Comparison of *in vitro* Cytotoxicity and Genotoxicity of Condensates Derived from E-vapor Products and Combustible Cigarette

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Abstract

The FDA draft guidance (2016) on premarket tobacco application for e-vapor products recommends full toxicity assessment including *in vitro* genotoxicity and cytotoxicity. Herein, we collected the condensates from e-vapor products (MarkTen® with menthol and non-menthol flavors) and reference (3R4F) cigarettes, on a Cambridge filter followed by an impinger filled with ethanol at 0°C (in an ice water bath). The condensates were then tested using standard in vitro assays: neutral red uptake (NRU) for cytotoxicity, *Salmonella* mutagenicity (Ames), and micronuclei (MN) for genotoxicity. E-liquids used for e-vapor products contained aerosol formers (propylene glycol [PG] and glycerol), water, nicotine (up to 2.5%) and flavor mixtures. The condensates (up to 47 mg total particulate matter (TPM)/mL) were analyzed for key formulation components and carbonyls, stored frozen at -70 °C, tested *in vitro* within 48 hrs of collection, and assessed for stability up to 8 weeks in storage. The 3R4F condensate tested positive in the NRU assay (IC_{50} of 0.048±0.004 mg/mL TPM), whereas the e-vapor condensates showed viability > 80% (IC_{50} could not be estimated). The 3R4F condensate tested positive in the Ames assay in strains TA1537 and TA98, while the e-vapor condensates did not in any of the five strains tested. The 3R4F condensate also tested positive in the MN assay but no significant effect was observed with the e-vapor condensates. Key formulation components as well as carbonyls were detected in e-liquid condensates and were stable for up to 8 weeks at -70° C. In summary, results from this study are consistent with the literature findings that e-vapor product aerosols have substantially lower biological activity than combustible cigarettes.

Objective

The objective of the study was to collect e-vapor aerosols as condensates, characterize analytically, and test their biological activity using battery of standard in vitro toxicity assays: the *Salmonella* mutagenicity (Ames), neutral red uptake cytotoxicity and micronucleus (MN) genotoxicity assays. Aerosol condensates from a reference 3R4F cigarettes were also collected as a reference.

Materials & Methods

Test Articles

A total of 8 MarkTen® products (ENDS), containing various e-vapor formulations [up to 2.5% nicotine, flavors and different ratios of aerosol formers (propylene glycol, glycerol)] and a reference cigarette 3R4F (University of Kentucky), were used for the study.

Aerosol Condensate of E-liquids

Aerosols were generated from ENDS devices using a modified Health Canada intense regimen (55mL puff volume, 30 second interval, 5 second duration using a square puff wave profile) on a linear smoking machine. One hundred and forty (140) puffs were collected from each of two ENDS on a non-conditioned 55 mm Cambridge filter pad (CFP) followed by impinger filled with 30 mL USP-grade ethanol cooled in an ice water bath. The CFP was extracted with the impinger contents and then filtered using sterile cheesecloth to produce the condensates (concentration of 39-47 mg/mL of collected aerosol mass (TPM) in ethanol). The condensate samples were analyzed for nicotine, menthol, propylene glycol (PG), glycerol (G) and carbonyls (formaldehyde, acetaldehyde and acrolein) immediately after collection and at several time points during 8 weeks storage to track its stability. The condensates were subjected to in vitro assays within 48-72 hrs of collection. To determine the collection efficiency, similar analytical characterization was performed on e-vapor aerosol, which were either collected on CFP alone (for nicotine, menthol, PG & G determination) or on CFP followed by impinger containing 2,4-dinitrophenylhydrazine (DNPH) (for carbonyl determination) using the same puffing regimen as condensates.

Aerosol Condensates of 3R4F

Mainstream cigarette smoke was generated from ISO conditioned reference 3R4F cigarettes according to Health Canada Official Method T-115 (55 mL puff volume, 30-second interval, 2-second duration with 100% of the ventilation holes blocked, using sine wave profile) on a rotary smoking machine. Total particulate matter (TPM) from 20 cigarettes was collected on a conditioned 92 mm CFP connected in series to an impinger filled with 30 mL USP-grade ethanol cooled in an ice water bath. The CFP was extracted with impinger contents and then filtered using sterile cheesecloth to produce the condensate (concentration of 28.1 mg TPM/mL in ethanol).

Neutral Red Uptake (NRU) Assay Using BALB/c 3T3 Cells

BALB/c 3T3 cells were incubated either in presence of the vehicle control (ethanol) or increasing concentrations of positive control (sodium lauryl sulfate) or the aerosol condensates for ~48 hrs according to OECD 129[1]. The maximum concentration of condensates was up to 0.5% (v/v).

Salmonella Mutagenicity (Ames) Assay

Condensates were tested in five *Salmonella typhimurium* strains: TA1537, TA98, TA100, TA1535, and TA102 according to OECD 471[2]. Cytotoxicity was checked to set the testing concentration, with the maximum concentration tested up to 100 µL/plate. The testing was performed in triplicate in presence and absence of metabolic activation (S9). Ethanol was used as the vehicle control.

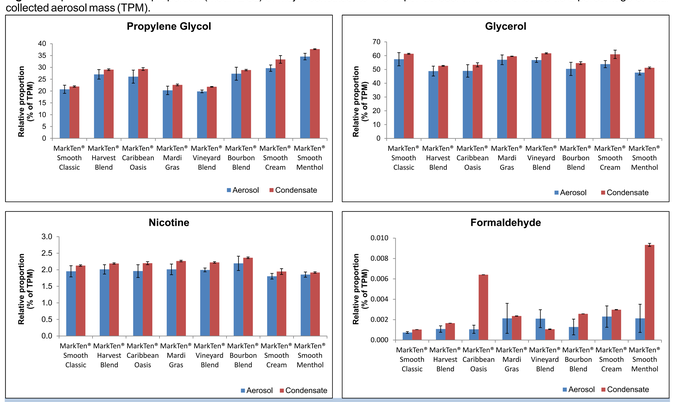
In Vitro Micronucleus (MNvit) Assay Using TK6 Cells

The aerosol condensates were evaluated for micronucleus induction according to OECD 487[3] in TK6 cells during short (4 hrs) incubations with and without S9, and long (27 hrs) incubations without S9 followed by an extended recovery of 40 hrs. Cytotoxicity was checked to set the testing concentration, with the maximum concentration tested up to 1 % (v/v).

Results

Collection Efficiency

Fig 1. Comparison of relative proportion (mean ± sd) of major constituents in e-vapor aerosol and condensates as a percentage of total collected aerosol mass (TPM).



- Relative to collected aerosol mass (TPM), the proportions of key aerosolized components were comparable between aerosol and condensates.
- Comparable results were observed in proportions of formaldehyde between aerosol and condensates except for two formulations; further investigation is ongoing.

Stability of E-Vapor Condensates

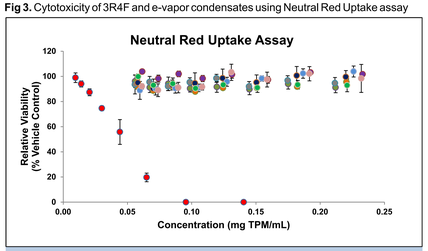
Table 1. Relative percentage (mean ± sd) of key aerosol constituents in the e-vapor condensates following 8 weeks of storage.

	% Remaining Following 8 weeks Storage (Relative to Time 0)				
Formulation	Propylene Glycol	Glycerol	Nicotine	Menthol	Formaldehyde
MarkTen® Smooth Classic	97.1 ± 0.5	96.3 ± 0.3	91.5 ± 0.0	-	94.3 ± 1.5
MarkTen® Harvest Blend	97.3 ± 0.7	97.7 ± 0.8	92.2 ± 1.0	-	94.8 ± 1.7
MarkTen® Caribbean Oasis	97.8 ± 0.9	97.6 ± 1.5	93.8 ± 1.0	-	97.3 ± 2.6
MarkTen® Mardi Gras	98.4 ± 0.8	97.7 ± 1.1	92.7 ± 1.0	-	94.7 ± 1.3
MarkTen® Vineyard Blend	99.7 ± 1.1	99.7 ± 1.0	93.6 ± 1.1	-	104.9 ± 1.5
MarkTen® Bourbon Blend	98.7 ± 1.6	97.4 ± 1.3	93.3 ± 1	-	102.9 ± 2.4
MarkTen® Smooth Cream	97.8 ± 2.7	98.6 ± 2.5	98.9 ± 0.0	-	113.5 ± 0.8
MarkTen® Smooth Menthol	100.3 ± 1.4	101.5 ± 0.8	103.4 ± 3.4	101.6 ± 0.0	105.7 ± 1.3

- Concentrations of the major constituents within the condensates were found to be stable for up to 8 weeks under the storage conditions.

NRU Assay

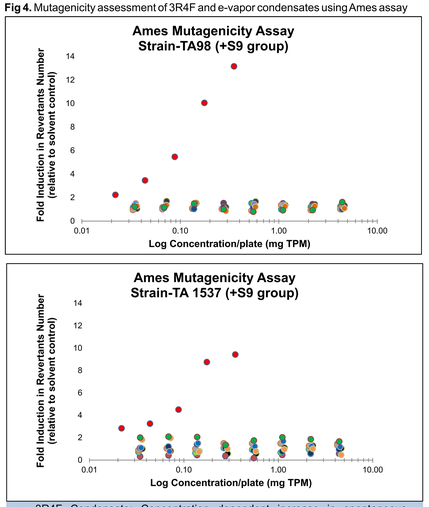
Fig 3. Cytotoxicity of 3R4F and e-vapor condensates using Neutral Red Uptake assay



- 3R4F Condensate: Positive response (IC_{50} of 0.048 ±0.004 mg/mL TPM)
- E-vapor condensates: Viability >80% (IC_{50} could not be estimated)

Ames Assay

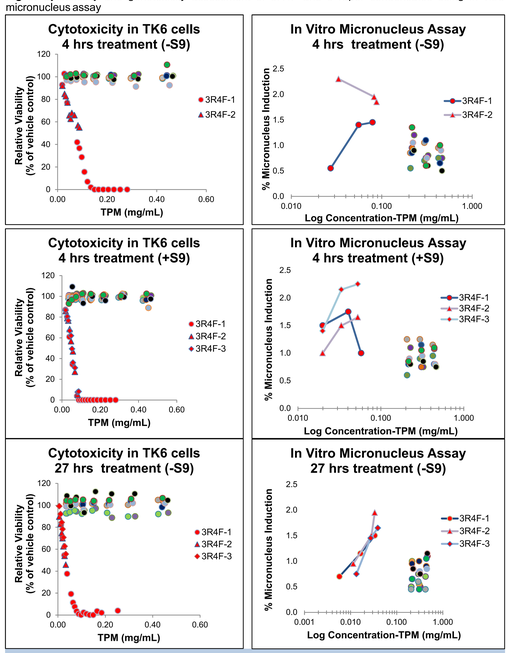
Fig 4. Mutagenicity assessment of 3R4F and e-vapor condensates using Ames assay



- 3R4F Condensate: Concentration dependent increase in spontaneous revertants per plate in strains TA1537 and TA98 in presence of metabolic activation relative to vehicle control
- E-vapor Condensates: No response in any of the five strains tested

in vitro Micronucleus Assay

Fig 5. Cytotoxicity and genotoxicity assessment of 3R4F and e-vapor condensates using *in vitro* micronucleus assay



- 3R4F Condensate: Concentration dependent decrease in viability and weak but reproducible and significant (both statistically and for trend) induction in micronucleus relative to vehicle control
- E-vapor Condensates: No toxicity or induction in micronucleus in TK6 cells

Conclusion

Under the test conditions, e-vapor product aerosols had substantially lower biological activity than combustible cigarettes.

Literature

1. OECD. (2010). OECD Guidance Document on Using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests.
2. OECD. (1997). OECD Guideline for Testing Chemicals Test Guideline 471, Bacterial Reverse Mutation Test.
3. OECD. (2016). OECD Guideline for Testing Chemicals Test Guideline 487, In vitro Mammalian Cell Micronucleus Test.