ASSESSMENT OF *IN VITRO* TOXICITY/GENOTOXICITY OF ENDS AND COMBUSTIBLE PRODUCTS

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Abstract

In May 2016, the U.S. Food and Drug Administration Center for Tobacco Products (CTP) issued draft guidance for Premarket Tobacco Product Applications (PMTA) for Electronic Nicotine Delivery Systems (ENDS), which recommends inclusion of *in vitro* toxicology testing (e.g., genotoxicity and cytotoxicity studies). Specific to genotoxic potential, the draft guidance suggests using assays/methods outlined in International Conference on Harmonization (ICH) S2(R1) and Organization for Economic Cooperation and Development (OECD) guidelines. The draft guidance also recommends testing multiple test article concentrations in each assay, and using comparator products for hazard identification. We present an example of *in vitro* toxicology testing to support an ENDS PMTA.

Bacterial mutagenicity (Ames test), *in vitro* micronucleus and cytotoxicity assays were conducted on ENDS e-liquids, ENDS product aerosols and U.S. market comparators in general accordance with applicable guidelines (ICH S2(R1); OECD 487 and 471; NIH Publication 07-4519 [2006] and Health Canada Official methods (T-501/T-502/T-503). Aerosol total particulate matter (TPM) and gas vapor phase (GVP) from ENDS products and a combustible comparator were generated under intense puffing/smoking regimens for testing. Neat e-liquid samples were diluted and applied to the test systems. Test samples were evaluated up to concentrations resulting in toxicity or up to limits inherent in the assays.

The ENDS e-liquids, TPM and GVP were either negative or demonstrated limited response at the highest concentrations tested in each of the assays, indicating a lack of toxicity and genotoxicity of the ENDS products under the experimental conditions tested. In contrast, with the exception of GVP in the Ames assay, the combustible market cigarette comparator was consistently positive, confirming that the assays conducted are sensitive to potential toxicants/genotoxicants found in combustible tobacco product emissions.

Introduction

Electronic nicotine delivery systems (ENDS), or e-cigarettes, typically contain e-liquids with the following components: nicotine, flavorings, propylene glycol, vegetable glycerin and water. Since the e-liquid is vaporized and tobacco is not burned as with a traditional combustible cigarette, lower comparative toxicant levels would be anticipated with ENDS due to the lack of pyrolysis (Royal College of Physicians, 2016). ENDS aerosols have significant reductions in chemical constituents and *in vitro* toxicity when compared to combustible cigarette smoke (National Academies of Sciences, Engineering and Medicine, 2018). Therefore, ENDS represent great potential for reducing the harm traditionally associated with combustible tobacco product use.

Many health-related effects of cigarette smoking have been linked to genetic and cell viability changes. Several mechanisms underlie these changes, including DNA damage, cytotoxicity and changes in chromosomal structure/function. For decades, standardized *in vitro* toxicological methods have been used to assess and compare the potential genotoxic, mutagenic and cytotoxic effects of combustible cigarettes and other traditional tobacco products. In recent years, external recommendations or requirements for assay batteries supporting toxicity testing of tobacco products have grown, including recommendations by the CORESTA Task Force (2004) on *In Vitro* Toxicity Testing (Ames mutagenicity assay, the Neutral Red Uptake [NRU] cytotoxicity assay and a mammalian genotoxicity assay [chromosome aberration, *in vitro* micronucleus (IVMN) or mouse lymphoma [MLA]) as well as Canadian Tobacco Reporting Regulations (2005; Ames, NRU, IVMN). Currently, Draft Guidance from the FDA Center for Tobacco Products recommends *in vitro* assays for Premarket Tobacco Product Applications (PMTA), Modified Risk Tobacco Product Applications (MRTPA) and PMTA for ENDS, further supporting a multi-assay approach for *in vitro* toxicity assessment of tobacco products.

In this study, the tests selected for evaluation of biological responses to e-liquids, ENDS aerosol and combustible cigarette aerosol were the Ames bacterial reverse mutation assay, the IVMN genotoxicity assay, and the NRU cytotoxicity assay. Studies included market combustible and ENDS products. The total particulate matter (TPM) and the gas vapor phase (GVP) of the products were generated using an intense puffing regimen, and were collected and assayed separately in order to analyze the highest concentrations.

Key References

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- Health Canada Official Method T-503, Second Edition (2004-11-01), In Vitro Micronucleus Assay for Mainstream Tobacco Smoke.
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- Health Canada Official Method T-115, (1999-12-31), Determination of "Tar", Nicotine& Carbon Monoxide in Mainstream Tobacco Smoke.
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Results

Figure 1: Examples of positive response with market combustible vs. example ENDS (ENDS-1) in Ames TA98 (+S9), NRU and MN

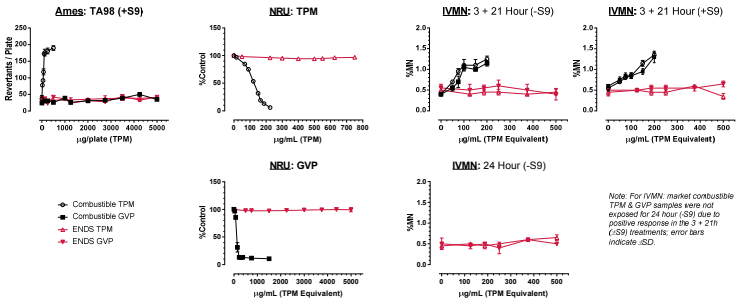


Figure 2: Market combustible TPM but not GVP samples demonstrate mutagenicity in Ames assay; all ENDS samples are negative

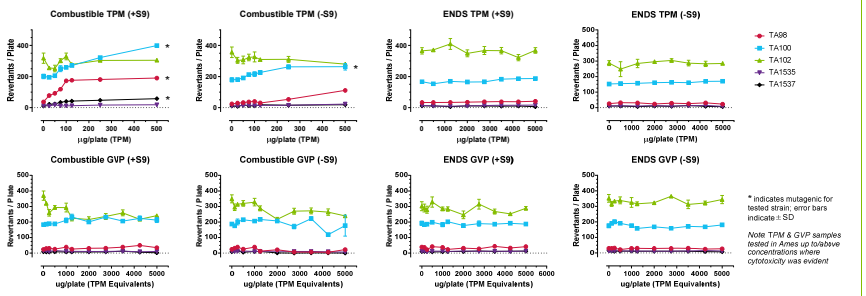


Figure 3: ENDS E- liquids are negative in IVMN

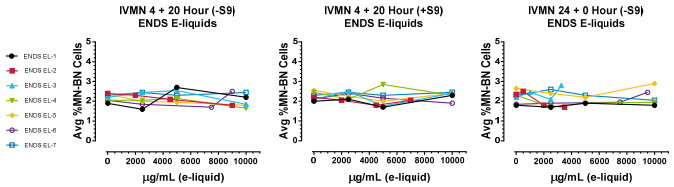


Figure 4: Representative Ames (+S9) results with e-liquid

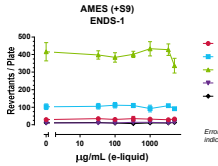


Table 1: Summary of results for in vitro mutagenicity, cytotoxicity and mammalian genotoxicity testing

	Ames EL[1]		Ames TPM[2]		Ames GVP[3]		NRU EL[1]	NRU TPM[2]	NRU GVP[3]	IVMN EL[1]				IVMN TPM[2]				MN GVP[3]			
Treatment Condition	+S9	-S9	+S9	-S9	+S9	-S9				3h +S9	3h -S9	24h -S9		3h +S9	3h -S9	24h -S9		3h +S9	3h -S9	24h -S9	
Market Combustible	NA	NA	+	+	-	-	NA	+	+	NA	NA	NA		+	+	NT		+	+	NT	
ENDS-1	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-		-	-	-	
ENDS-2	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-		-	-	-	
ENDS-3	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-		-	-	-	
ENDS-4	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-		-	-	-	
ENDS-5	-	-	-	-	-	-	+	-	-	-	-	-		-	-	-		-	-	-	
ENDS-6	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-		-	-	-	
ENDS-7	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-		-	-	-	

EL=e-liquid; TPM=total particulate matter; GVP=gas vapor phase; NA=not applicable; NT=not tested; **1**= tested up to 5,000 µg/mL e-liquid; **2**= tested up to 5,000 µg/mL TPM or TPM equivalent for ENDS; **3**= tested up to 5,500 µg/mL e-liquid; **4**=tested up to 750 µg/mL TPM and 5000 µg/mL TPM equivalent for ENDS; **5**=tested up to 10,000 µg/mL e-liquid; **6**=tested up to 500 µg/mL TPM or TPM equivalent for ENDS

Materials and Methods

Generation/Preparation of Test Matrices

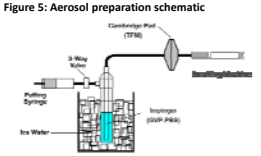
Seven different flavor variants of a single ENDS product were assessed under GLP conditions at three separate contract research laboratory facilities using standard nonclinical regulatory toxicology procedures. Three different test matrices from each flavor variant were used for the *in vitro* assessments, including e-liquid, and two aerosol fractions: TPM and GVP. The neat e-liquids were diluted with DMSO, and the TPM and GVP were generated as described below.

Table 2: Puffing regimes

	RAIS ENDS Intense*	Market Combustible HCI T-115
Puff Volume	80 mL	55
Puff Frequency	15 sec	30
Puff duration	4 sec	2 sec
Puff Profile	Square wave	Bell shape
Vent blocking	Not Applicable	Fully blocked

* RAIS-specified intense regime based on modified CRM 81 intense regimen and review of puffing topography, machine limitations and product design and delivery

Figure 5: Aerosol preparation schematic



For each combustible cigarette or ENDS test article, using an automated constant-volume rotary smoking machine, TPM was collected on 92mm Cambridge filter pads and the mainstream GVP was bubbled into a cooled glass impinger containing phosphate-buffered saline (PBS). DMSO was used to elute the TPM from the pads to a stock concentration of 50 mg/mL for ENDS and either 10 (Ames), 15 (NRU) or 20 (IVMN) mg/mL TPM stock solution for the combustible cigarette. Calcium-magnesium free (CMF)-PBS was added to the collected GVP to generate 50 mg TPM equivalent/mL GVP stock solution for ENDS and either 50 (Ames), 15 (NRU) or 20 (IVMN) mg TPM equivalent/mL GVP stock solution for the combustible cigarette. The TPM extracts were either applied to the test system within 1 hour of generation (NRU) or stored at -80°C until required for testing (Ames and IVMN) and the GVP samples were tested in each of the biological assays within 1 hour of generation. In order to avoid potential artifactual positive response, the highest concentration of test article assessed in these assays was limited to one that did not produce excessive toxicity, precipitation in the culture medium, or marked changes in pH or osmolality.

Ames bacterial reverse mutation assay

E-liquid, TPM and GVP test sample exposures were conducted to the principles of OECD 471 and HC T-501, using 5 bacterial (*Salmonella typhimurium)* strains: TA98, TA100, TA1535, TA1537 and TA102; each with and without metabolic activation (5% S9 mix). Results are reported in terms of the average number of observed revertants (i.e. colony mutations) per plate following exposure to test sample.

Neutral red uptake (NRU) cytotoxicity assay

Cytotoxicity assessment following exposure to e-liquid, TPM and GVP test samples was conducted according to the HC T-502 guidance, using Chinese hamster ovary (CHO) cells. Results are expressed as the average % viability of test sample treated cultures compared with vehicle (negative) controls.

***In vitro* micronucleus genotoxicity assay**

In alignment with the principles of OECD 487 and HC T-503, e-liquid, TPM and GVP test sample genotoxicity was determined as follows. CHO cells were exposed to test sample in one of three conditions: ± S9 with short 3-4h exposure and 20-21h recovery or a longer 24h exposure (-S9). In cases where a positive response was observed in one of the short exposure schedules, the longer exposure schedule was not performed. Due to laboratory-specific differences in validated GLP methods for IVMN conduct, experiments with the e-liquid test samples utilized a cytokinesis block method (CytoB) and experiments with the TPM and GVP test samples did not. Thus, results for the IVMN assay are presented as either the (average) % MN observed, or the average % micronucleated binucleated cells (%MN-BN cells) observed following exposure to test sample.

Sample Characterization and Stability

Characterization and bulk stability testing were performed for all test samples by quantifying concentrations of nicotine, glycerin, propylene glycol and water content at 3 different time-points over the course of biological assay conduct (data not shown). Acceptance criteria for solvent standard solutions and analyses at all time-points were met and each test sample was determined to be stable over the course of study conduct.

Summary & Conclusions

- Across all the *in vitro* assays conducted, a clear positive response was observed with market combustible TPM.
- A clear positive response was observed with market combustible GVP in the NRU and MN assays, but not in the Ames assay.
- When tested up to maximum dose concentrations inherently appropriate for the respective biological assay systems, and at doses equivalent to or higher than the market combustible, exposure to ENDS TPM and GVP test samples resulted in little or no activity, and no positive overall results, in any of the *in vitro* assays conducted.
- Overall, consistent with general consensus, ENDS aerosols tested in this *in vitro* test battery showed lower toxicity than a combustible comparator aerosol.
- ENDS e-liquids representing seven different flavor variants of the same ENDS product demonstrated little or no activity in the Ames and MN assays when tested up to concentration dose limits (with considerations for pH and osmolality) or recommended maximum levels of the respective test systems.
- In the NRU cytotoxicity assay, only one of seven different e-liquid flavor variants tested demonstrated a positive response (e-liquid only) at the highest concentration tested. However, the exposure level at which toxicity was observed for the e-liquid is considered not biologically relevant. When TPM and GVP generated using the same e-liquid were tested in the NRU assay, results were negative.

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