POTENTIAL TOXICITY OF ELECTRONIC CIGARETTE LIQUIDS AND AEROSOLS AS MEASURED BY FOUR *IN VITRO* ASSAYS

Leverette, R.D., Misra, M., Cooper, B.T. and Bennett, M.B.

Lorillard Tobacco Company, A.W. Spears Research Center, Greensboro, NC 27405, USA

ABSTRACT #1015

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The popularity of electronic cigarettes (E-cig) continues to increase worldwide, with several major tobacco companies entering the E-cig market. A typical E-cig delivers a flavored propylene glycol or glycerol based aerosol, with or without nicotine, via vaporization by a battery-powered heating element. Currently, data regarding the potential toxicity of E-cigs is limited. To further our understanding, an *in vitro* battery of established assays was used to examine the mutagenicity (Ames), cytotoxicity (Neutral Red Uptake; NRU), genotoxicity (Micronucleus; MN) and inflammatory (IL-8 release) response of a set of glycerol-based commercial E-cigs, specifically the neat E-cig liquids, pad-collected aerosols and freshly generated whole aerosols. Pad-collected smoke condensates and whole smoke from traditional tobacco burning cigarettes (3R4F, 1R5F and one commercial cigarette) as well as aqueous extracts from smokeless tobacco and nicotine replacement therapy products were included for comparison. All E-cigs and traditional cigarettes were smoked under Canadian Intense parameters (55 mL puff volume, 2 second puff duration, 30 second puff interval, 100% blocked air dilution). At the levels tested, exposures with neat E-cig liquids and pad-collected aerosols showed no-to-extremely low activity in the Ames, NRU, MN and IL-8 assays when compared to responses from the traditional tobacco burning cigarettes. Results from E-cig samples without nicotine were very similar in all assays, indicating that the presence of nicotine, at the levels tested, did not significantly contribute to any cytotoxic and genotoxic effects observed at high doses. Whole smoke and whole E-cig aerosols were tested in the Ames, NRU and IL-8 assays, again resulting in no-to-extremely low activity when compared to traditional tobacco burning cigarettes. Overall, under the experimental conditions used to evaluate traditional tobacco burning cigarettes, E-cigs did not produce any meaningful toxicological effects as measured by four *in vitro* endpoints: Ames, NRU, IL-8, and Micronucleus.

MATERIALS & METHODS

CIGARETTE SMOKE PREPARATIONS & EXPOSURES:
Tobacco burning cigarettes (3R4F, 1R5F, Marlboro Gold) conditioned (60% relative humidity, ~23°C) at least 18 hours prior to smoking.

Electronic Cigarettes (E-cig): blu™ E-cigs, Classic Tobacco (CT) and Magnificent Menthol (MM). Nicotine @ 0 mg / mL (Ø; rechargeable) or 24 mg / mL (High; disposable). Batteries charged immediately prior to vaping (rechargeable only). Control E-cig: disposable E-cig containing a glycerol / water mixture, no flavors or nicotine.

All cigarettes & E-cigs smoked on a VITROCELL® VC10 smoking robot under Canadian Intense (CI) puff profile: 55 mL puff volume, 2 sec draw, 30 sec puff interval, 100% blocked air dilution.

Wet Total Particulate Matter (WTPM) and E-cig aerosols collected on Cambridge filter pads, extracted in dimethylsulfoxide (DMSO) to a final concentration of 40 mg / mL, stored at -80°C prior to analysis.

Whole Smoke (WS) and Whole Aerosol (WA) exposures performed on a VITROCELL® VC10, VITROCELL® Ames Modules, with smoke dilution air flows ranging from 0.1 – 8.0 L / min.

Smokeless Tobacco aqueous extracts prepared from commercially available products (Marlboro SNUS, Copenhagen Snuff, Nicorette® Lozenge Original 4 mg Nicotine). Samples suspended in Phosphate Buffered Saline @ 80 mg / mL (Dulbecco's PBS, Gibco #14040, +$MgCl_2$ +$CaCl_2$), incubated @ 37°C for 21-24 hrs @ 150 rpm shaking, centrifuged @ 12K g for 10 min to remove particulates, filter sterilized, aliquoted and stored @ -80°C prior to analysis[1].

AMES[2,3]:
S9-Mix: 33mM KCl, 8mM $MgCl_2$, 5mM Glucose-6-phosphate, 4mM NADP, sodium phosphate buffer (0.1M, pH 7.4), S9 fraction @ 5% v/v (Aroclor 1254-induced male Sprague-Dawley rat liver in 0.15M KCl; Moltox; Boone, NC).

Preincubation assays: 100 µL of *Salmonella* strains TA98 or TA100, 500 µL S9-Mix (5% v/v), 25 µL sample, 20 min preincubation @ 37°C, 250 rpm shaking followed with the addition of 0.05 mM Histidine / Biotin top agar (2.5 mL) and plated onto minimal glucose agar plates.

WS & WA Exposures: TA98 or TA100 @ ~2 – 4 X 10^9 bacteria / mL in 200 µL S9-Mix (5% v/v) were spread on fresh 0.4% minimal glucose agar plates (35 mm) supplemented with 0.05 mM Histidine / Biotin, exposed to WS or WA from 3 tobacco burning cigarettes or 200 puffs from E-cig.

Revertant colonies counted after 48 hrs of incubation @ 37°C.

Activity reported as revertants per mg was calculated from the linear portion of the dose response curve and compared using GraphPad Prism v. 5.04 (slope analysis, two tailed; for comparisons, statistical significance @ p < 0.05).

NEUTRAL RED UPTAKE (NRU)[4,5] & IL-8[6]
A549 (human lung epithelial carcinoma cell line, ATCC# CCL-185) cells seeded (~15K cells / well) and incubated (complete media: F-12K + 10% FBS) in 96-well plates overnight (37°C, 5% CO_2) prior to exposures.

Cells exposed to increasing doses of samples (E-cig liquids, smokeless extracts, pad-collected smoke and aerosols) and incubated for 20 – 24 hrs @ 37°C, 5% CO_2 followed by NRU[4,5] and IL-8[6] analyses.

WS & WA Exposures: A549 cells seeded in 35 mm culture dishes @ 400K cells / dish in 2 mL complete media and incubated overnight (37°C, 5% CO_2). Just prior to exposures, complete media removed and cells washed 1X in DPBS. Added 1 mL incomplete media (-FBS) and exposed to either 5 tobacco burning cigarettes (8 puffs / cigarette) or 200 puffs from E-cig. After exposure added 1 mL complete media and incubated for 20 – 24 hrs @ 37°C, 5% CO_2 followed by NRU[4,5] and IL-8[6] analyses.

EC50 (NRU) and IL-8 (pg / mL; % control) calculated and compared using GraphPad Prism v. 5.04 (two tailed; for comparisons, statistical significance @ p < 0.05).

MICRONUCLEUS (MN)
Chinese hamster ovary (CHO-K1, ATCC# CCL-61) cells seeded (~2500 cells / well) and incubated (complete media: F-12K media + 10% FBS) in 96-well plates overnight (37°C, 5% CO_2) prior to exposures.

Cells exposed (absence of S9) to increasing doses of samples (E-cig liquids, smokeless extracts, pad-collected smoke and aerosols) and incubated for 20 hrs @ 37°C, 5% CO_2 followed by treatment with cytochalasin B (cytokinesis blocking agent) for 27.5 hrs @ 37°C, 5% CO_2 (Cellomics® Micronucleus Kit K11-0001-1; Thermo Scientific). Cell viability determined by cytokinesis-block proliferation index (CBPI).

MN frequency (%MN) determined using Cellomics® ArrayScan® VTI (Micronucleus Bioapplication, V.4). MN activity reported as % control and compared using GraphPad Prism v. 5.04 (two tailed; for comparisons, statistical significance @ p < 0.05).

SMOKE & E-CIG AEROSOL DELIVERIES
WS & WA: VITROCELL® Laser Photometers positioned between dilution system and exposure module with 35 mm plates containing 4 mL DMSO (dH_2O for WA). Dilution air flow rates 0.1 – 8.0 L / min. For WS, Ex / Em: 355nm / 485nm measurements of smoke-exposed DMSO extrapolated to WTPM standard curve[2]. WA (E-cig) dose correlated to glycerol captured in aerosol-exposed dH_2O quantified with Free Glycerol Reagent (Sigma # FG0100). Plot of laser photometer values (Volt*sec, Area Under Curve) versus quantified smoke particulate (WS) or glycerol (WA) used to calculate delivered doses.

RESULTS

E-CIG LIQUIDS & SMOKELESS EXTRACTS

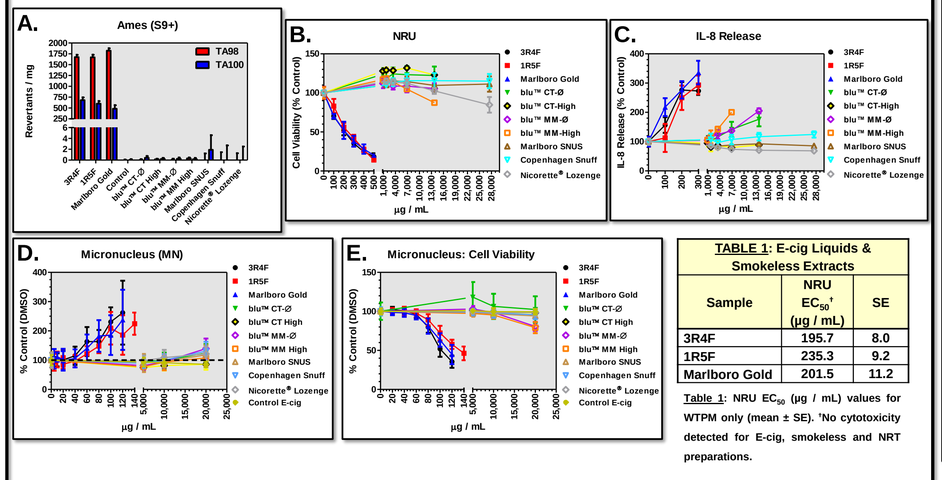

TABLE 1: E-cig Liquids & Smokeless Extracts

Sample	NRU EC_{50}† (µg / mL)	SE
3R4F	195.7	8.0
1R5F	235.3	9.2
Marlboro Gold	201.5	11.2

Table 1: NRU EC_{50} (µg / mL) values for WTPM only (mean ± SE). †No cytotoxicity detected for E-cig, smokeless and NRT preparations.

Figure 1: *In vitro* activity of E-cig liquids, smokeless tobacco and lozenge aqueous extracts, and pad-collected smoke particulate (WTPM) in Ames (A), NRU (B), IL-8 (C), and MN (D, E). Data points in each plot represent the mean values ± SD from a minimum of two (2) independent experiments. MN cell viability (E) shown to verify lack of MN induction is not due to cytotoxicity at higher doses. All activities for E-cig liquids and smokeless tobacco extracts significantly different when compared to WTPM from traditional tobacco burning cigarettes (p < 0.001).

PAD-COLLECTED SMOKE & E-CIG AEROSOLS

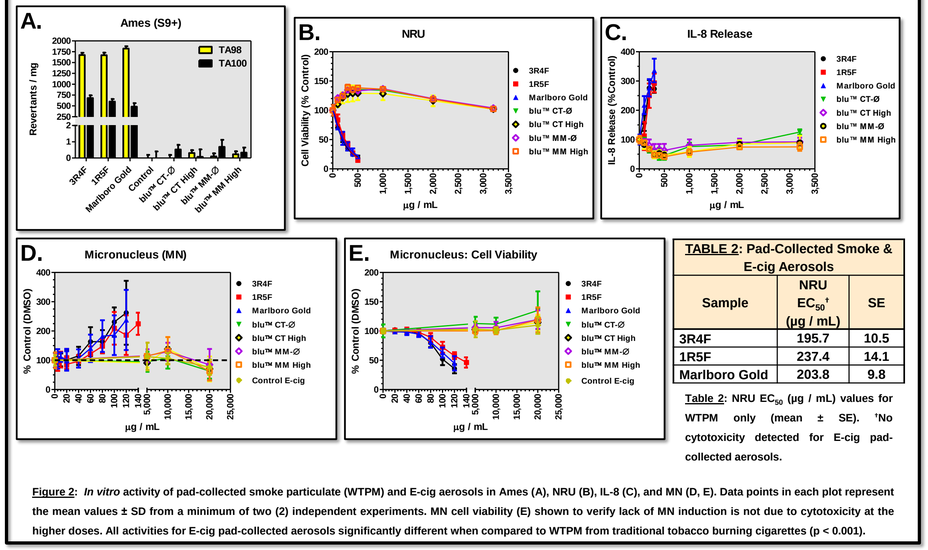

TABLE 2: Pad-Collected Smoke & E-cig Aerosols

Sample	NRU EC_{50}† (µg / mL)	SE
3R4F	195.7	10.5
1R5F	237.4	14.1
Marlboro Gold	203.8	9.8

Table 2: NRU EC_{50} (µg / mL) values for WTPM only (mean ± SE). †No cytotoxicity detected for E-cig pad-collected aerosols.

Figure 2: *In vitro* activity of pad-collected smoke particulate (WTPM) and E-cig aerosols in Ames (A), NRU (B), IL-8 (C), and MN (D, E). Data points in each plot represent the mean values ± SD from a minimum of two (2) independent experiments. MN cell viability (E) shown to verify lack of MN induction is not due to cytotoxicity at the higher doses. All activities for E-cig pad-collected aerosols significantly different when compared to WTPM from traditional tobacco burning cigarettes (p < 0.001).

WHOLE SMOKE & WHOLE E-CIG AEROSOLS

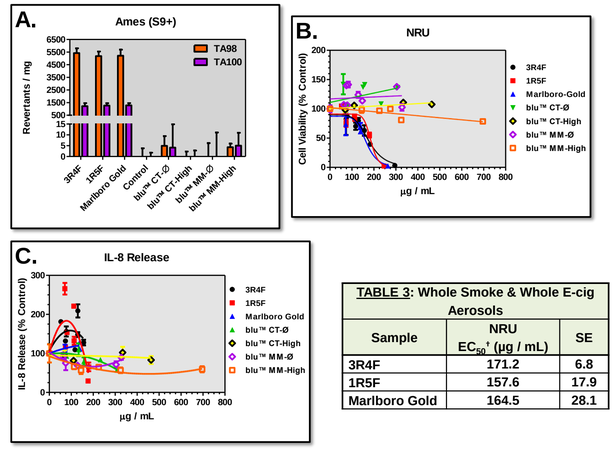

TABLE 3: Whole Smoke & Whole E-cig Aerosols

Sample	NRU EC_{50}† (µg / mL)	SE
3R4F	171.2	6.8
1R5F	157.6	17.9
Marlboro Gold	164.5	28.1

Figure 3: *In vitro* activity of whole smoke and whole E-cig aerosols in Ames (A), NRU (B) and IL-8 (C). MN not done for whole smoke and whole aerosols. Data points in each plot represent the mean values ± SD from a minimum of two (2) independent experiments. All E-cig whole aerosol activities significantly different when compared to whole smoke from traditional tobacco burning cigarettes (p < 0.001). Micronucleus methods for whole smoke / aerosol exposures under development.

Table 3: NRU EC_{50} (µg / mL) values for tobacco burning cigarette whole smoke only (mean ± SE). †No cytotoxicity detected for E-cig whole aerosols.

SUMMARY

- **E-cigs VS. Tobacco WTPM: At doses up to 100 X higher than typical cigarette smoke exposures, E-cig liquids and pad-collected aerosols had no-to-extremely low *in vitro* activity (Ames, NRU, IL-8 & MN) when compared to WTPM from tobacco burning cigarettes (WTPM activity was up to ~ 6000 X higher than E-cigs).**
- **E-cigs VS. Smokeless & NRT: E-cig liquids demonstrated similar no-to-extremely low *in vitro* activity as aqueous extracts from a commercial nicotine lozenge and commercial smokeless tobacco products (SNUS & Snuff).**
- **E-cigs VS. Tobacco Whole Smoke: Direct exposure of freshly generated E-cig aerosols did not produce any significant levels of *in vitro* activity (Ames, NRU & IL-8) when compared to fresh whole smoke from tobacco burning cigarettes (cigarette whole smoke activity up to ~ 1300 X higher than E-cigs). Micronucleus whole smoke / aerosol exposure methods currently under development.**
- **Effect of Nicotine: *In vitro* activities (Ames, NRU, IL-8 & MN) measured for E-cig exposures, with and without nicotine, were similar for all sample types, indicating that the presence of nicotine, at the levels tested, did not significantly contribute to any toxicological effects.**
- **Effect of Flavors: *In vitro* activities (Ames, NRU & MN) for the commercial E-cigs were indistinguishable from control (glycerol / water); indicating these flavors (CT & MM), at the levels tested, had no detectable impact on the cytotoxicity and genotoxicity endpoints utilized in this study.**
- **Liquid VS. Aerosol: *In vitro* results for E-cigs, in this study, were similar for the different exposure methods (liquids, pad-collected & freshly generated whole aerosols); demonstrating no detectable impact on the *in vitro* toxicological responses when the liquids were aerosolized.**
- **Overall: Under the experimental conditions used to evaluate traditional tobacco burning cigarettes, E-cigs did not produce any meaningful toxic effects as measured by four *in vitro* endpoints: Ames (bacterial mutagenicity), NRU (cytotoxicity), IL-8 (inflammation) and Micronucleus (genotoxicity). These results demonstrate the potential for E-cigs to significantly reduce the toxicological impact when compared to traditional tobacco burning cigarettes.**

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