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In vitro toxicological evaluation of nicotine concentration and flavor variants of Vuse Alto ENDS

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ABSTRACT

In vitro toxicological evaluations are recommended for determination of the appropriateness for the protection of public health (APPH) of tobacco products seeking marketing authorization in USA. In this manuscript, we assessed mutagenicity, genotoxicity, and cytotoxicity by Ames, *in vitro* micronucleus, and neutral red assays, respectively of 13 Vuse Alto electronic nicotine delivery system (ENDS) products that differed in nicotine concentration and flavor, using established regulatory toxicological assays. Market comparator products for cigarettes and ENDS were also included in these evaluations. The Vuse Alto ENDS test products were non-genotoxic and non-mutagenic in the *in vitro* micronucleus and Ames assays, respectively, while the cigarettes elicited positive responses in both the assays. Whole smoke generated from cigarettes and whole aerosol from the Vuse Alto ENDS test products was used to test for potential cytotoxicity. While most Vuse Alto ENDS were non-cytotoxic, 3 test products were determined to be cytotoxic, with a markedly (>200 fold) higher IC_{50} values compared to cigarettes. Overall, our results show that the Vuse Alto ENDS evaluated in this study are non-genotoxic and non-mutagenic, and either non-cytotoxic or exhibit minimal cytotoxicity, compared to cigarettes.

1. Introduction

Electronic nicotine delivery systems (ENDS), popularly known as e-cigarettes, are non-combustible inhalable nicotine delivery products which have gained popularity in tobacco marketplace in the US (Ali et al., 2023a; Ali et al., 2023b; Center for Disease Control and Prevention, 2023). ENDS are an evolving category of tobacco products, ranging from the earlier "cig-a-like" products to a more contemporary "pod--mod" devices (Center for Disease Control and Prevention, 2024). Fundamentally, ENDS vaporize e-liquids, which contain nicotine, propylene glycol, glycerol and flavorings, and produce nicotine-containing aerosol which is inhaled by users (DeVito and Krishnan-Sarin, 2018). Thus, the aerosol from ENDS is chemically far less complex (Cunningham et al., 2020; Margham et al., 2016, 2021) than the smoke from cigarettes which contains thousands of toxicants arising from combustion. Many of the toxicants present in the cigarette smoke are identified as harmful and potentially harmful constituents (HPHCs) by the United States Federal Drug Administration (FDA) (Food and Drug Administration, 2012).

The HPHCs consist of several classes of chemicals, including carbonyls, tobacco specific nitrosamines (TSNAs), polycyclic aromatic hydrocarbons (PAHs), and others (United States Public Health Service Office of the Surgeon et al., 2010). At a cellular level, cigarette smoke toxicants cause cytotoxicity, mutations and genotoxicity, among other toxic effects (United States Public Health Service Office of the Surgeon et al., 2010). These cellular perturbations eventually progress to smoking-related diseases, such as cancer, COPD, and cardiovascular diseases in susceptible chronic smokers. Although nicotine is one of the HPHCs identified by the FDA, it is not considered to be a causative agent of smoking-related diseases (Food and Drug Administration, 2022; Gottlieb and Zeller, 2017).

Extensive research has demonstrated the existence of a tobacco product risk continuum, with cigarettes as the highest risk products, and abstinence being the safest option to reduce harm from smoking (Zeller and Hatsukami, 2009). Non-combustible tobacco products, such as smokeless tobacco and ENDS are placed at the lower end of the risk continuum, and nicotine-containing pharmaceutical products are recognized as minimally risky (Abrams et al., 2018; Institute of Medicine, 2001; 2001). Recently, the FDA has acknowledged the tobacco

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Abbreviations

NRU	Neutral Red Assay
TPM	Total Particulate Matter
ENDS	Electronic Nicotine Delivery System
GVP	Gas Vapor Phase
ACM	Aerosol Collected Matter
TPM + GVP	Total Particulate Matter combined with Gas Vapor Phase
FDA	United States Food and Drug administration
WS	Whole Smoke
WA	Whole Aerosol
TSNAs	Tobacco Specific Nitrosamines
APPH	Appropriate for the Protection of Public Health
PAHs	Polycyclic Aromatic Hydrocarbons
THR	Tobacco Harm Reduction
PMTA	Premarket Tobacco Product Application; ivMN Premarket Tobacco Product Application; ivMN*in vitro* micronucleus
CMF-PBS	Calcium–Magnesium Free Phosphate Buffer Saline
CF	Cambridge Filter Pad
mCRM	Modified CORESTA Reference Method

product risk continuum with ENDS and other smokeless tobacco products having generally lower health risks than combustible cigarettes. (Food and Drug Administration, 2024b). No tobacco product is safe or risk free, the best way for adult smokers to achieve risk reduction is to quit. Based on the relative risks of combustible and non-combustible tobacco products, a comprehensive tobacco harm reduction (THR) strategy in an effort to minimize/reduce the harm from cigarette smoking (Institute of Medicine 2001; 2001). Tobacco harm reduction (THR) is an overall approach to reduce harm from cigarette smoking. THR is about educating adult smokers who are uninterested in quitting about alternatives to combustible cigarette. Various studies have been performed to assess the toxicity of combustible tobacco products with non-combustible tobacco products. For example, several studies have shown that aerosols from ENDS contain far fewer toxicants (Margham et al., 2016, 2021). The PAHs (benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[k]fluoranthene, benzo[*a*]pyrene, dibenz[a,h]anthracene, and benzo[g,h,i]perylene) detected in seven different ENDS aerosols were >10 fold less than cigarette smoke (Alshutairi et al., 2024). The HPHCs detected in ENDS aerosols are at several fold lower levels in magnitude than in cigarette smoke (Keyser et al., 2024c; Son et al., 2020; Talih et al., 2019, 2023), and users are exposed to significantly lower levels of HPHCs (including PAHs) as reflected in biomarker studies (Kanobe et al., 2022, 2023). However, there are non-clinical and clinical reports indicating some adverse effects of ENDS (Chhor et al., 2023; Gong et al., 2023; Jasper et al., 2021; Rasmussen et al., 2023). Hence, evaluation of ENDS products continues to be an active research area (Anic et al., 2022; Christensen et al., 2021; Holt et al., 2023)

The FDA reviews and authorizes marketing of new products through the premarket tobacco product application (PMTA) process (Food and Drug Administration, 2023). There is a wide array of scientific studies recommended for the evaluation of new tobacco products including but not limited to non-clinical/*in vitro* toxicological studies. Assessment of genotoxicity and cytotoxicity are some of the widely employed *in vitro* toxicology tools. Non-clinical studies offer insight into the mechanisms of disease incidence caused by a tobacco product and, more generally, provide context for the data obtained from human studies regarding health risks (Food and Drug Administration, 2023). Assessment of mutagenicity by Ames assay, genotoxicity by *in vitro* micronucleus (ivMN) assay, and cytotoxicity by neutral red uptake (NRU) are three widely used methods for regulatory assessments of tobacco products. Numerous studies have demonstrated that preparations of cigarette smoke induce mutagenic, genotoxic, and cytotoxic responses in these assays (Johnson et al., 2009; United States Public Health Service Office of the Surgeon et al., 2010). The ivMN assay has been shown to be predictive of head and neck cancer, breast cancer, cervical, and lung cancer, and the Ames assay is 77 %–~90 % predictive of rodent carcinogenicity including airborne particles (Bolognesi et al., 2021; DeMarini and Linak, 2022; El-Zein et al., 2014; Thomas et al., 2023). However, *in vitro* regulatory assessment of diverse tobacco product categories presents some technical challenges (Moore et al., 2023).

Cigarette smoke is a dynamic aerosol, and it is difficult to replicate human smoking under *in vitro* conditions. A vast majority of studies to date have utilized preparations of total particulate matter (TPM) as test samples, and they have provided valuable information on the toxicological effects of cigarette smoking (DeMarini, 2004; Johnson et al., 2009). Subsequently, preparations of gas vapor phase (GVP) have also been developed to complement the TPM studies (Johnson et al., 2009; Moore et al., 2023). Combined use of TPM and GVP (TPM + GVP) as a singular test sample has been recommended to capture the more wholistic effects of cigarette smoke for regulatory purposes (Health Canada, 2017a; Lauterstein et al., 2020). While the widely used NRU assay utilizes non-human cells (OECD, 2019), incorporation of human cell-based test systems have been advocated for regulatory assessment of tobacco products (Johnson et al., 2009; Lauterstein et al., 2020). Furthermore, the development of whole smoke (WS) or whole aerosol (WA) technology, using smoking robots, has allowed exposure to freshly generated cigarette smoke or ENDS aerosols and facilitated dosimetric evaluations of test articles (Cao et al., 2021; Miller-Holt et al., 2022). Refinement of these WS/WA systems from tobacco product evaluations, which may be used as alternative test systems for *in vivo* studies, is an active area of research (Moore et al., 2023).

Selected Vuse Solo, Vuse Ciro, Vuse Vibe, and Vuse Alto ENDS products have been authorized for marketing under the PMTA pathway (Food and Drug Administration, 2024a). This manuscript has assessed the *in vitro* toxicology of Vuse Alto brand of ENDS which differed in nicotine concentration and flavor characteristics.

2. Materials and Methods

2.1. Test products

The Vuse Alto ENDS products tested in this study include several variants with different nicotine concentration and flavors. Vuse Alto ENDS consist of closed e-liquid cartridges (referred to as pods). The Vuse Alto pods are non-refillable and are comprised of e-liquids of flavor components, propylene glycol, glycerin, and salt-based nicotine. In this manuscript we tested a total of 13 Vuse Alto ENDS variants at 3 nicotine concentrations (1.5 %, 2.4 % or 5.0 %) across 9 flavors (Table 1). The following variants of Vuse Alto products at 1.5 % nicotine concentration were tested: Tropical Coconut, Berry Cream, Glacier Menthol, Unflavored, Menthol, Rich Tobacco, Smooth Tobacco, Aromatic Tobacco, and Golden Tobacco. The Berry Cream and Golden Tobacco flavored Vuse Alto product at 5 % nicotine, and the Menthol flavored Vuse Alto ENDS at 2.4 % and 5 % nicotine were also tested.

Marlboro Gold (85 mm Box King Size) cigarettes, a market leading style of non-menthol cigarettes in the United States, were concurrently tested as a comparator for cigarettes. Similarly, NJOY ACE, menthol flavor at 5 % nicotine concentration, which recently received a marketing granting order from the US FDA, was used a market comparator for ENDS. The comparator products were selected as representatives in their respective categories at the time of the study (Center for Disease Control and Prevention, 2017; Herzog and Kanada, 2018).

Table 1
Description of Study products: This study evaluated 13 Vuse Alto test products which spanned 3 different nicotine concentrations and 9 flavors. In addition, market comparator products for cigarettes and ENDS were also included in the assessments.

Study product	Nicotine concentration	Purpose
Vuse Alto Tropical Coconut 1.5 %	1.5 %	Test Product
Vuse Alto Glacier Menthol 1.5 %	1.5 %	Test Product
Vuse Alto Unflavored 1.5 %	1.5 %	Test Product
Vuse Alto Rich Tobacco 1.5 %	1.5 %	Test Product
Vuse Alto Smooth Tobacco 1.5 %	1.5 %	Test Product
Vuse Alto Aromatic Tobacco 1.5 %	1.5 %	Test Product
Vuse Alto Menthol 1.5 %	1.5 %	Test Product
Vuse Alto Menthol 2.4 %	2.4 %	Test Product
Vuse Alto Menthol 5 %	5 %	Test Product
Vuse Alto Golden Tobacco 1.5 %	1.5 %	Test Product
Vuse Alto Golden Tobacco 5 %	5 %	Test Product
Vuse Alto Berry Cream 1.5 %	1.5 %	Test Product
Vuse Alto Berry Cream 5 %	5 %	Test Product
NJOY Ace Menthol 5 %	5 %	ENDS comparator
Marlboro Gold	N/A	Cigarette comparator

2.2. Generation of total particulate matter (TPM), aerosol collected matter (ACM) and gas vapor phase (GVP)

Cigarettes were conditioned prior being smoked on a rotary smoking machine (Borgwaldt RM20/CSR; Körber Technologies Instruments GmbH, Hamburg, Germany) under a Health Canada Intense (HCI) puffing regimen of 55 mL puff volume, 30 s puff interval, 2 s puff duration (International Standards Organization, 2018) (Supplementary Table S1). Vuse Alto ENDS products and the ENDS comparator NJOY ACE were placed in a vertical position on a linear smoking machine (SM410 or SM450RH; Cerulean, Richmond, VA, USA). Aerosol from ENDS was generated using an intense puffing regimen of 80 mL puff volume, 5 s puff duration, 15 s puff interval from 120 (Vuse Alto ENDS) or 140 puffs (NJOY ACE Menthol) per cartridge (CORESTA, 2018; International Standards Organization and Organization, 2018a). Even though the puff profiles were different between the product types, there was no difference in the capturing of the whole aerosol (ENDS) or whole smoke (combustible cigarette).

TPM, particulate collected material from a combustible cigarette or ACM, aerosol matter from ENDS aerosol (Keyser et al., 2024a; Moore et al., 2023) and GVP samples were prepared using International Standards Organization (ISO) and Health Canada guidelines as described previously (Keyser et al., 2024a, 2024c; Moore et al., 2023). TPM and ACM are collected in the same procedure. The differences between TPM and ACM is a combustible cigarette contains particulates due to combustion, with the captured matter termed TPM; however, since ENDS produce an aerosol, the captured matter is termed ACM. Briefly, TPM and ACM were collected on a Cambridge filter pad (CF) and eluted into dimethyl sulfoxide (DMSO) at desired concentration (mg/mL) (Health Canada, 2004b). The smoke/aerosol passing through the CF was termed GVP (Johnson et al., 2009). The GVP phase was collected by bubbling the smoke into calcium-magnesium free phosphate buffer saline (CMF-PBS). The volume of CMF-PBS used to capture the GVP was adjusted with ice-cold CMF-PBS to equal the volume of DMSO used to extract the TPM or ACM from the CF. Combined TPM + GVP and ACM + GVP test samples were prepared by mixing equal volumes (1:1 v/v) of TPM or ACM (in DMSO) and the respective GVP (in CMF-PBS) fractions (Health Canada, 2004). Freshly prepared (<1 h) combined TPM/ACM + GVP preparations were used in the toxicological assays.

2.3. Generation of whole smoke (WS) and whole aerosol (WA)

Human exposure to these test articles is via WS/WA; therefore, *in vitro* exposure using WS/WA could reflect an *in vivo* response. At the time of these studies, the linear smoking/vaping machine (robot) was not validated (for whole smoke) and the rotary machine used for the combustible comparator could not use the 80/15/5 regimen due to technical limitations of the system for smoke generation and delivery into the high throughput exposure system. Cigarettes were smoked according to the HCI puffing regimen using Vitrocell® VC10® smoking robot (Vitrocell Systems, Waldkirch, Germany) (Supplementary Figure S1) (International Standards Organization, 2018). ENDS were puffed under ISO 20768:2018 (55 mL puff volume, 30 s puff interval, 3 s puff duration) using Vitrocell® VC1/7 smoking robot (International Standards Organization and Organization, 2018a); ENDS were placed on the robot in a vertical position and 120 puffs were taken per pod (Table 2). The whole smoke/whole aerosol is then immediately directed to the exposure module.

Exposure to different doses of WS/WA was achieved by altering the diluting airflow. For all experiments, diluting airflows were expressed in terms of liters per minute (L/min). The vacuum rate was fixed at 5 mL per minute (mL/min). WS from cigarettes was exhausted from two ports of the Vitrocell® VC10® and directed to either the first (Port A) or fifth (Port B) row in the dilution system (Supplementary Figure S1). For the ENDS products, WA from each of seven products was exhausted from seven individual ports of the Vitrocell® VC1/7 and directed in a linear process to the dilution system. The WS/WA was then diluted with air via air inlets to achieve the desired concentration of smoke/aerosol doses; the exception was row seven for the ENDS product which was undiluted. This exposure module has been characterized in the laboratory previously (Keyser et al., 2022).

2.4. Chemical characterization of test samples

TPM/ACM quantification was performed per Health Canada method T501 (Health Canada, 2004). Nicotine in TPM/ACM fractions was analyzed per Health Canada test method T-115 (Health Canada, 1999), whereas the four carbonyls (acrolein, acetaldehyde, crotanaldehyde and formaldehyde) were analyzed in the GVP fractions using Health Canada test method T-304 (Health Canada, 2017b).

Chemical analyses of WS and WA were performed from the CMF-PBS samples placed in the dosimetric modules of the smoking robots. The WS/WA-conditioned CMF-PBS was used for the analysis of nicotine and carbonyl compounds. Nicotine was determined as described previously, briefly Samples were processed on a Thermo Endura LC-MS/MS with a Dionex Ultimate 3000 low pressure quaternary analytical HPLC system (Waltham, MA, USA) fitted with a Waters XBridge BEH Shiled RD18 (2.5 μm) 3.0 × 500 mm analytical column (Milford, MA, USA) (Keyser et al., 2024a, 2024c). Acetaldehyde, acrolein, crotonaldehyde, and formaldehyde were determined as described previously, briefly, the trapped carbonyls in the condition medium were derivatized with 2, 4-dinitrophenylhydrazine (DNPH). Samples were performed using a Thermo Endura LC-MA/MS with a Dionex Ultimate 3000 low pressure quaternary analytical HPLC system (Waltham, MA, USA) fitted with a Waters Acquity BEH Shield C18 (1.7 μm) 2.1 × 500 mm analytical column (Milford, MA, USA) (Keyser et al., 2024a, 2024c).

2.5. Normalization of exposure concentration

Since the emissions from cigarette and ENDS vastly differ in their overall chemistry profiles, nicotine content of the WA and WS was used to normalize dosing, as reported in previous publications (Keyser et al., 2024a, 2024c; Rayner et al., 2019, 2021, 2022). Thus, results from TPM/ACM + GVP or WS/WA exposures are presented in terms of μg of nicotine equivalents. Nicotine equivalents were calculated by multiplying the volume of the dosimetry well in the exposure module by the

Table 2
Generation of WS/WA and exposure conditions for ALI: Whole Smoke and whole aerosols were generated using Vitrocell smoking robots, per the indicated smoking regimens. Different dosing concentration of WS and WA were achieved using diluting airflows (L/min), with undiluted aerosol (0 L/min) representing the most concentrated exposure. The dosing for WA ranged from undiluted aerosol (0) to 4 L/min, whereas for the WS varied from a maximal dose of 0.5 L/min to the lowest dose of 8 L/min. HCI, Health Canada Intense; ISO, International Standards Organization, N/A, not applicable; freq, frequency.

Study Products	Smoking Robot	Method	Puff Volume (mL)	Puff Duration (sec)	Puff Freq (sec)	Puff Profile	Exhaust Duration (sec)	Vent Blocking	Puffing Position	Number of puffs/ ENDS/ Cigarette	Exposure Time (min)	Diluting Airflows (L/min)
Marlboro Gold	Vitrocell VC10	HCI	55	3	30	Bell shaped	8	100 %	Horizontal	8	24	8, 6, 5, 4, 2, 1, 0.5
Vuse and NJOY Ace ENDS	Vitrocell VC1/7	ISO 20768, 2018	55	2	30	Square Wave	8	N/A	Vertical	120	180	4, 3, 2, 1, 0.5, 0.25, 0

chemical determination of nicotine following the completion of the experiment for the WS/WA exposures.

2.6. Regulatory toxicology assays with TPM/ACM + GVP

Combined TPM + GVP from the Marlboro Gold comparator cigarettes, ACM + GVP from the Vuse Alto ENDS test products and the NJOY ACE comparator ENDS were used to assess for *in vitro* mutagenicity in bacterial reverse mutation (Ames) and genotoxicity in the *in vitro* micronucleus (ivMN) assays and were conducted under GLP. These toxicology assays were performed per the OECD guidelines and Health Canada regulations as described previously (Keyser et al., 2024a, 2024c). The Ames assay was conducted according to OECD 471 using the preincubation method (OECD, 2020). The TPM/ACM + GVP was preincubated for 20 min at 37 °C prior to mixing with the overlay agar. The Vuse Alto ENDS test products and the comparator ENDS were tested at various concentrations of ACM + GVP ranging from 0 to 10,000 μg of ACM + GVP equivalents/plate, which corresponds to approximately 0–200 μg of nicotine equivalents/plate, or equivalent volume of DMSO + PBS (vehicle control) (i.e., 100 μL Ames; 200 μL ivMN). The concentrations of TPM + GVP fractions from the comparator cigarette ranged from 0 to 1000 μg TPM + GVP equivalents/plate, which corresponds to 0–200 μg of nicotine equivalents/plate. The highest concentration for each test article was selected by the concentration in which toxicity (lawn thinning) was observed. The criteria for a positive mutagenic response have been described previously, (i) a concentration-related increase in revertant colony count; (ii) a statistically significant increase (Dunnett's test, $\alpha = 0.01$) in mean revertant colonies/plate, and; (iii) revertant colony count higher than the historical background count values at the testing laboratory (Keyser et al., 2024a).

For the ivMN assay, Chinese Hamster Ovary (CHO-WBL) cells were cultured as described previously (Keyser et al., 2024b). OECD 487 and Health Canada T-503 lists this cell line as one that can be selected for these test guidances (OECD, 2016)(Health Canada, 2017d). The top dose tested in each treatment schedule is shown in Supplementary Table S2; top concentration was selected as to induce cytotoxicity in the range of 55 ± 5 % which was determined by Relative Increase in Cell Counts (RICC). Following each exposure of 3 h (schedule I and II) or 24 h (schedule III and IV), flasks were rinsed with CMF-PBS, fresh growth media only (no cytochalasin B) was added to each flask, then the flasks were given a recovery period of 21 h (schedule I and II), 24 h (schedule IV), or none (schedule III) prior to the detection of micronuclei. The Vuse Alto ENDS test products were tested from 0 to 2000 of ACM + GVP/flask, which corresponds to approximately 40 μg of nicotine equivalents/flask, or equivalent volume of DMSO + PBS (vehicle control). The TPM + GVP extracts from the comparator cigarette were tested from 0 to 200 μg/mL of TPM + GVP/plate, which corresponds to approximately 0–4 μg of nicotine equivalents/flask. The criteria for determining a positive genotoxic response of a study product in a replicate assay (i.e., two flasks per concentration) was as per standard criteria, which is as follows: (i) A concentration-related increase in the number of micronuclei in 2000 scored cells (MN) (1000 scored cells per flask). (ii) A statistically significant increase ($\alpha = 0.01$) in the mean frequency of micronuclei (%MN) for at least one concentration compared to the vehicle control using the Dunnett's test. (iii) Number of micronuclei (at any assay dose) outside (i.e., greater than) testing laboratory's historical vehicle control results. Genotoxicity slopes for each test article replicate was determined using Poisson-based regression model (generalized linear model with Poisson distribution and identity link function) Only concentrations with ≤60 % cytotoxicity were considered for model fitting. A study product which did not give a reproducible genotoxic response across the three replicate assays was considered overall as non-genotoxic.

2.7. Neutral red uptake cytotoxicity assay with WS/WA

In vitro cytotoxicity of WA from Vuse Alto ENDS products and ENDS comparator, and WS from the combustible comparator was assessed in monolayer cultures of NCI–H292 cells using NRU assay as described previously and conducted under GLP (Keyser et al., 2024c). Briefly, NCI–H292 cells were maintained in RPMI medium supplemented with 10 % FBS and 0.52 % penicillin/streptomycin at 37 °C with 5 % CO_2. Approximately 48 h prior to exposure, NCI–H292 cells were seeded on to 24 mm Transwells™ using the cell culture media as previously described. Diluting airflows of 0 (undiluted) to 4 L/min were used for the ENDS while airflows of 0.5–8 L/min were used for the cigarette comparator (Table 2), or exposed to laboratory air (vehicle control). A test study product which gave a cytotoxic response across at least two experiments (i.e., at least a 50 % reduction in neutral red absorbance compared to the ALI control) was considered overall as cytotoxic. Flowing air experiments were conducted (data not shown) to determine the longest exposure time until cytotoxicity was observed, which was determined to be 3 h. Hence, the highest ENDS exposure concentration was the undiluted airflow for 3 h.

2.8. Data analysis and statistics

Statistical analysis was performed as described previously (Keyser et al., 2024a). Briefly, NRU for each test article in which a 50 % reduction in mean survival relative to the air control was achieved, IC_{50} was a sigmoidal model with the top parameter fixed at 100 as detailed below:

$$\% \text{ of ALI control} = 100/[(1 + (C/D)\ \hat{}\ \text{Slope})]$$

Parameter C is the nicotine equivalents for which the percentage of ALI survival is halfway between the two asymptotes (top and bottom parameters). Parameter D is the nicotine equivalents for the concentration Comparison of IC_{50} values between the market combustible and each study product was performed using t-tests of mean log-transformed IC_{50} values with a $p < 0.05$ being considered statistically significant. Statistical comparisons between the market combustible and other test articles could not be performed for ivMN or Ames assays because only

the combustible cigarette was met the assay criteria to be classified as positive. All statistical analyses were performed using SAS analytical software (SAS Institute, Inc, Cary, NC USA).

3. Results

In this study we comparatively evaluated mutagenicity and genotoxicity of a total of 13 variants of differing flavor and/or nicotine concentrations of Vuse Alto ENDS using established toxicology assays (OECD, 2016, 2020).

3.1. Characterization of test samples

TPM (from cigarettes), ACM (from ENDS) and GVP (from cigarettes and ENDS) were generated using Health Canada Intense (for cigarettes) and modified CORESTA reference (mCRM) methods, as described in Supplementary Table S1. The yields of TPM/ACM, and the content of nicotine and glycerol from the study products were quantified. The GVP was analyzed for four HPHC carbonyl compounds: formaldehyde, acetaldehyde, acrolein and crotanaldehyde according to Health Canada test method T502 (Health Canada, 2017c). The TPM, nicotine and glycerol contents/cigarette were 44.4, 1.6 ± 0.04 and 1.46 ± 0.05 mg, respectively (Table 3). For Vuse Alto ENDS, the ACM content ranged from 1152 (Vuse Alto Berry Cream, 1.5 %) to 1497 mg (Vuse Alto Menthol, 2.4 %)/cartridge. The nicotine content (per cartridge) for the 1.5 % Vuse Alto ENDS ranged from 12.53 ± 0.25 mg (Vuse Alto unflavored) to 15.86 ± 0.11 mg (Vuse Alto Tropical Coconut), and 28.76 ± 0.61 mg for Vuse Alto Menthol 2.4 %. For the 5 % Vuse Alto ENDS, nicotine content (per cartridge) was highest for Vuse Alto Golden Tobacco, 5 % (57 ± 1.94 mg) followed by and Vuse Alto Berry Cream, 5 % (45.16 ± 2.33 mg) and Vuse Alto Menthol, 5 % (43.73 ± 0.2 mg). The glycerol content was for the Vuse Alto ENDS (per cartridge) was between 525.33 ± 2.86 mg/cartridge of Vuse Alto Menthol, 5 % to 706.33 ± 24.13 mg for Vuse Alto Golden Tobacco, 5 %. The ACM, nicotine, and glycerol for the NJOY ACE Menthol, 5 % were generally similar to the 5 % Vuse Alto ENDS (Table 3).

The GVP fraction from the cigarette comparator contained readily quantifiable levels of formaldehyde (2.16 μg/mL), acetaldehyde (164.66 μg/mL), acrolein (18.82 μg/mL), and crotanaldehyde (9.63 μg/mL) (Table 4). The levels of these four aldehydes were either lacking or significantly lower in the Vuse Alto ENDS test products. While the formaldehyde levels were somewhat higher in the comparator ENDS (4.74 μg/mL compared to the cigarette comparator (2.16 μg/mL), the other three HPHCs were lower (Table 4).

The WS (from cigarettes) and WA (from ENDS) test samples were prepared according HCI and ISO methods (Supplementary Table S1 and Table 2). The WS from the comparator Marlboro Gold cigarette contained quantifiable nicotine (22.89 ± 7.8 μg) and the four carbonyl compounds (Table 5). The highest levels of the carbonyls (in μg/mL) in WS were formaldehyde (0.83 ± 0.22), acetaldehyde (6.75 ± 1.87), acrolein (0.91 ± 0.22) and crotanaldehyde (0.61 ± 0.19).

Parallel chemical analyses of WA from the Vuse Alto ENDS test products and the comparator ENDS were performed using the samples generated from WA. The nicotine content of Vuse Alto ENDS in the dosimetry well at the 1.5 % products ranged from 191 ± 26.85 μg/mL (Unflavored) to 419.33 ± 364.37 μg/mL (Golden Tobacco 1.5 %) (Table 5). The samples from Vuse Alto Menthol, 2.4 % had 273.3 ± 123 μg/mL of nicotine, and the three 5 % Alto ENDS samples contained 409.6 ± 343.61 μg/mL (Vuse Alto Berry Cream) to 796 ± 387.32 μg/mL (Vuse Alto Menthol, 5 %) of nicotine. The levels of formaldehyde, acetaldehyde, acrolein, and crotanaldehyde were found to be at the LOQ or LOD levels in all the Vuse Alto ENDS. The ENDS comparator NJOY ACE Menthol, 5 % samples had 450.66 ± 159.87 μg/mL of nicotine and the four carbonyls at LOD, or LOQ levels.

3.2. Regulatory toxicology

3.2.1. Bacterial reverse mutation (Ames) assay

Mutagenic potential of Vuse Alto ENDS was assessed by the Ames assay, using TA98, TA100, TA102, TA1535, and TA1537 strains of *S. typhimurium* in the presence or absence of S9 extracts. Toxicity was observed for all four bacterial strains with all test product samples at 10,000 μg ACM + GVP (200 μg of nicotine) equivalents for ENDS and 1000 μg ACM + GVP (200 μg of nicotine) equivalents for Marlboro Gold which were the highest tested concentrations tested.

Mutagenic responses were observed for the TPM + GVP preparations generated from the Marlboro Gold cigarettes in Salmonella strains TA98 (+S9), TA100 (-S9), TA100 (+S9) and TA1537 (+S9) (Fig. 1). Mutagenicity was not observed with any of the *S. typhimurium* bacterial strains with or without exogenous metabolic activation within the concentration ranges tested for ACM + GVP preparations from Vuse Alto ENDS test products (Fig. 1). Similarly, the comparator ENDS also did not elicit

Table 3
Chemical analyses of TPM and ACM extracts: Chemical analyses of TPM/ACM extracts from Vuse Alto ENDS test products and comparator products. Mean and standard deviation values from mean of three experiments are presented.

Study Products	Puff Count [per cig/ENDS cartridge]	ACM/TPM [mg/[per cig/ENDS cartridge]]	Nicotine [mg/[per cig/ENDS cartridge]]	Glycerol [mg/[per cig/ENDS cartridge]]
Vuse Alto Tropical Coconut 1.5 %	120	1325	15.86 ± 0.11	667.0 ± 7.76
Vuse Alto Berry Cream 1.5 %	120	1152	13.26 ± 0.66	570.0 ± 28.6
Vuse Alto Glacier Menthol 1.5 %	120	1407	16.20 ± 0.62	668.7 ± 28.02
Vuse Alto Unflavored 1.5 %	120	1244	12.53 ± 0.25	546.0 ± 12
Vuse Alto Menthol 1.5 %	120	1192	14.86 ± 0.25	597.0 ± 10.53
Vuse Alto Rich Tobacco 1.5 %	120	1317	15.36 ± 0.23	649.7 ± 9.71
Vuse Alto Smooth Tobacco 1.5 %	120	1275	15.16 ± 0.23	646.7 ± 14.01
Vuse Alto Aromatic Tobacco 1.5 %	120	1381	16.00 ± 0.30	678.7 ± 14.01
Vuse Alto Golden Tobacco 1.5 %	120	1344	14.43 ± 0.30	620.0 ± 10.81
Vuse Alto Menthol 2.4 %	120	1497	28.76 ± 0.61	697.0 ± 15.01
Vuse Alto Menthol 5 %	120	1449	43.73 ± 0.20	525.3 ± 2.86
Vuse Alto Golden Tobacco 5 %	120	1444	57.00 ± 1.94	706.3 ± 24.13
Vuse Alto Berry Cream 5 %	120	1162	45.10 ± 2.33	579.0 ± 29.13
NJOY ACE Menthol 5 %	140	1562	60.26 ± 2.00	648.66 ± 20.66
Marlboro Gold	9.9	44.4	1.60 ± 0.04	1.46 ± 0.05

Table 4
Chemical analyses of GVP extracts: Chemical analyses of GVP extracts from Vuse Alto ENDS test products and comparator products. Data from representative runs is presented. LOQ of crotonaldehyde 0.016 μg/mL.

Study Product	Puff Count [per cig/ENDS]	ACM/TPM [mg/cig/ENDS]	Formaldehyde [μg/mL]	Acetaldehyde [μg/mL]	Acrolein [μg/mL]	Crotonaldehyde [μg/mL]
Vuse Alto Tropical Coconut 1.5 %	120	2563	0.50	1.42	0.62	<LOQ
Vuse Alto Berry Cream 1.5 %	120	2497	1	1.70	0.53	<LOQ
Vuse Alto Glacier Menthol 1.5 %	120	2664	0.36	0.91	0.24	<LOQ
Vuse Alto Unflavored 1.5 %	120	2379	0.78	1.07	0.33	<LOQ
Vuse Alto Menthol 1.5 %	120	2567	0.30	0.73	0.29	<LOQ
Vuse Alto Rich Tobacco 1.5 %	120	2546	0.41	0.67	0.19	<LOQ
Vuse Alto Smooth Tobacco 1.5 %	120	2744	0.39	1.07	0.26	<LOQ
Vuse Alto Aromatic Tobacco 1.5 %	120	2292	0.58	1.07	0.35	<LOQ
Vuse Alto Golden Tobacco 1.5 %	120	2923	0.11	0.98	0.23	<LOQ
Vuse Alto Menthol 2.4 %	120	2628	0.23	0.82	0.27	<LOQ
Vuse Alto Menthol 5 %	120	2774	0.28	1.87	0.59	<LOQ
Vuse Alto Berry Cream 5 %	120	2346	0.90	4.04	0.64	<LOQ
Golden Tobacco 5 %	120	2923	0.11	0.98	0.23	<LOQ
NJOY ACE Menthol 5 %	120	2673	4.71	5.97	2.04	0.03
Marlboro Gold	9.9	44.4	2.16	164.66	18.82	9.63

Table 5
Chemical analyses of whole smoke and whole aerosols from the study products: WS/WA test samples were generated using Vitrocell smoking robots (Table 2; Supplementary Figure S1) and were analyzed for nicotine and the four carbonyl HPHCs. The WS-conditioned samples from 0.5 L/min of airflow were used for the chemical analyses, whereas The WA samples were generated from undiluted aerosol (0 L/min of diluting air flow). Mean and standard deviation values from mean of three experiments are presented. <LOD for carbonyl HPHCs: 0.1 μg/mL; <LOQ for carbonyl HPHCs: 0.3 μg/mL.

Study Product	Analytes (μg/mL)				
	Nicotine	Formaldehyde	Acetaldehyde	Acrolein	Crotanaldehyde
Vuse Alto Tropical Coconut 1.5 %	280 ± 24.54	<LOQ	<LOD	<LOD	<LOD
Vuse Alto Berry Cream 1.5 %	277 ± 82.24	<LOQ	<LOD	<LOD	<LOD
Vuse Alto Glacier Menthol 1.5 %	306 ± 21	<LOQ	<LOD	<LOD	<LOD
Vuse Alto Unflavored 1.5 %	191 ± 26.85	<LOQ	<LOD	<LOD	<LOD
Vuse Alto Menthol 1.5 %	201.33 ± 43.6	<LOQ	<LOD	<LOD	<LOD
Vuse Alto Rich Tobacco 1.5 %	226.66 ± 27.46	<LOQ	<LOQ	<LOD	<LOD
Vuse Alto Smooth Tobacco 1.5 %	226.66 ± 40.91	<LOQ	<LOD	<LOD	<LOD
Vuse Alto Aromatic Tobacco 1.5 %	206.33 ± 54.16	<LOQ	<LOQ	<LOD	<LOD
Vuse Alto Golden Tobacco 1.5 %	419.33 ± 364.37	<LOQ	<LOD	<LOD	<LOD
Vuse Alto Menthol 2.4 %	273.3 ± 123	<LOQ	<LOD	<LOD	<LOD
Vuse Alto Menthol 5 %	796 ± 387.32	<LOQ	<LOD	<LOD	<LOD
Vuse Alto Golden Tobacco 5 %	428.66 ± 280	<LOQ	<LOD	<LOD	<LOD
Vuse Alto Berry Cream 5 %	409.6 ± 343.61	<LOQ	<LOD	<LOD	<LOD
NJOY Ace Menthol 5 %	450.66 ± 159.87	<LOQ	<LOD	<LOD	<LOD
Marlboro Gold	7.63 ± 2.6	0.83 ± 0.22	6.75 ± 1.87	0.91 ± 0.22	0.61 ± 0.19

mutagenic responses. Thus, the Vuse Alto ENDS, independent of nicotine concentration or tested flavors were non-mutagenic.

3.2.2. *In vitro micronucleus assay*

Genotoxicity was assessed by ivMN assay under four treatment schedules, as described in Materials and Methods and listed in Supplementary Table S2, with the concentrations ranging from 0 to 200 μg/mL (combustible cigarette) or 0–2000 μg/mL (ENDS). The TPM + GVP samples from Marlboro Gold cigarette comparator were both cytotoxic and genotoxic. Cytotoxicity was observed for 150 and 200 μg/mL in Schedule (i), at 150, 175, and 200 μg/mL in Schedule (iii) and 125, 150, 175, and 200 μg/mL in Schedule (iv) assay of TPM + GVP equivalents/mL for Marlboro Gold. The TPM + GVP preparations were deemed genotoxic under treatment schedules (i), (ii), (iii), and (iv) over the dose ranges tested (Fig. 2).

Cytotoxicity (≥60 % relative to the vehicle control by relative increase in cell counts [RICC]) was not observed at any ACM + GVP concentrations tested for any Vuse Alto ENDS test products in any of the four treatment schedules. Genotoxicity was not observed in any of the four treatment schedules at the concentration range tested for ACM + GVP preparations from Vuse Alto ENDS test products or the NJOY ACE Menthol ENDS market comparator. The Vuse Alto ENDS test products were non-genotoxic.

3.3. *Whole smoke and whole aerosol cytotoxicity studies*

Cytotoxicity of Vuse Alto test products was assessed by exposure to whole aerosol, along with the market comparators for cigarettes and ENDS in monolayer cultures of H292 cells. Exposure of the H292 cells was performed by whole smoke/aerosol generated by a Vitrocell smoking robot and then was diluted by various amounts of clean air to alter the concentration delivered to the cells. One of the exposure wells was filled with PBS and following the exposure, chemical analysis was performed. Chemistry results from the PBS filled well at the highest concentration of whole smoke (0.5 L/min diluting air) or whole aerosol (0 L/min diluting air, undiluted) are shown in Table 4. Whole smoke from the Marlboro Gold cigarette elicited cytotoxic responses over the dose range tested (0.11–10.2 μg nicotine equivalents/mL), with a mean IC_{50} value of 0.8 μg nicotine equivalents/mL (Fig. 3).

Whole aerosol from the following 1.5 % nicotine concentration

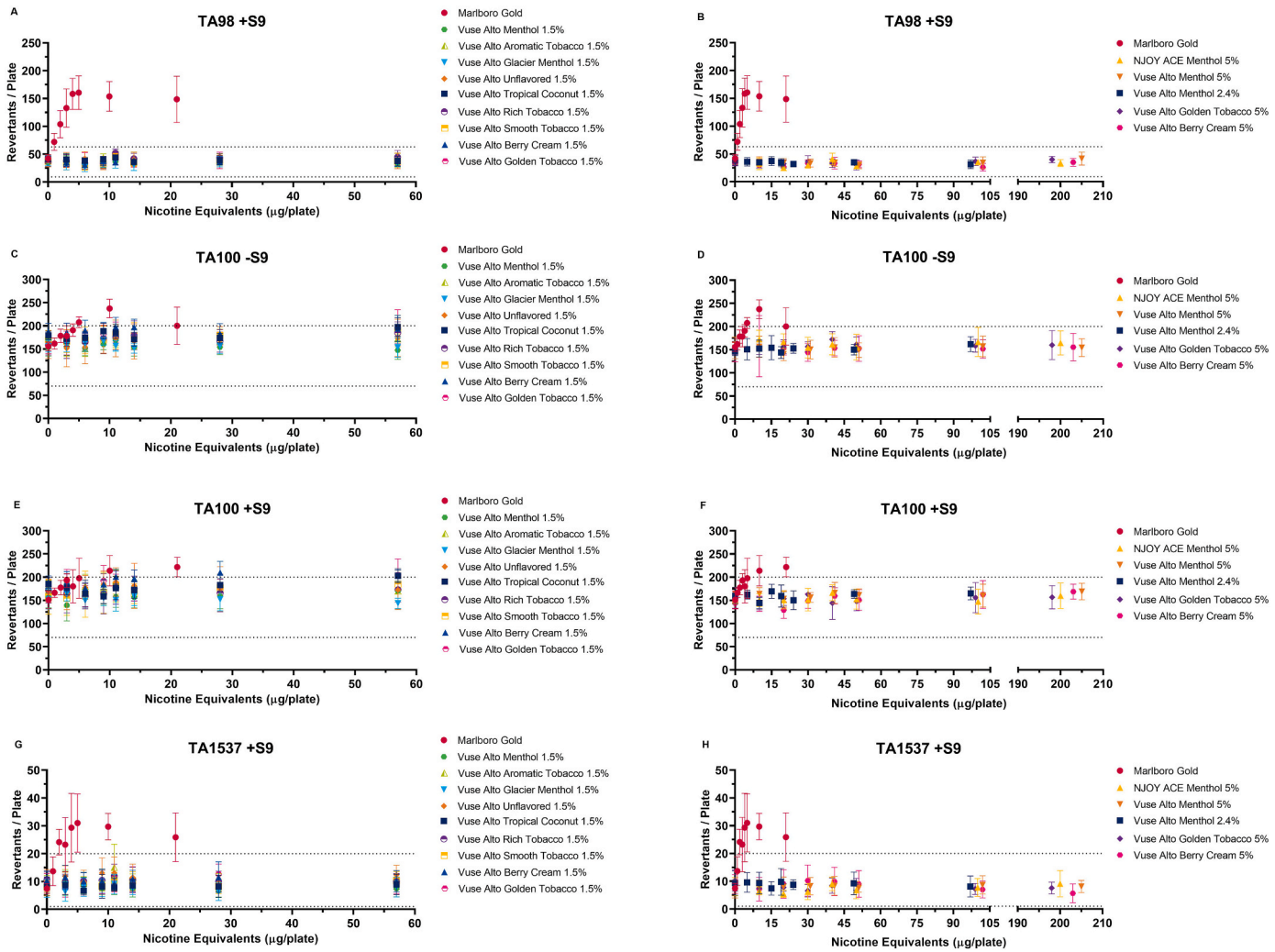

Fig. 1. Bacterial reverse mutagenesis (Ames assay): Vuse Alto ENDS test products and the market comparators for cigarettes (Marlboro Gold) and ENDS (NJOY ACE, Menthol 5 %) were assessed for mutagenic potential in the Ames assay. Five Salmonella tester strains, with or without metabolic activation (±S9), were used in this assay (Materials and Methods). TPM/ACM + GVP extracts were used as test samples and their dosage is given as Nicotine Equivalents (µg/plate). Mutagenic responses observed for any one of the test products are shown. Panels A, C, E, and G show results from Vuse Alto 1.5 %, nicotine concentration products, along with Marlboro Gold. Panels B, D, F, and H show results from Vuse Alto 5 %, nicotine concentration products, along with Marlboro Gold and NJOY ACE, Menthol 5 %. The dotted horizontal lines in the panels represent the historical range of background revertants observed at the testing laboratory. Marlboro Gold elicited mutagenic responses, whereas none of the Vuse Alto test products were mutagenic. Cell survival (mean and standard deviation) was determined from three independent experiments with three replicates per concentration. Panels A, C, E, and G: Circle, Marlboro Gold; green octagon, Vuse Alto Menthol 1.5 %; half-filled triangle, Vuse Aromatic Tobacco 1.5 %; blue nabla, Vuse Alto Glacier Menthol 1.5 %; diamond, Vuse Alto Unflavored 1.5 %; blue square, Vuse Alto Tropical Coconut 1.5 %; filled semi-circle, Vuse Alto Rich Tobacco 1.5 %; half-filled square Vuse Alto Smooth Tobacco 1.5 %; blue triangle, Vuse Alto Berry Cream 1.5 %; half-octagon, Vuse Alto Golden Tobacco 1.5 %. Panels B, D, F, and H: Circle, Marlboro Gold; NJOY ACE Menthol 5 %; orange nabla, Vuse Alto Menthol 5 %; dark blue square, Vuse Alto Menthol 5 %; blue diamond, Vuse Alto Golden Tobacco 5 %; fuchsia octagon, Vuse Alto Berry Cream 5 %. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

products was non-cytotoxic under the experimental conditions: Vuse Alto Tropical Coconut; Vuse Alto Berry Cream; Vuse Alto Glacier Menthol, Vuse Alto Unflavored; Vuse Alto Rich Tobacco; Vuse Alto Smooth Tobacco; Vuse Alto Golden Tobacco; Vuse Alto Menthol. Additionally, Vuse Alto Menthol, 2.4 % and Vuse Alto Berry Cream, 5 % were also non-cytotoxic. Therefore, IC_{50} values for these Vuse Alto test products could not be determined (Fig. 3).

However, three Vuse Alto ENDS yielded inconsistent results and IC_{50} values in replicate experiments. The lowest IC_{50} value is presented in Fig. 3, as it represents the maximal cytotoxic potential. While the WA from Vuse Alto Golden Tobacco, 5 % produced vastly different cytotoxic responses in replicate experiments, the lower value of 596 µg equivalents of nicotine is taken as the IC_{50} value. Further, Vuse Alto Menthol, 1.5 % and Vuse Alto Aromatic Tobacco, 1.5 % also produced cytotoxic responses in two of three replicate experiments. The lower IC_{50} values for Vuse Alto Menthol 1.5 % and Vuse Alto Aromatic Tobacco 1.5 % were 498 µg and 939 µg nicotine equivalents, respectively. While these 3 Vuse Alto ENDS showed evidence of some degree of cytotoxicity, their potency, based on the IC_{50} values, is several hundred-fold lower compared to the Marlboro Gold cigarette comparator. The ENDS comparator NJOY Ace, Menthol, 5 % was also non-cytotoxic. These results show that the Vuse Alto ENDS tested in this study are either non-cytotoxic or minimally cytotoxic in H292 cells *in vitro*.

4. Conclusions and discussion

An important purpose of our overall research is to support the goals of tobacco harm reduction by developing alternate tobacco products that are appropriate for APPH. In this manuscript, we assessed *in vitro* toxicological effects of several flavor and nicotine concentration

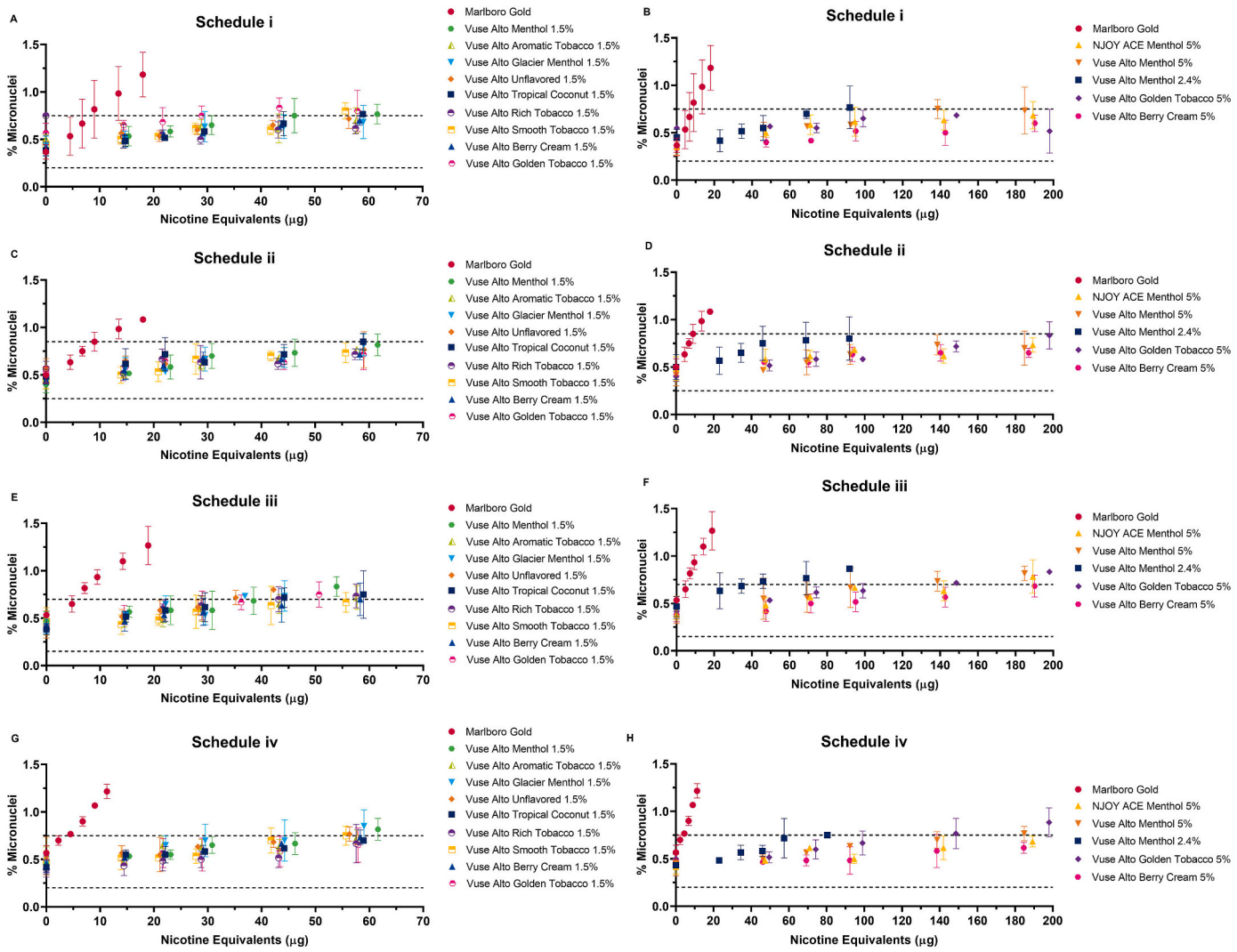

Fig. 2. ***In vitro*** **micronucleus assay:** Vuse Alto ENDS test products and the market comparators for cigarettes (Marlboro Gold) and ENDS (NJOY ACE Menthol 5 %) were assessed for genotoxicity. The ivMN assay was performed in CHO cells under 4 treatment schedules (Materials and Methods). TPM/ACM + GVP extracts were used as test samples and their dosage is given as Nicotine Equivalents (μg/plate). Genotoxic responses observed for any one of the test products are shown. Panels A, C, E, and G show results from Vuse Alto 1.5 %, nicotine concentration products, along with Marlboro Gold. Panels B, D, F, and H show results from Vuse Alto 5 %, nicotine concentration products, along with Marlboro Gold and NJOY ACE, 5 % Menthol. The dotted horizontal lines in the panels represent the historical background range of %micronuclei observed at the testing laboratory. Marlboro Gold elicited genotoxic responses under all 4 treatment schedules, whereas none of the Vuse Alto test products were genotoxic. Cell survival (mean and standard deviation) was determined from three independent experiments with three replicates per concentration. Panels A, C, E, and G: Circle, Marlboro Gold; green octagon, Vuse Alto Menthol 1.5 %; half-filled triangle, Vuse Aromatic Tobacco 1.5 %; blue nabla, Vuse Alto Glacier Menthol 1.5 %; diamond, Vuse Alto Unflavored 1.5 %; blue square, Vuse Alto Tropical Coconut 1.5 %; filled semi-circle, Vuse Alto Rich Tobacco 1.5 %; half-filled square Vuse Alto Smooth Tobacco 1.5 %; blue triangle, Vuse Alto Berry Cream 1.5 %; half-octagon, Vuse Alto Golden Tobacco 1.5 %. Panels B, D, F, and H: Circle, Marlboro Gold; NJOY ACE Menthol 5 %; orange nabla, Vuse Alto Menthol 5 %; dark blue square, Vuse Alto Menthol 5 %; blue diamond, Vuse Alto Golden Tobacco 5 %; fuchsia octagon, Vuse Alto Berry Cream 5 %. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

variants of Vuse Alto ENDS. A key finding from this work is the ENDS tested in this study are non-genotoxic and non-mutagenic under the conditions of these assays. Further, the ENDS were either non-cytotoxic or substantially less cytotoxic compared to the market comparator for cigarettes. The lack of demonstrable activity in these *in vitro* toxicological studies was independent of nicotine concentration or flavor.

The FDA reviews and authorizes marketing of new tobacco products based on whether they meet the criteria of APPH (Food and Drug Administration, 2023). Considering the abundance of combustion-derived toxicants in cigarette smoke, non-combustible tobacco products, such as e-cigarettes have been advocated as alternatives for smokers who are unwilling to quit for reducing harm from cigarette smoking (National Academies of SciencesE. and Medicine, 2018). With the recognition that no tobacco product is safe, several public health researchers and FDA have accepted the relative risk continuum among tobacco products and acknowledged ENDS as potentially lower risk tobacco products compared to cigarettes (Food and Drug Administration, 2024b; Gottlieb and Zeller, 2017). No tobacco product is safe or risk free, the best way for adult smokers to achieve risk reduction is to quit. However, ENDS are a highly heterogeneous category of products and the APPH of candidate products must be demonstrated to gain FDA's marketing authorization (Toll et al., 2024).

The design features of ENDS, such as e-liquid composition, the power of the battery units and other additives such as flavors, among other factors, have been reported to influence the generation of toxic aldehydes in the emissions of ENDS (Geiss et al., 2016; Kosmider et al., 2014; Noël and Ghosh, 2022). It has been noted that methodological inconsistencies also account for the varied levels of carbonyls reported in

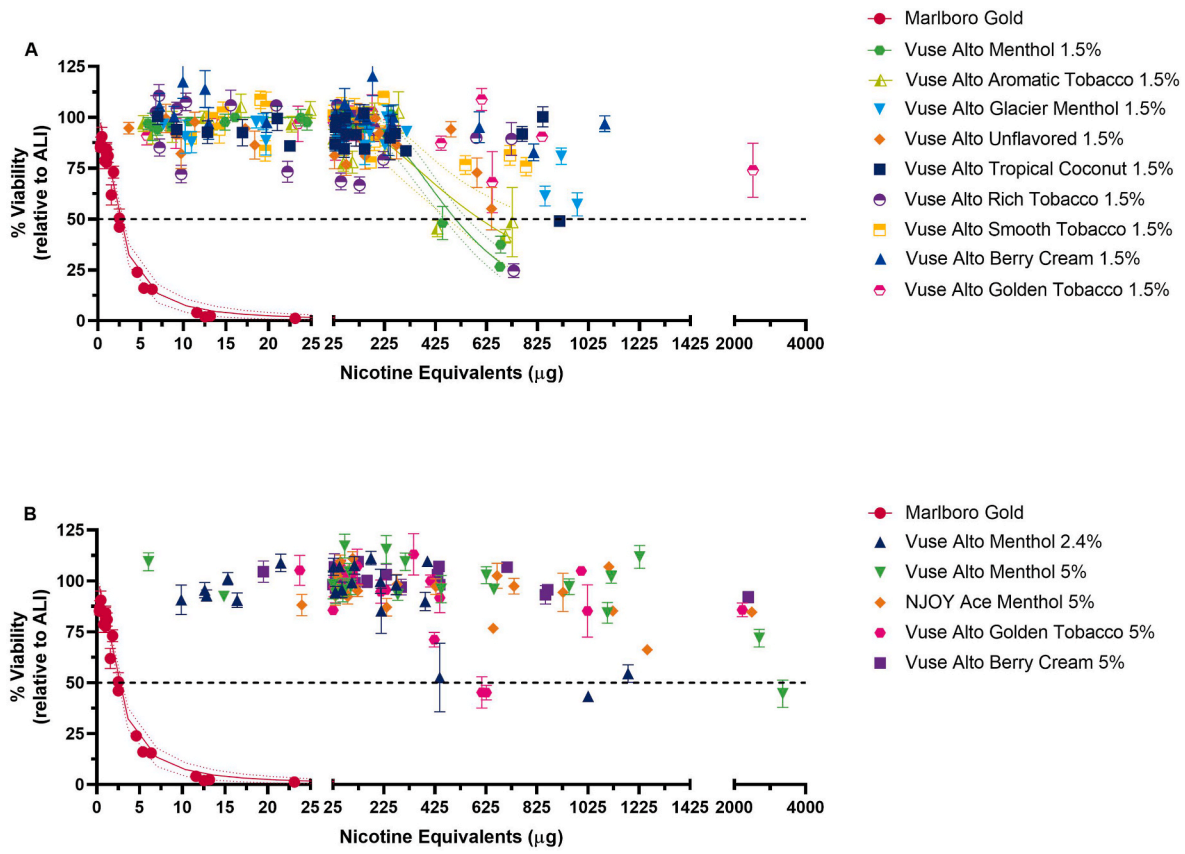

Fig. 3. Cytotoxicity, NRU assay: The whole aerosol generated from VC1/7 smoking robot was used to assess the cytotoxicity of Vuse Alto ENDS test products and the comparator ENDS using monolayer H292 cells at ALI. WS from the comparator cigarette generated from the VC10 smoking robot was also tested in parallel. Panel A shows viability data for the Vuse Alto ENDS with 1.5 % nicotine concentration, whereas Panel B shows the data for Vuse Alto ENDS with 5 % nicotine concentration and the comparator ENDS. Data for Marlboro Gold cigarette is included in both panels. A viability response curve with a 95 % CI (dotted line) was plotted for any test article that induced a 50 % reduction in cell viability in at least two experiments. A: Circle, Marlboro Gold; green octagon, Vuse Alto Menthol 1.5 %; half-filled triangle, Vuse Aromatic Tobacco 1.5 %; blue nabla, Vuse Alto Glacier Menthol 1.5 %; diamond, Vuse Alto Unflavored 1.5 %; blue square, Vuse Alto Tropical Coconut 1.5 %; filled semi-circle, Vuse Alto Rich Tobacco 1.5 %; half-filled square Vuse Alto Smooth Tobacco 1.5 %; blue triangle, Vuse Alto Berry Cream 1.5 %; half-octagon, Vuse Alto Golden Tobacco 1.5 %. B: Circle, Marlboro Gold; NJOY ACE Menthol 5 %; orange nabla, Vuse Alto Menthol 5 %; dark blue square, Vuse Alto Menthol 5 %; blue diamond, Vuse Alto Golden Tobacco 5 %; fuchsia octagon, Vuse Alto Berry Cream 5 %. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ENDS emissions (Farsalinos and Gillman, 2017; Travis et al., 2023). Therefore, we have adhered to Health Canada methods for the evaluation of a heated product (Keyser et al., 2024a) and variants of Vuse Alto ENDS (Leverette et al., in preparation). Additionally, we implemented CORESTA-recommended methods for WS/WA assessments, including performing regular performance qualifications of the whole smoke equipment and normalizing delivered concentration to compare between product types (Thorne et al., 2021).

In this study, we assessed 13 Vuse Alto ENDS that varied in nicotine concentration (1.5 %, 2.4 % and 5 %) and 9 flavors (Table 1), using established regulatory toxicological assays along with market comparators for cigarette (Marlboro Gold) and ENDS (NJOY ACE Menthol, 5 %). ACM + GVP mixtures from ENDS, and TPM + GVP mixtures from the cigarette comparator were prepared and used as the test samples per the Health Canada and ISO (CORESTA, 2018; Moore et al., 2023). The current set of 13 Vuse Alto ENDS test products exposures had higher levels of nicotine and glycerol relative to the comparator cigarettes, and those two compounds are not likely to contribute to toxicity endpoints as assessed in this study. Importantly, the ENDS contained lower levels of formaldehyde, acetaldehyde, acrolein, and crotanaldehyde relative to the cigarette comparator. These ENDS were non-mutagenic and non-genotoxic in the *in vitro* regulatory toxicology assays.

Additional suggested improvements for the assessment of tobacco products include use of freshly generated smoke/aerosol for dosing, implementation of exposure dosimetry and use of human cell-based assays (Lauterstein et al., 2020), and those methods are currently under development for regulatory acceptance (Cao et al., 2021; Miller-Holt et al., 2022; Moore et al., 2023). We and others have utilized smoking robots for the generation of WS and WA and adapted cytotoxicity assays using human cell systems (Fields et al., 2017; Keyser et al., 2019, 2022, 2024a, 2024c). The WA from the ENDS test products contained lower levels of the four HPHC aldehydes compared to WS from the comparator cigarettes.

The ENDS variants tested in this study were non-mutagenic, non-genotoxic and were mostly non-cytotoxic or exhibited lower cytotoxicity compared to cigarettes. These results align with our previous work (Keyser et al., 2024c) (Leverette et al., in preparation). In a related series of studies with a similar Vuse Alto ENDS products, Keyser et al. comparatively assessed the *in vitro* toxicology of 6 Vuse Alto ENDS that varied in nicotine concentration (1.8 %, 2.4 % and 5 %) and flavors (Golden Tobacco, Menthol, Mixed Berry 5 % and Rich Tobacco 5 %) (Keyser et al., 2024c). These ENDS were found to be non-cytotoxic in the NRU assay in monolayer cultures, MTT, and lactate dehydrogenase assays using EpiAirway tissues. Additionally, these ENDS products either not induce oxidative stress or elicited marginal levels of oxidative stress compared to cigarette smoke (Keyser et al., 2024c). In the current study, for some replicate experiments involving three variants of Vuse Alto (Golden Tobacco 5 %, Menthol 1.5 %, and Aromatic Tobacco 1.5 %) the

cytotoxicity results were inconsistent due to some technical (model fit) limitations. Hence, the IC_{50} values could not be calculated. The reason (s) for the methodological inconsistencies that led to equivocal cytotoxicity results for these three variants of Vuse Alto ENDS is unclear at present. However, we conservatively report the lowest obtained IC_{50} values in any of the replicate experiments and classified them as cytotoxic. The reported IC_{50} values for Vuse Alto Golden Tobacco, 5 %, Vuse Alto Menthol, 1.5 % and Vuse Alto Aromatic Tobacco, 1.5 % were several hundred-fold higher, compared to Marlboro Gold cigarettes, thus attesting to their markedly lower cytotoxicity.

Similar to our findings discussed herein, several reports suggest a lack of (or a markedly reduced) *in vitro* toxicological effects (e.g., Ames, ivMN, NRU) of e-cigarettes (Caruso et al., 2023; Czekala et al., 2019; Emma et al., 2023; Thorne et al., 2018, 2019). Those findings include positive results in these assays with a combustible cigarette with minimal to no activity when compared to the ENDS products that were evaluated. Although the role of flavored e-cigarettes in smoking cessation continues to be an active area of research, some reports have shown that flavors may augment adaption of e-cigarettes and reduce smoking (Cobb et al., 2019; Gades et al., 2022; Li et al., 2021; Mok et al., 2022). Therefore, we have undertaken a comprehensive evaluation of ENDS products and shown in this manuscript that the ENDS test products exhibit reduced toxicity in the *in vitro* studies.

Some of the strengths of this research are the use of established toxicological methods and a wholistic relative assessment of the toxic effects of the mainstream emissions (i.e., emissions from the device to be inhaled by the user) from the Vuse Alto ENDS and the market comparators, using TPM/ACM + GVP extracts as well as the whole aerosol exposures furthering the use of *in vitro* assays to replace/reduce the use of animals for regulatory toxicity testing. One of the limitations this study is the partial analyses of toxicants in the test samples, which was limited to the four carbonyl HPHCs.

In summary, the 13 Vuse Alto ENDS products across nicotine strength and multiple flavors were non-mutagenic and non-genotoxic. A majority of the Vuse Alto ENDS products were also non-cytotoxic. Three Vuse Alto ENDS exhibited lower cytotoxicity compared to cigarette comparator, as reflected by higher IC_{50} values.

CRediT authorship contribution statement

Brian M. Keyser: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Robert Leverette:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Reagan McRae:** Writing – review & editing, Project administration, Formal analysis, Conceptualization. **John Wertman:** Writing – review & editing, Project administration, Methodology, Data curation. **Tom Shutsky:** Writing – review & editing, Project administration, Formal analysis, Data curation. **Kristen G. Jordan:** Writing – review & editing, Supervision, Funding acquisition. **Ken Szeliga:** Writing – review & editing, Project administration. **Patrudu Makena:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

Brian Keyser, Robert Leverette, John Wertman, Kristen Jordan, Ken Szeliga and Patrudu Makena are full time employees of RAI Services Company (RAIS). Thomas Shutsky and Reagan McRae are former employees of RAIS and were full time employees during the time of these studies. RAIS is a wholly owned subsidiary of Reynolds American, Inc., which is a wholly owned subsidiary of British American Tobacco plc (BAT).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2025.115640.

Data availability

The data that has been used is confidential.

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