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Authors

Omaiye, Esther E
Luo, Wentai
McWhirter, Kevin J
et al.

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Flavor chemicals, synthetic coolants, and pulegone in popular mint and menthol flavored e-cigarettes

Esther E. Omaiye[1,3] MS, Wentai Luo[2] Ph.D., Kevin J. McWhirter[2] BS, James F. Pankow[2] Ph.D., and Prue Talbot[3*] PhD

[1] Environmental Toxicology Graduate Program, University of California, Riverside, Riverside, CA, United States

[2] Departments of Chemistry and Civil & Environmental Engineering, Portland State University, Portland, Oregon, United States

[3]Department of Molecular, Cell and Systems Biology, University of California, Riverside, Riverside, CA, United States

Correspondence to

Dr. Prue Talbot

Dept. of Molecular, Cell and Systems Biology,

University of California Riverside,

Riverside, CA, United States.

talbot@ucr.edu

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ABSTRACT

Background. The FDA recently banned flavors from pod-style electronic cigarettes (e-cigarettes), except for menthol and tobacco. JUUL™ customers quickly discovered that flavored disposable e-cigarettes from other manufacturers, such as Puff, were readily available. Our goal was to compare flavor chemicals, synthetic coolants, and pulegone in mint/menthol-flavored e-cigarettes from JUUL™ and Puff, evaluate the cytotoxicity of the coolants and perform a cancer risk assessment for pulegone, which was present in both JUUL™ pods and disposable Puff product.

Methods. Identification and quantification of chemicals were performed using gas chromatography/mass spectrometry. Cytotoxicity of the coolants was evaluated with BEAS-2B cells using the MTT assay. The cancer risk of pulegone was calculated using the Margin of Exposure (MOE).

Results. Menthol was the dominant flavor chemical (> 1 mg/mL) in all products from both manufacturers. Minor flavor chemicals (< 1 mg/mL) differed in the JUUL™ and Puff fluids and may produce flavor accents. The concentrations of WS-3 and WS-23 were higher in Puff than in JUUL™. WS-23 was cytotoxic in the MTT assay at concentrations 90 times lower than concentrations in Puff fluids. The risk for cancer (MOE < 10,000) was greater for mint than menthol products and greater for Puff than JUUL.

Conclusions. Switching from JUUL™ to Puff e-cigarettes may expose users to increased harm due to the higher levels of WS-23 and pulegone in Puff products. Cancer risk may be reduced in e-cigarettes by using pure menthol rather than mint oils to produce minty flavored e-cigarette products.

INTRODUCTION

JUUL™ was the first popular pod-style e-cigarette with a large share of its sales going to middle and high school students.[1-5] JUUL™ initially marketed eight flavors of pods, including "Cool Mint" and Classic Menthol", which were later replaced by "Mint" and "Menthol", respectively.[6] The rapid spike in JUUL™ popularity concerned parents, public health officials, and regulatory agencies, leading JUUL™ in 2019 to remove all flavors from their product line in the US, except for "Classic Tobacco," "Virginia Tobacco," and "Menthol." Puff products, which appear similar to JUUL™, did not fall under the Food and Drug Administration's limitations on flavors, and many JUUL™ users switched to Puff , which rapidly became a dominant e-cigarette brand.[7-9] In spite of their popularity, we know little about the relative safety of Puff and JUUL™ products.

This study compares three classes of chemicals in Puff and JUUL™ e-fluids. These include flavor chemicals, in particular menthol, two synthetic coolants, and pulegone, a potential carcinogen that has been reported in mint-flavored e-cigarettes.[10,11] Because the use of menthol is permitted by the Family Smoking Prevention and Tobacco Control Act of 2009,[12] it is one of the most widely used flavor chemicals in tobacco products,[13] sometimes appearing in e-cigarettes that are not explicitly labeled "mint" or "menthol".[14] The cooling properties and pleasant minty flavor of menthol may make smoking initiation easier among novice users.[15,16] Although generally regarded as safe (GRAS) for ingestion by the Flavor and Extract Manufacturers Association (FEMA).[17] menthol is often used in e-cigarette products at high concentrations,[14] which are cytotoxic in vitro.[14,18,19]

The synthetic coolants WS-3 (N-ethyl-p-menthane-3-carboxamide; CAS # 39711-79-0) and WS-23 (2-isopropyl-N,2,3-trimethylbutyramide; CAS # 51115-67-4) are popular cooling

agents, were initially developed by Wilkinson Sword Ltd. in the 1970s.[20] These coolants are considered safe for ingestion by FEMA and are used extensively in consumer products, including breath fresheners, confectionaries, and cosmetics.[21-23] WS-3 and WS-23 activate the TRPM8 and TRPA1 receptors, creating a cool relaxing sensation,[24] while imparting little or no flavor to products that are ingested. WS-23 has been reported in JUUL™ pods purchased in the European Union,[25] but was not found in JUUL™ pods purchased in the US.[6] Bloggers have discussed the addition of coolants to e-cigarette fluids, suggesting they are more widely used than generally recognized.[26-28] However, apart from one report on JUUL™,[25] very little is known about the identities and concentrations of coolants used in e-fluids, and the range of concentrations of these coolants in JUUL™ and Puff e-cigarette has not previously been compared.

Mint oil, which is often used in e-cigarettes to create "mint" flavor, can contain pulegone,[29,30] a known carcinogen.[31,32] In several recent studies, a Margin of Exposure (MOE) analysis found pulegone to be sufficiently high in some e-cigarettes to present a cancer risk,[10,11] which motivated us to examine pulegone in JUUL™ and Puff products.

This study compares menthol, WS-3, and WS-23, and pulegone in menthol and minty-flavored products made by JUUL™ and Puff to gain insight into their relative safety. Specifically, we have compared the following: (1) concentrations of the flavor chemicals, (2) the concentrations and cytotoxicity of WS-3 and WS-23, and (3) the MOEs, which predict cancer risk.

METHODS

Sample Acquisition

In 2018 and 2019, JUUL™ "Cool Mint", "Classic Menthol", and their replacements "Mint", and "Menthol" were purchased online (www.juul.com) and from local stores in

Riverside, CA, and Portland, OR. Of the four minty/menthol-flavored pods produced by JUUL™, only "Menthol" is currently available. JUUL™ "Cool Mint", "Classic Menthol", "Mint", and "Menthol" pods were analyzed to compare chemical composition in all minty/menthol JUUL™ pods. All pods were stored in the dark and analyzed close to the time of purchase.

Two types of disposable Puff devices were purchased; the 1.3 mL Puff Bar "Menthol" labeled to deliver 300 puffs/device and the 3.2 mL Puff Plus "Cool Mint" labeled to deliver 800 puffs/device. Puff devices were purchased at vape shops in Los Angeles, CA, and Riverside, CA, in 2020. All devices were stored in the dark and analyzed close to the time of purchase.

Identification and Quantification of Chemicals Using (GC/MS)

E-cigarette fluids were extracted from the pods and devices, and 50 µL was dissolved in 0.95 mL of isopropyl alcohol (Fisher Scientific, Fair Lawn, NJ). Chemical analysis was performed with an Agilent 5975C GC/MS system (Santa Clara, CA) using internal standard-based calibration procedures and methods previously described in detail.[6,33] The method analyzes 180 flavor chemicals plus nicotine.

Culturing of BEAS-2B Cells

Human bronchial epithelial cells (BEAS-2B) from American Type Culture Collection (ATCC), Manassas, VA were cultured in a growth medium made with 500 mL of Airway Epithelial Cell Basal Medium supplemented with 1.25 mL HLL supplement containing human serum albumin (500 µg/mL), linoleic acid (0.6 µM), and lecithin (0.6 µg/mL), 15 mL of L-glutamine (6 mM), 2 mL of extract P (0.4%), and 5.0 mL Airway Epithelial Cell Supplement containing epinephrine (1.0 µM), transferrin (5 µg/ml), T3 (10 nM), hydrocortisone (0.1 µg/ml), rh EGF (5 ng/mL), and rh Insulin (5 µg/mL) from ATCC, Manassas, VA. Nunc T-25 tissue culture flasks (Thermo Scientific, Waltham, MA.) were coated overnight with a coating medium made with basal

medium (69.3%) (ATCC, Manassas, VA), collagen (29.7%) (Sigma-Aldrich, St Louis, MO), bovine serum albumin (0.99%) (Sigma-Aldrich, St Louis, MO), and fibronectin (0.01%) (Sigma-Aldrich, St Louis, MO) before culturing and passaging cells. At 85 - 90% confluency, cells were harvested using Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium (Lonza, Walkersville, MD) for washing and incubated with a trypsin solution containing Trypsin-EDTA (0.25% trypsin/0.53 mM EDTA) from ATCC, Manassas, VA, and 0.5% poly-vinyl-pyrrolidone (Sigma-Aldrich, St Louis, MO), for 3 mins at 37ºC to allow detachment. Cells were cultured in T-25 flasks at 75,000 cells/flask, and the medium was replaced every other day. Cells were then plated at 10,000 cells/well in pre-coated 96-well tissue culture plates (Thermo Scientific, Waltham, MA.) and allowed to attach overnight before a 24-hour treatment.

MTT Cytotoxicity of WS-3 and WS-23

The effects of WS-3 and WS-23 on mitochondrial reductases were evaluated in concentration-response experiments. BEAS-2B cells were seeded, allowed to attach overnight, and treated with 0.5 – 5 mg of each coolant/mL of culture medium for 24 hours at 37 ºC. After treatment, 20 µL of MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St Louis, MO) dissolved in 5 mg/mL of DPBS (Fisher Scientific, Chino, CA) were added to wells and incubated for 2 hours at 37 ºC. Solutions were removed from wells, and 100 µl of dimethyl sulfoxide (DMSO) (Fisher Scientific, Chino, CA) were added to each well and gently mixed on a shaker to solubilize formazan crystals. Absorbance readings of control and treated wells were taken against a DMSO blank at 570 nm using an Epoch microplate reader (Biotek, Winooski, VT). The MTT assay quantifies the conversion of a yellow tetrazolium salt (MTT) to purple formazan. For each coolant tested, three independent experiments on different passages of the same culture were performed.

The Margin of Exposure (MOE) Calculations for Pulegone

To assess the cancer risk associated with pulegone in pod/device fluids, the MOE was calculated using the no-observed adverse effect level (NOAEL) of pulegone and the estimated exposure dose (EED) from pods/devices. Regulatory agencies, including the FDA use the MOE to assess the cancer risk of food additives.[31] Chemicals with MOE values below 10,000 require strategies to limit exposure. The risk associated with pulegone content in JUUL™ and Puff e-cigarettes was evaluated using a daily EED of 1 – 3 mL,[34-37] a NOAEL of 13.39 mg/kg and an adult body weight of 60 kg.[31,32]

Data Analysis and Statistics

For GC/MS data, and the means and standard deviations for at least three pods/devices were plotted using Prism software (GraphPad, San Diego, CA). For the MTT assay, treatment groups were expressed as percentages of the negative control. IC_{50}s were computed using the log inhibitor vs. normalized response-variable slope in GraphPad Prism, and IC_{70}s were evaluated visually. Statistical significance in the MTT assay was determined in GraphPad using a one-way analysis of variance (ANOVA) on the raw data. When means were significant ($p < 0.05$), treated groups were compared to the untreated control using Dunnett's post hoc test.

RESULTS

Concentrations of Flavor Chemicals in JUUL™ and Puff E-cigarettes

Menthol was the dominant flavor chemical in the JUUL™ and Puff samples (concentration range 5 - 14 mg/mL) (figure 1A). Menthol concentrations were similar in all products, except Puff Bar "Menthol" in which the concentration was lower. Other flavor chemicals were generally < 1 mg/mL (figures 1B and C), except for triacetin and p-menthone, which were >1 mg/mL in Puff

Plus “Cool Mint” and Puff Bar “Menthol”, respectively (figure 1B). In JUUL™ fluids, minor flavor chemicals (< 1 mg/mL) were generally present in the two “mint” flavors from JUUL™ but absent or lower in concentration in the “menthol” flavors. Puff products had more minor flavor chemicals than JUUL™ (figures 1B and C). In Puff, minor flavor chemicals were generally higher in the “Menthol” devices (figure 1B and 1C). Estimated concentrations of flavor chemicals identified at levels below the LOQ (20 µg/mL for 50 µl samples) are shown in supplementary table S1.

WS-3 and WS-23 Concentrations in JUUL™ and Puff

While WS-3 was absent in all JUUL™ pods, WS-23 was present in JUUL™ “Menthol” pods at an average concentration of 0.1 mg/mL (figure 2A). Both coolants were in Puff fluids at much higher concentrations. WS-23 in Puff Plus “Cool Mint” averaged 36 mg/m/L with one device having 45 mg/mL of WS-23. In the other Puff groups, the average concentrations of WS-3 and WS-23 were similar and ranged between 4.3 - 7.2 mg/m/L.

Cytotoxicity of WS-3 and WS-23

The cytotoxicity of WS-3 and WS-23 was evaluated using the MTT assay in conjunction with ISO protocol #10993-5, which measures mitochondrial reductase activity (figure 2B).[38] BEAS-2B cells were tested using concentrations of coolant that were lower than those found in the e-cigarettes. While concentrations of WS-3 below 5 mg/mL produced little to no response in the MTT assay, BEAS-2B cells were adversely affected by all concentrations of WS-23 that were tested (IC_{70} = 0.59).

Hazard Analysis of Pulegone in JUUL™ and Puff E-Cigarettes

The concentrations of pulegone in JUUL™ pods and disposable Puff fluids ranged from 0.002 – 0.2 mg/mL and were higher in the "mint" labeled products (figure 1). For "menthol" products from both manufacturers, only the 3 mL/day exposure scenario for Puff Bar "Menthol" generated a MOE < 10,000, which is below the safety threshold (figure 3A). In contrast, for all "mint" flavored samples, most scenarios produced a MOE < 10,000 (figure 3B). For all scenarios for both mint and menthol-flavored products, the MOEs for Puff were consistently lower than those for JUUL™, suggesting a greater risk with Puff.

Concentrations of Flavor Chemicals in Edible Consumer Products

Synthetic coolants and menthol in edible consumer goods were compared to concentrations in JUUL™ and Puff e-cigarette fluids (figure 4). Concentrations of menthol in JUUL™ and Puff were similar but between 14 to 543 times higher than in other consumer products (figure 4A). WS-23 in Puff was 450 times higher than concentrations in JUUL™ pods, and 23 to 4500 times higher than the concentration in edible consumer products (figure 4B). WS-3, which was absent in JUUL™ pods, was 2 to 688 times higher in Puff when compared to edible products (figure 4C).

DISCUSSION

Four main observations come from our comparison of three classes of chemicals in JUUL™ and Puff e-cigarettes. First, in both brands, menthol was the dominant flavor chemical in mint and menthol-flavored fluids, which likely have similar, although not identical, minty flavors. Secondly, while low concentrations of WS-23 were present in JUUL™ "Classic Menthol", both WS-3 and WS-23 were present at much higher concentrations in Puff products with the concentration of WS-23 exceeding that of menthol in Puff Plus "Cool Mint". Third, WS-23 was cytotoxic in the MTT assay at concentrations well below those found in Puff

devices. Fourth, pulegone concentrations in mint products from JUUL™ and Puff were high enough to present a cancer risk based on MOE evaluations. While the FDA flavor ban has reduced sales of JUUL™ to minors, young users appear to have rapidly adopted other brands, such as Puff,[22,] which has high concentrations of WS-23 and concerning levels of pulegone. Ironically, the flavor ban may have caused youth to migrate to a potentially more harmful e-cigarette.

Since the dominant flavor chemical in mint and menthol-flavored JUUL™ and Puff products was menthol, banning the sale and distribution of mint-flavored pods may not adequately address the widespread use of this popular flavor. While current federal regulations limit the distribution and sale of flavored cartridge-based pod products, such as JUUL™, they do not solve the problem that "menthol" flavored e-cigarettes are apparently similar, although not identical to "mint." Consequently, a minty flavor is still sold by JUUL™ as "Menthol" and is also available as "mint" in disposable devices from other manufacturers, such as Puff. Although our study deals only with JUUL™ and Puff, any e-cigarette manufacturer can produce menthol-flavored pods or cartridges that may be an acceptable substitute for "mint."

FEMA has designated menthol and synthetic coolants (WS-3 and WS-23) as GRAS (generally regarded as safe) for ingestion, and they are widely used in food and cosmetic products.[17] As pointed out previously, the concentrations of flavor chemicals in e-cigarettes are often very high.[14,39] Menthol and WS-23 concentrations in both brands exceeded those used in most edible consumer products (figure 4).[22,23] While acceptable exposure to GRAS chemicals is based on ingestion data, the acceptable exposures when inhaled are generally unknown and are likely to be much lower, [40,41] raising concerns about the delivery of coolants in e-cigarettes.

Unlike the US, several countries (Canada and Germany) have avoided potential problems with coolants by banning their use in tobacco products. [42,43]

The concentrations of menthol in JUUL™ and Puff are high enough to affect cell health. In numerous studies with various cell types, menthol inhibited proliferation and/or caused cell death.[44,45] Menthol concentrations in JUUL™ and Puff would be cytotoxic in the MTT assay based on prior reports with BEAS-2B cells (IC_{70} = 1.38 mg/mL) and A549 cells (IC_{50} = 0.98 mg/mL – aerosol data).[14,18] Even at concentrations below the MTT NOAEL, menthol, when delivered in a PG aerosol using an e-cigarette, binds to TRPM8 receptors on BEAS-2B cells allowing calcium influx and downstream activation of oxidative stress and inflammatory responses.[46] The reported adverse effects of menthol in humans have generally been derived from studies comparing mentholated vs. non-mentholated tobacco cigarettes and have ranged from it being an irritant to causing cancer, although the data supporting the latter claim have been ambiguous.[44] In 2011, it was concluded by the FDA's Tobacco Products Scientific Advisory Committee (TPSAC) that menthol is not a carcinogen.[47] Nevertheless, the inhalation of menthol does have an effect on humans. For example, inhalation of a high dose of menthol by a 13-year-old boy resulted in adverse central nervous system effects.[48] Workers in a throat lozenge manufacturing plant reported that menthol was an irritant that affected their eyes, nasal passages, throats and larynxes.[49] Ingestion of menthol at high doses has resulted in abdominal discomfort, convulsions, nausea, vertigo, ataxia, drowsiness and coma. [49,50] In future studies, it will be important to determine if the high concentrations of menthol inhaled in the context of EC aerosols produce health effects that have not yet been recognized.

High concentrations of WS-23 and WS-3 appeared in our EC fluid data for the first time in Puff and are likewise concerning, as they produce cytotoxic effects in the MTT assay at

concentrations below those in Puff e-cigarettes. In contrast, the concentration of WS-23 in JUUL™ "Classic Menthol" was not high enough to produce an IC_{70} in the MTT assay. The cytotoxicity that could be ascribed to menthol in the six products we tested would be roughly equivalent. However, the toxicity ascribable to WS-23 would be many times greater in the Puff products than in JUUL™, suggesting that the removal of most JUUL™ flavors inadvertently motivated users to try other products, such as Puff, that may be more harmful.

Pulegone in EC fluids is a concern because of its known carcinogenicity.[31,32] Our data are based on acute exposures and do not directly assess the long-term effects of e-cigarette chemicals on human health. Calculation of the MOE enables a prediction to be made about the possibility of cancer developing with long-term exposure to individual chemicals and is useful to regulatory agencies in prioritizing their cancer risk.[31,51-53] As MOE values fall below 10,000, the possibility of cancer developing increases. Products labeled "menthol" had concentrations of pulegone that produced MOEs above 10,000, indicating they are not likely to cause cancer in users. However, Puff Bar "Menthol" was much closer to the 10,000 cut off than the JUUL™ products, which ranged from 100,000 to >300,000. In contrast, products labeled "mint" generally had MOEs below 10,000, and in all cases, MOEs for Puff were lower than those for JUUL™. These data are consistent with the interpretation that the mint products were flavored with mint oil, which usually contains pulegone,[29,30] while menthol-flavored products were likely made from crystalline menthol, which would have higher purity and lower concentrations of pulegone. These data support the idea that using pure menthol rather than mint oil in e-fluids would reduce the risk of developing cancer, which could provide a basis for the regulation of additives to mint/menthol-flavored products. Since our MOE calculations are based on pulegone ingestion,

our values probably underestimate inhalation exposure, which generally produces a stronger effect to toxicants, including carcinogens.[40,41]

Our data are based on concentrations of chemicals in e-liquids, which we have previously shown generally correctly predicts the cytotoxicity of aerosols.[18] The concentrations of flavor chemicals and coolants received by a user will depend on the transfer efficiency of each chemical to the aerosol and its retention by the user. Therefore, the actual doses inhaled during vaping may be lower than the concentrations we report in the e-liquid. The frequency of vaping will also affect the overall exposure a user receives. These factors will eventually need to be determined to understand the concentrations of flavor chemicals, coolants fully, and pulegone users of JUUL™ and Puff products receive.

In summary, flavor chemicals in JUUL™ "Cool Mint," "Mint," "Classic Menthol," and "Menthol," and in Puff Plus "Cool Mint" and Puff Bar "Menthol" were similar, but not identical, with menthol being the dominant flavor chemical in all products tested. Synthetic coolants are being added to e-cigarettes, sometimes at high concentrations that exceed those used in other consumer products and produced in vitro cytotoxicity. Regulation of mentholated e-cigarettes is now complicated by the sale of "mint-like" flavors under the name "menthol," the lack of regulation of flavor chemicals in disposable e-cigarettes, the presence of cytotoxic concentrations of synthetic coolants in menthol and mint e-cigarettes, and the presence of pulegone in mint-flavored products at concentrations that may be a cancer risk.

What This Paper Adds

- We compared the flavor chemicals, coolants (WS-3 and WS-23), and pulegone in mint and menthol-flavored Puff (disposable) and JUUL™ (pod) e-cigarettes.

- Menthol was the dominant flavor chemical in all products suggesting users may interchange mint and menthol products to achieve a "minty" flavor.
- Unlike JUUL™, Puff products contained cytotoxic concentrations of the synthetic coolant WS-23 and concentrations of pulegone that present a greater cancer risk based on MOE analysis.
- Restriction of JUUL™ flavors may have inadvertently caused a migration of users to a potentially more harmful product.
- The use of pure menthol instead of mint oil in e-cigarette fluids may reduce cancer risk.

Contributors. EEO and PT formed the conception and design of the study. WL, KJM, and JFP were involved in the GC/MS analysis. EEO performed the cell culture experiment. EEO, WL, KJM, and PT were involved in the data analysis and interpretation. EEO and PT drafted the manuscript. All authors critically reviewed, edited, and approved the final manuscript.

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Competing Interests. None declared

Ethics approval. Not required

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Figure Legend

Figure 1. Flavor chemicals in JUUL™ and Puff "mint" and "menthol" e-cigarette fluids. (A) Menthol was the dominant flavor chemical in all six products. (B) Chemicals present at concentrations ranging 0.1 - 2 mg/mL. (B) Chemicals present at concentrations lower than 0.1 mg/mL. Data are means ± the standard deviations of at least three samples for each group.

Figure 2. Synthetic coolant concentrations in e-cigarette fluids and their toxicities. (A) WS-23 and WS-3 were higher in Puff fluids than in JUUL™ pods. (B) Cytotoxicity of WS-3 and WS23 in the MTT assay. Data are the means ± the standard deviations of at least three independent biological experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 3. The Margin of Exposure (MOE) for pulegone in JUUL™ and Puff products. (A) MOE for "menthol" labeled JUUL™ and Puff e-cigarette fluids. (B) MOE for "mint" labeled JUUL™ and Puff e-cigarette fluids. MOEs below the threshold of 10,000 indicate a high carcinogenic potential and concern for human health.

Figure 4. Concentrations of flavor chemicals in JUUL™ and Puff e-cigarette fluids and edible consumer products. (A) Menthol. (B) WS-23 (C) WS-3.