Using *In Vitro* Assays, the Direct Peptide Reactivity Assay (DPRA), KeratinoSens™ Assay (KS), and Human Cell Line Activation Test (h-CLAT) to Assess Skin Sensitization Potential of Electronic Cigarette Liquids

Abstract #3374

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Abstract

Three regulatory accepted *in vitro* assays were evaluated in a proof-of-concept project to determine skin sensitization potential of electronic cigarette liquids (e-liquids). These assays measure molecular initiating events and initial cellular responses prescribed in the OECD Integrated Testing Strategy (ITS) describing key events in the adverse outcome pathway (AOP) leading to skin sensitization. Briefly, DPRA measures ability of materials to bond with proteins (lysine and cysteine), KS determines keratinocyte activation, and h-CLAT determines dendritic cell activation potential by measuring CD86/CD54 surface markers. Two e-liquid base formulations [1:1 ratio glycerol:propylene glycol ± nicotine (6% w/w), without flavors] were tested to confirm assay compatibility and sensitization potential in DPRA and KS. Base formulations, both determined as non-sensitizers in DPRA and KS, were spiked with a known sensitizer, cinnamic aldehyde (CA), at 1% w/w to determine whether these assays can identify spiked formulations as sensitizers. For DPRA, the initial test with 1% CA was negative, but two higher CA concentrations (10% and 50%) in subsequent assays were confirmed as sensitizers. For KS, the CA spike concentration of 1% was identified as a sensitizer, but two lower CA spike concentrations of 0.1% and 0.01% were not identified although, the 0.1% CA approached a 1.5-fold induction threshold. The responses of both e-liquids (± nicotine) were similar in DPRA and KS. The h-CLAT experiments are ongoing. The initial results of spiking experiments indicate that potential skin sensitizers in e-liquid formulations might be identified in these *in vitro* assays. Based on these results, further efforts are warranted to test additional known sensitizers, accurately establishing the sensitivity of these assays, and formulating an efficient testing strategy (e.g. establishing optimal spike concentrations for each assay) leading to a robust *in vitro* sensitization testing program for possible new flavorings in e-liquids.

Material and Methods

Table 1: Experimental E-Liquids:

E-Liquid	Component (% w/w)			
	Glycerin	Propylene Glycol	Nicotine	Water
16AH13	44.5%	44.5%	6.0%	5.0%
16AH14	47.5%	47.5%	0.0%	5.0%

Direct Peptide Reactivity Assay[1]:

- Experimental e-liquid dilutions prepared in acetonitrile at 100mM assuming MW = 200 g/mole.
- Cinnamic Aldehyde "spiked" into experimental e-liquids at 1.0%, 10% & 50% (w/w).
- Samples mixed with synthetic peptides (cysteine & lysine) and incubated in the dark at R.T. for 24 hours.
- Cysteine and lysine depletion determined by HPLC with UV detection.

KeratinoSens™ Assay[2]:

- Experimental e-liquid dilutions prepared in DMSO (w/v: µg/mL)
- A known strong sensitiser, Cinnamic Aldehyde (CA), or weak sensitiser, Ethylene Glycol Dimethacrylate (EGD), were "spiked" into e-liquid at various levels: 0.01%. 0.1%, 1.0% (w/w).
- 96-well plates were seeded at ~1.0 x 10^4 cells / well; incubated 24 hours prior exposure (37ºC, 5% CO_2).
- After 48 hours exposure (37ºC) wells were washed with CMF-DPBS, followed by the addition of ONE-Glo™ Reagent, incubated 5 min at R.T. and luminescence read at 565nm.
- Cytotoxicity determined by MTT.

h-CLAT Assay[3]:

- Experimental e-liquid dilutions and Cinnamic Aldehyde prepared in DMSO at 500 mg/mL and 20 mg/mL, respectively.
- 24-well plates were seeded at ~1.0 x 10^6 cells/well and exposed to 8 concentrations of the test articles or CA for 24 hours.
- After exposure, cells were rinsed and treated with anti-CD54 or anti-CD86 antibody labeled with the APC fluorophore.
- Mean fluorescence intensity for each cell population was determined using flow cytometry.

References

1. OECD (2015) Guideline for the Testing of Chemicals: *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA) TG 442C
2. OECD (2015) Guideline for the Testing of Chemicals: *In Vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method (KeratinoSens™) TG 442D
3. OECD (2016) Guideline for the Testing of Chemicals: *In Vitro* Skin Sensitisation: human Cell Line Activation Test (h-CLAT) TG 442E
4. OECD (2012) The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins, Part 1: Scientific Evidence

Results

Table 2: Results from KeratinoSens™ Assay (CA, n = 3; EGD, n = 2)

KeratinoSens™ Assay Test Samples	EC 1.5[a] (µg/mL)	EC 1.5[b] (µM)	Potential Sensitizer[c]
16AH13	>400	0	No
16AH13 + 1.0% CA	83.07	6.27	Yes
16AH13 + 0.1% CA	>400	>3.02	No
16AH13 + 0.01% CA	>400	>0.302	No
16AH14	>400	0	No
16AH14 + 1.0% CA	118.04	8.91	Yes
16AH14 + 0.1% CA	>400	>3.02	No
16AH14 + 0.01% CA	>400	>0.302	No
Cinnamic Aldehyde (CA)	NC	7.21	Yes
16AH13 + 1.0% EGD	326.12	16.45	Yes
16AH14 + 1.0% EGD	1290.55	65.11	Yes
Ethylene Glycol Dimethacrylate (EGD)	NC	43.43	Yes

NC = Not Calculated

[a] The EC 1.5 (µg/mL) is the effective concentration (mixture as a whole) for gene induction above the threshold (1.5 fold) as compared to the DMSO solvent controls.

[b] The EC 1.5 (µM) refers to the induction based upon the molarity of CA or EGD in the mixture.

[c] A single component test article is predicted to have sensitization potential if:
1) The EC 1.5 value falls below 200 µg/mL (or 1000 µg/mL, EGD) in all 3 repetitions (or at least 2/3).
2) Cell viability >70% @ the lowest concentration with a gene induction above 1.5.
3) There should be an apparent overall dose response which is similar between repetitions.

Table 3: Direct Peptide Reactivity Assay Results (n = 3)

DPRA Test Samples	[CA]	Mean Peptide Depletion of Cys & Lys	Reactivity (Cys & Lys)	Potential Sensitizer
Cinnamic Aldehyde (CA)	100 mM	67.99%	High	Yes
16AH13	0 µM	0.13%	Minimal	No
16AH13 + 1.0% CA	1.41 µM	3.15%	Minimal	No
16AH13 + 10.0% CA	14.1 µM	26.38%	Moderate	Yes
16AH13 + 50.0% CA	70.4 µM	59.85%	High	Yes
16AH14	0 µM	0.57%	Minimal	No
16AH14 + 1.0% CA	1.41 µM	4.25%	Minimal	No
16AH14 + 10.0% CA	14.1 µM	24.84%	Moderate	Yes
16AH14 + 50.0% CA	70.4 µM	60.32%	High	Yes

Mean of Cysteine + Lysine % Depletion	Reactivity Class	Prediction
0% < Mean % Depletion < 6.38%	Minimal Reactivity	Non-sensitizer
6.38% < Mean % Depletion < 22.62%	Low Reactivity	Sensitizer
22.62% < Mean % Depletion < 42.47%	Moderate Reactivity	Sensitizer
42.47% < Mean % Depletion < 100%	High Reactivity	Sensitizer

Table 4: h-CLAT Assay Results (n = 2)

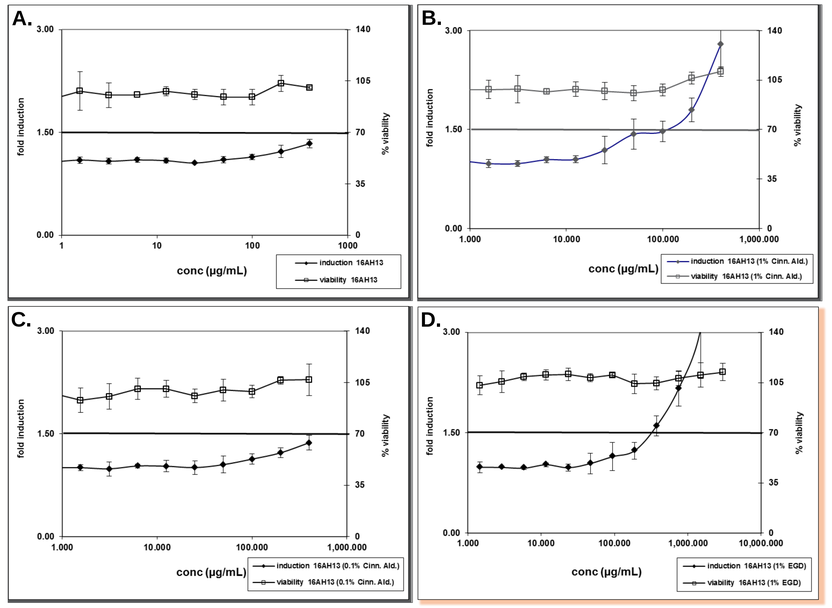
h-CLAT Test Samples	CD54 EC 200[a] (µg/mL)	CD86 EC 150[b] (µg/mL)	Potential Sensitizer[c]
Cinnamic Aldehyde (CA)	17.2	26.2	Yes
16AH13	> 1000	> 1000	No
16AH14	> 1000	> 1000	No

[a] CD54: EC 200 (µg/mL) is the effective concentration (mixture as a whole) showing a Relative Fluorescence Intensity (RFI) of 200% when compared to the solvent control.

[b] CD86: EC 150 (µg/mL) is the effective concentration (mixture as a whole) showing a RFI of 150% when compared to the solvent control.

[c] A single component test article is predicted to have sensitization potential if:
1) CD86: RFI is ≥ 150% at any tested concentration (with cell viability ≥50%).
2) CD54: RFI is ≥ 200% at any tested concentration (with cell viability ≥50%).
3) NOTE: maximum dose tested (16AH13 & 16AH14) was 1000 µg/mL.

Figure 1: KeratinoSens™ Assay, sample 16AH13 (A) "spiked" with Cinnamic Aldehyde (B & C) or EGD (D).



Summary and Conclusions

- **Experimental e-liquids were non-sensitizers in these assays.**
- **DPRA detected CA "spiked" into the e-liquids at 10% & 50% (w/w).**
- **KeratinoSens™ Assay detected CA and EGD "spiked" into the e-liquids at 1.0% w/w; however, for EGD a higher dose range was required.**
- **h-CLAT experiments ongoing with known sensitizers.**

CONCLUSIONS

- **Further research and method development is needed for optimization of the DPRA for use in e-liquid testing.**
- **Further method development of the KeratinoSens™ Assay is needed to enhance sensitivity for the assessment of e-liquids.**
- **For mixtures (e-liquids), higher doses allowed by solubility should be considered to maximize assay sensitivity.**
- **In the DPRA and KeratinoSens™ assays, nicotine did not appear to have an effect on the sensitization of CA in the e-liquids, *in vitro*.**
- **This proof of concept study indicates these *in vitro* assays, indicative of 3 key events on the Skin Sensitisation Adverse Outcome Pathway (AOP)[4], could be useful in an integrated testing strategy of e-liquids.**