The *In Vitro* assessment of Respiratory Sensitisation Potential of Electronic Cigarette Liquids

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British Toxicology Society Annual Congress 2018

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1. Introduction and Objectives

There is a general consensus amongst the scientific and public health community that e-cigarettes constitute a less harmful source of nicotine than combustible cigarettes, and that flavours play a critical role in attracting and retaining smokers into the vaping category. Due to the dynamic nature of innovation with e-cigarettes, new assays are required to quickly determine the subtle biological response of these products for product stewardship activities. The size of this task is considerable as recent estimates state that more than 8,000 e-liquid flavours are on the market (Hartung, 2016). One particular toxicological endpoint which is of interest for the stewardship of e-liquids, is Respiratory Sensitisation.

Respiratory sensitization (RS) is an allergic type I hypersensitivity reaction of the upper and lower respiratory tract caused by an immune response triggered by low molecular weight compounds or other environmental proteins. Clinical symptoms of RS include asthmatic attacks, bronchoconstriction and wheezing upon repeated exposure to the same compound. However, respiratory sensitisers are rare, with around 100 well characterised substances described in the literature.

As a responsible manufacturer, it is Fontem Ventures' policy to screen all novel e-liquid ingredients for respiratory sensitising activities using published literature and *in silico* techniques. However, there is a need for alternative techniques to fill data gaps and add to a weight-of-evidence approach. Several *in vitro* assays have been described and validated to assess skin sensitisation, however for respiratory sensitization there are no validated predictive assays. It is of note that not all skin sensitizers are also respiratory sensitizers. In 2015, Basketter and Kimber concluded that "…airborne fragrance materials, including skin sensitising fragrance materials, do not pose a risk of the induction or elicitation of allergic reactions consequent upon exposure via the respiratory tract". Therefore, it is critical that any assays developed to determine the sensitising properties of a chemical can distinguish between dermal and respiratory activity.

The objective of this study was to assess experimental and commercial e-liquids in GARDair™; an assay which claims to detect respiratory sensitisers.

GARDair™ measures the genomic biomarker signature of a human myeloid leukemia-derived cell line exposed to test substances; making this technology in keeping with the 3Rs (Reduce, Replace and Refine) and Toxicity Testing in the 21st Century principles. Gene expression analysis is performed using Affymetrix microarray technology and a prediction model is used to classify each sample according to its respiratory sensitizing potential.

2. Materials and Methods

Test Materials

Three experimental e-liquids: Base Liquid (PG/VG: 50/50% W/W); Base Liquid + 2.4% Nicotine (PG/VG/Nic: 48/48/2.4% W/W); and Base Liquid + 4.5% Nicotine (PG/VG/Nic: 47.75/47.75/4.5% W/W). Two commercially available e-liquids (Commercial Sample 1: Blu Cherry 1.6% Nicotine and Commercial Sample 2 : 1.2% Nicotine).

Cell maintenance, chemical stimulations, phenotypic analysis and total RNA isolation

All GARD protocols for cell maintenance, cellular stimulation with chemicals, required phenotypical quality control of cells prior to chemical stimulation, and isolation of total RNA have been previously described (Forreryd *et al.*, 2015) and were followed without deviations in this study. The human myeloid leukemia-derived cell line is maintained in α-MEM (Thermo Scientific Hyclone, Logan, UT) supplemented with 20% (volume/volume) fetal calf serum (Life Technologies, Carlsbad, CA) and 40 ng/ml rhGM-CSF (Bayer HealthCare Pharmaceuticals, Seattle, WA).

Assessment of Cytotoxicity

Prior to commencing the assay the cytotoxic potential and solubility of the test samples was performed. For cytotoxic articles, the concentration yielding 90% relative viability (Rv90) is used for the GARD assay, the reason being that this concentration demonstrates bioavailability of the compound used for stimulation, while not impairing immunological responses. The concentration to be used for any given chemical is termed the 'GARD input concentration'. For further details of the GARD input concentration see Table 2.

Chemical exposure of cells for GARD

Once the GARD input concentration for chemicals to be assayed is established, the cells are stimulated again as described above, this time only using the GARD input concentration. All assessments of test substances are assayed in biological triplicates, performed at different time-points and using different cell cultures.

Preparation of benchmark controls

In addition to any test materials, samples exposed to a set of benchmark controls are created, for the purpose of prediction model calibration and estimation of prediction performance. For results of these benchmark controls see Table 1 and Figure 1.

Data analysis

For assessment of chemical RS, a Support Vector Machine (SVM) was modelled on a training data set corresponding to samples used for assay development. For a comprehensive overview of the training data set and methods, see Forreryd *et al.*, 2015. Each sample in the test set were assigned a decision value (DV), based on its transcriptional levels of the GRPS3 biomarker signature. Any test substance with a mean DV > 0 (n=3) is classified as a respiratory sensitizer. For GARDair predictions of the test articles see Figure 2.

3. Results

Table 1: Benchmark Controls

Substance ID	Figure annotation	Skin sensitization	Respiratory sensitization	CLP potency
2,4-dinitrochlorobenzene	DNCB	Sensitizer	Non-sensitizer	1A
p-phenylendiamine	PPD	Sensitizer	Non-sensitizer	1A
2-hydroxyethylacrylate	2-HA	Sensitizer	Non-sensitizer	1A
2-aminophenol	2-amino	Sensitizer	Non-sensitizer	1A
2-nitro-1,4-phenylendiamine	2-nitro	Sensitizer	Non-sensitizer	1A
Resorcinol	Resorcinol	Sensitizer	Non-sensitizer	1B
Geraniol	Geraniol	Sensitizer	Non-sensitizer	1B
hexyl cinnamic aldehyde	Ah cinnamicald	Sensitizer	Non-sensitizer	1B
chlorobenzene	chlorobenzene	Non-sensitizer	Non-sensitizer	No cat.
1-butanol	1-but	Non-sensitizer	Non-sensitizer	No cat.
DMSO	DMSO	Non-sensitizer	Non-sensitizer	No cat.
chloramine T	chloramine T	Non-sensitizer	Sensitizer	-
glutaraldehyde	glutaraldehyde	Non-sensitizer	Sensitizer	-
hexamethylen diisocyanate	hexamethylen diisocyanate	Non-sensitizer	Sensitizer	-
isophorone diisocyanate	isophorone diisocyanate	Non-sensitizer	Sensitizer	-
reactive orange	reactive orange	Non-sensitizer	Sensitizer	-
toluen diisocyanate	toluendiisocyanate	Non-sensitizer	Sensitizer	-
trimellitic anhydride	trimellitic anhydride	Non-sensitizer	Sensitizer	-
unstimulated cells	unstim	-	-	-

Table 1: Benchmark Controls: The list of chemicals used for prediction model calibration and estimation of prediction performance

Table 2: GARD Input Concentrations

Test substance ID	Vehicle	Max. screen[I]	Rv90[II]	GARD input concentration[III]
BL	Cell Culture Medium	5	3.5	0.55
BL + 1.6% Nictotine	Cell Culture Medium	5	2.5	0.55
BL + 4.5% Nicotine	Cell Culture Medium	5	1.125	0.55
Commercial E-liquid (1.6% Nicotine)	Cell Culture Medium	5	0.55	0.55
Commercial E-liquid (1.2% Nicotine)	Cell Culture Medium	5	1.125	0.55

I) The highest concentration (%) used in screening titration range.
II) Concentration (%) yielding 90% relative viability
III) Based on max. screen and Rv90

Table 2: GARD Input Concentrations: The test substances are mixtures containing a variety of compounds. For a fair comparison of the mixtures, it was decided that the test substance with Rv90 at the lowest concentration would be used for all other test substances, i.e. all substances were run at the same concentration.

GARDair Classification of Benchmark Controls

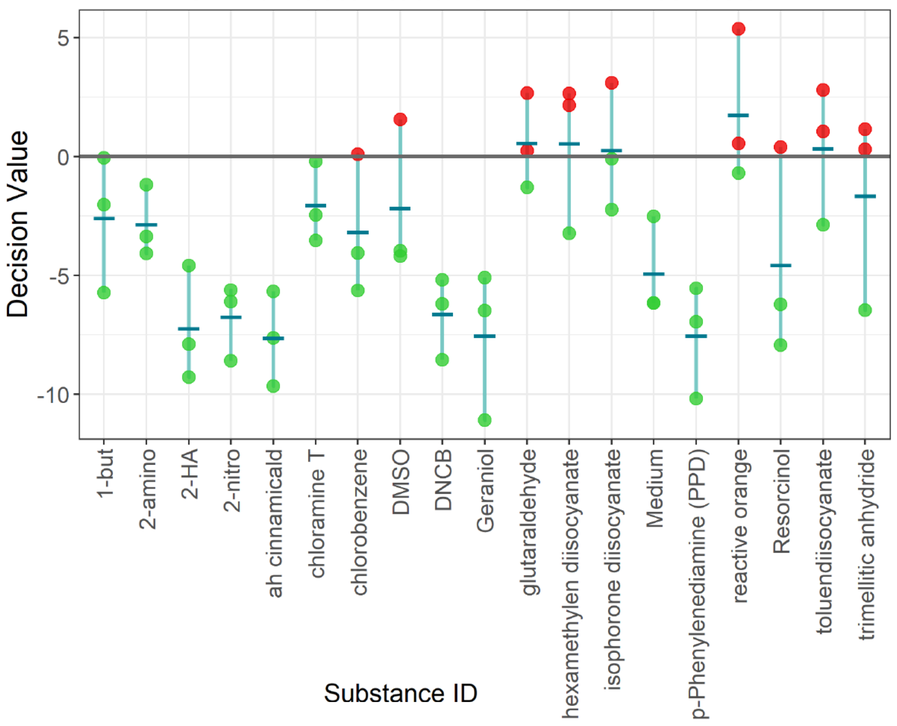

Figure 1: GARDair Decision Values of benchmark controls: Any test substance with a mean DV > 0 (n=3) is classified as a respiratory sensitizer. Out of the 7 assayed respiratory sensitizers, 5 are accurately classified as such by GARDair. No false positives were generated. Thus, the sensitivity and specificity are estimated to 71% (5/7) and 100% (12/12), respectively, with an overall predictive accuracy of 89% (17/19).

GARDair Classification of Test Materials

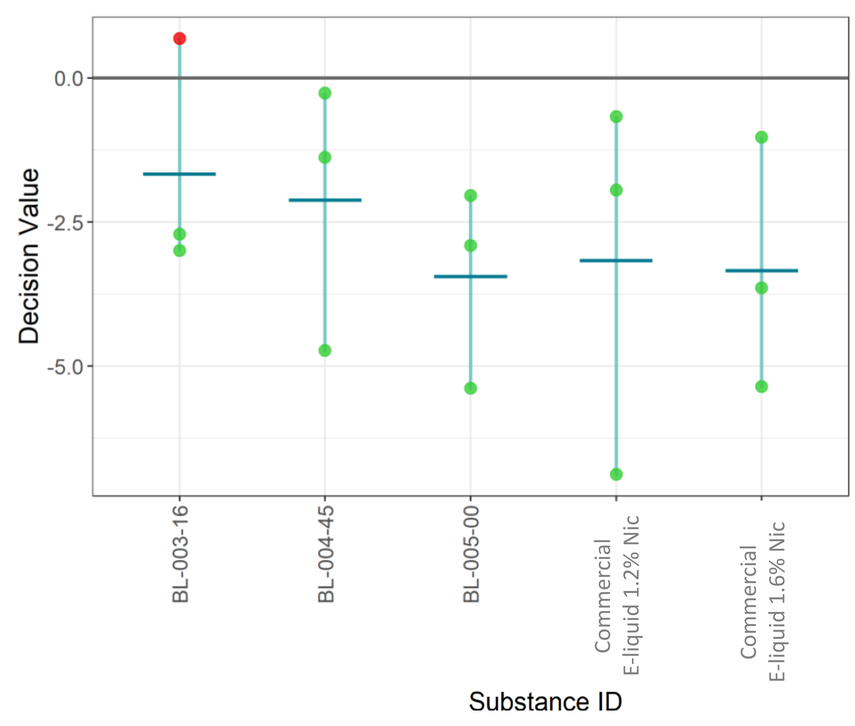

Figure 2: GARDair Decision Values of Test Materials: Any test substance with a mean DV > 0 (n=3) is classified as a respiratory sensitizer.
All Test Materials were classified as non-Sensitisers by GARDair.

4. Conclusion

- From the Benchmark Control data it was estimated that GARDair™ had a sensitivity and specificity of 71% and 100% respectively; with an overall predictive accuracy estimated as 89%.
- Extensive validation of this assay is ongoing, however, the lack of well characterised Chemical Respiratory Sensitisers may limit this.
- None of the experimental or commercial samples were classified as respiratory sensitisers.
- Further exploration of this assay is required, particularly its ability to detect low concentrations of sensitiser in complex mixtures and to ensure that the e-liquid matrix does not interfere with the detection of activity.

References

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