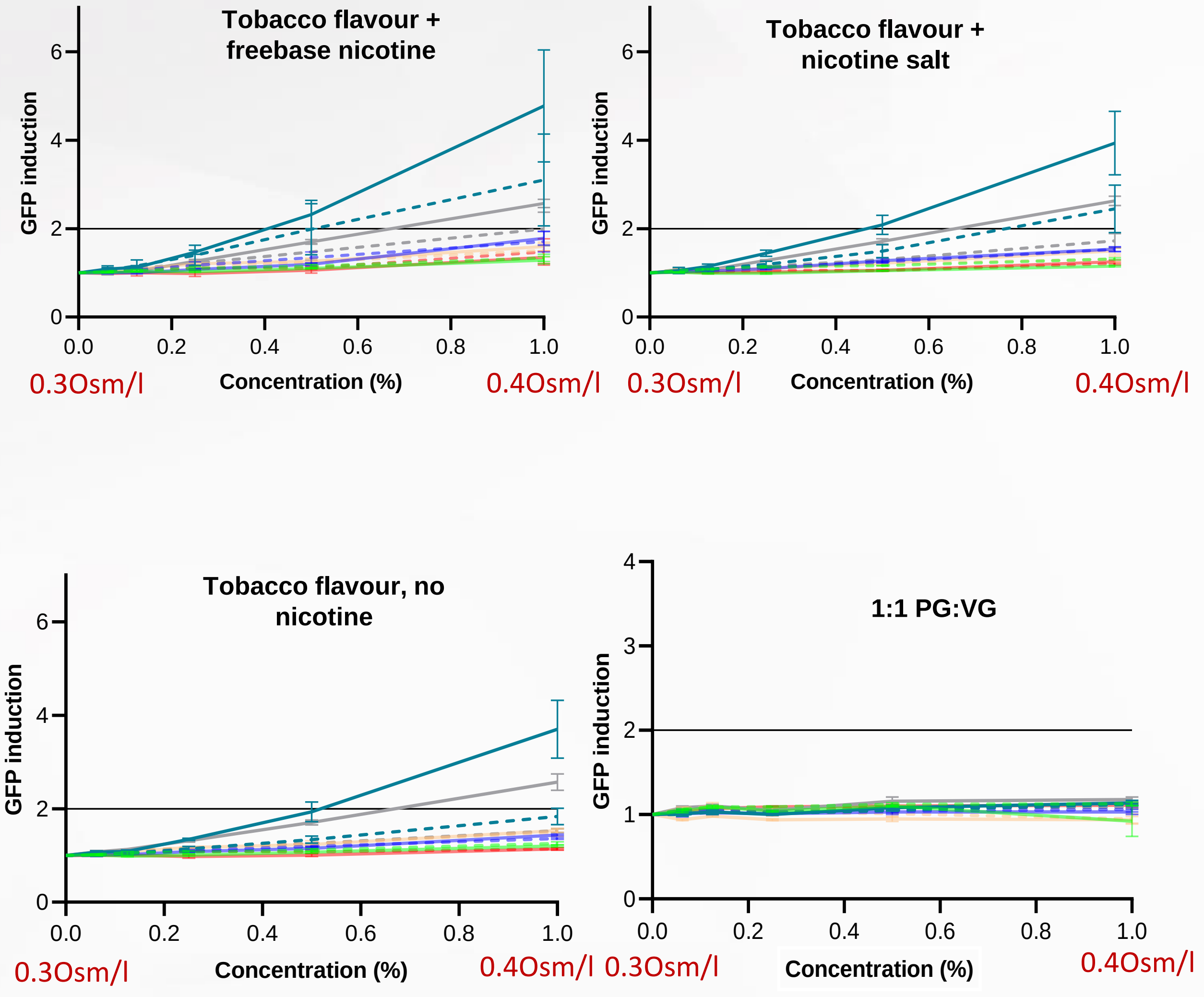
Positive induction	



- Common, published effect (Czekala *et al.*, 2019).
- Oxidative stress possibly due to artificial effects of e-liquids on osmolarity *in vitro* – PG and VG are hygroscopic
- Osmolality effect already a consideration in OECD guidelines
- In conclusion, from medium osmolarity measurement, neat e-liquids can be tested up to 1% to avoid effects of increasing osmolarity

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Confirmation of smoke/ aerosol trapping in PBS

- The next samples to be tested, PBS-trapped smoke/ aerosol, were analysed to confirm trapping of constituents
  - Nicotine
  - 8 carbonyls, found on regulator HPHC lists

Analyte	***1R6F-bPBS***		***EVP-bPBS-TF***		***EVP-bPBS-FB-TF***		***EVP-bPBS-NS-TF***		***EVP-bPBS-1:1 PG:VG***	
	µg/ml	***µg/puff***	***µg/ml***	***µg/puff***	***µg/ml***	***µg/puff***	***µg/ml***	***µg/puff***	***µg/ml***	***µg/puff***
Nicotine	112.2	62.33	<1	<0.25	167	41.75	187	46.75	<1	<0.25
Formaldehyde	9.7	5.39	4.4	1.10	2.1	0.53	9.0	2.25	2.3	0.58
Acetaldehyde	167.3	92.94	<LOQ	<LOQ	<LOQ	<LOQ	3.3	0.83	<LOQ	<LOQ
Acetone	19.5	10.83	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Acrolein	4.2	2.33	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Propionaldehyde	8.7	4.83	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Crotonaldehyde	4.4	2.44	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2-Butanone (MEK)	3.9	2.17	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
n-Butyraldehyde	3.5	1.94	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

- Nicotine levels in nicotine salt/ freebase nicotine EVP aerosols comparable to 1R6F smoke nicotine levels, indicating comparable nicotine delivery between the samples

- Increased formaldehyde in nicotine salt sample, compared to the other EVP samples, was observed, but this was still 2-fold lower per puff than in the 1R6F smoke bPBS

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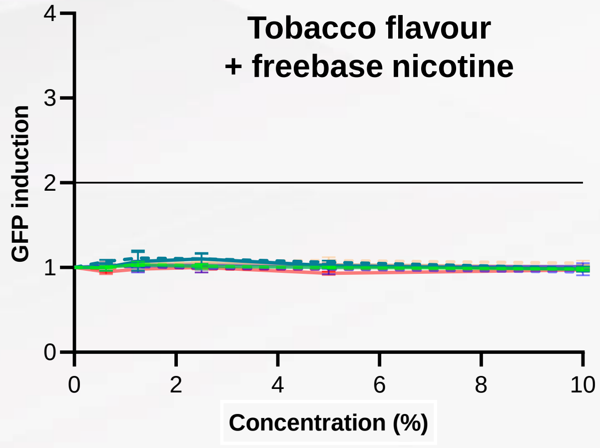
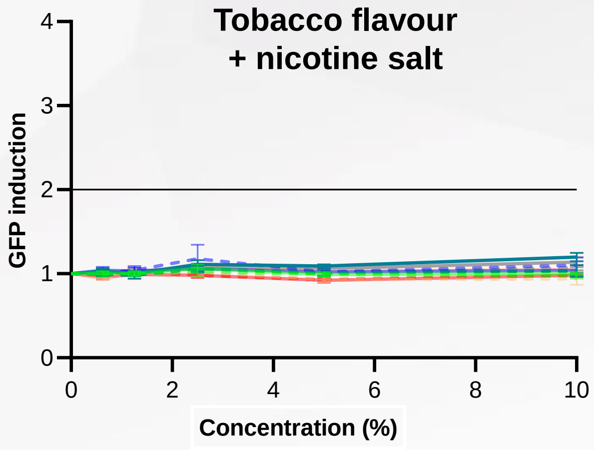
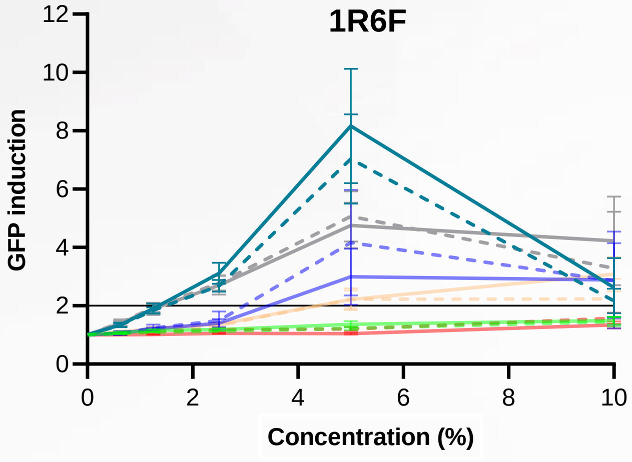
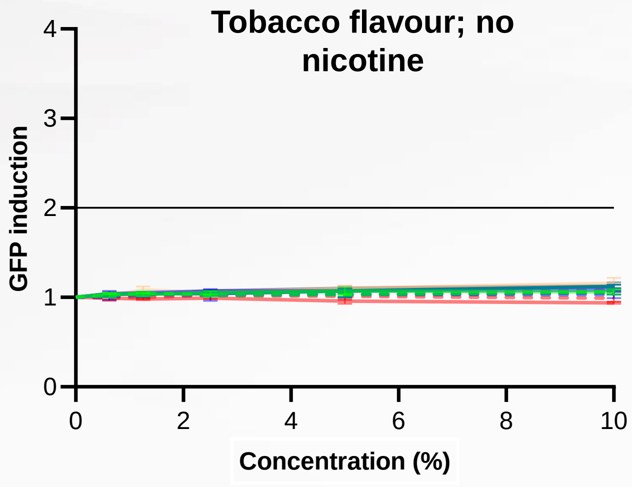
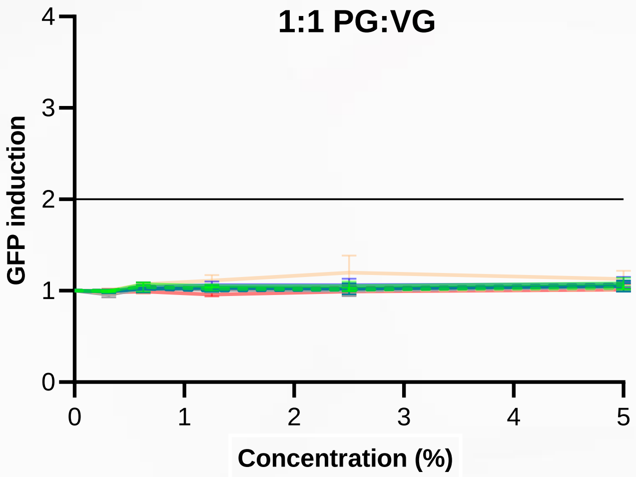
Effects of bPBS samples











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Protein damage	Ddit3 -S9 Ddit3 +S9
Positive induction	

- Complex response to 1R6F bPBS – due to unknown chemical fraction, requires further compositional analysis
  - Oxidative stress
  - Direct DNA damage
  - p53-related response

- On comparison with the 1R6F bPBS, the EVP bPBS samples did not cause cellular responses under the conditions of the test

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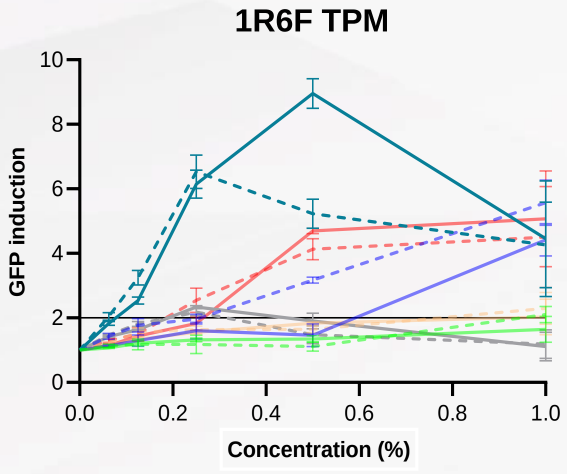
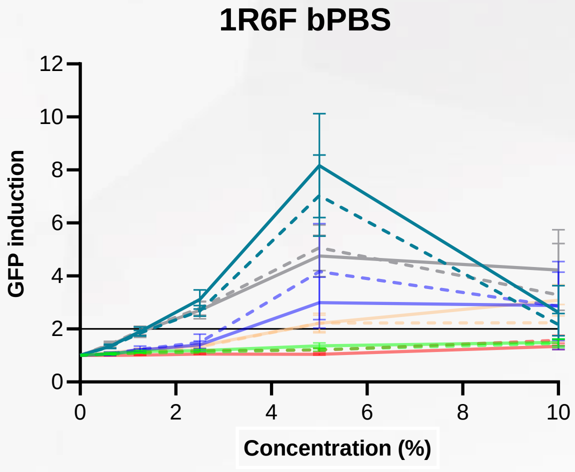
ToxTracker Aneugen Clastogen Evaluation (ACE) assay extension





- 1R6F TPM
  - Oxidative stress
  - Direct DNA damage
  - Protein damage
  - p53 stress response

Endpoint	Marker
DNA damage	Bscl2 -S9 Bscl2 +S9 Rtkn -S9 Rtkn +S9
p53 activation	Btg2 -S9 Btg2 +S9
Oxidative stress	Srxn1 -S9 Srxn1 +S9 Blvrb -S9 Blvrb +S9
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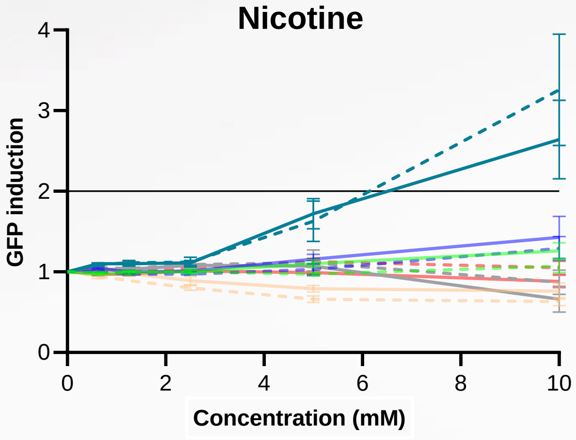

- Nicotine induced oxidative stress reporter…
- …only at a supraphysiological concentration, 10mM
- Up to 0.6µM nicotine in blood plasma, 10µM in saliva (Ginzkey *et al.*, 2014)

- Complex response to 1R6F samples, due to complex chemical mixture → further investigation with ACE extension
- Small changes to cell cycle were induced
- No changes to DNA content
- 1R6F samples not classed as aneugenic under the conditions of the assay

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Conclusions

- The ToxTracker assay can provide a quick indication of (geno)toxic mechanisms and has the potential to be incorporated fully into our assessment framework to supplement regulatory genotoxicity assay outcomes

  - PG/VG did not decrease cell sensitivity to the effects of additional components added to the cell system
  - Neat e-liquids can be tested up to a concentration of 1% with ToxTracker
  - bPBS is a suitable method of exposure in the assay
  - 1R6F smoke did not appear to have an aneugenic mode of action
  - Nicotine causes oxidative stress, only at a supraphysiological concentration
    - EVP and 1R6F smoke bPBS contained similar levels of nicotine
    - Responses to the nicotine-containing test articles in the assay not thought to be due to nicotine content

- Further evaluation of additional flavoured e-liquid derived samples, and characterisation of bPBS chemical content, is required

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References

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Questions?

Thank you

Imperial Brands
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