

## Original Investigation

# Chemical Adducts of Reactive Flavor Aldehydes Formed in E-Cigarette Liquids Are Cytotoxic and Inhibit Mitochondrial Function in Respiratory Epithelial Cells

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## Abstract

**Introduction:** Flavor aldehydes in e-cigarettes, including vanillin, ethyl vanillin (vanilla), and benzaldehyde (berry/fruit), rapidly undergo chemical reactions with the e-liquid solvents, propylene glycol, and vegetable glycerol (PG/VG), to form chemical adducts named flavor aldehyde PG/VG acetals that can efficiently transfer to e-cigarette aerosol. The objective of this study was to compare the cytotoxic and metabolic toxic effects of acetals and their parent aldehydes in respiratory epithelial cells.

**Aims and Methods:** Cell metabolic assays were carried out in bronchial (BEAS-2B) and alveolar (A549) epithelial cells assessing the effects of benzaldehyde, vanillin, ethyl vanillin, and their corresponding PG acetals on key bioenergetic parameters of mitochondrial function. The potential cytotoxic effects of benzaldehyde and vanillin and their corresponding PG acetals were analyzed using the LIVE/DEAD cell assay in BEAS-2B cells and primary human nasal epithelial cells (HNEpC). Cytostatic effects of vanillin and vanillin PG acetal were compared using Click-iT EDU cell proliferation assay in BEAS-2B cells.

**Results:** Compared with their parent aldehydes, PG acetals diminished key parameters of cellular energy metabolic functions, including basal respiration, adenosine triphosphate production, and spare respiratory capacity. Benzaldehyde PG acetal (1–10 mM) increased cell mortality in BEAS-2B and HNEpC, compared with benzaldehyde. Vanillin PG acetal was more cytotoxic than vanillin at the highest concentration tested while both diminished cellular proliferation in a concentration-dependent manner.

**Conclusions:** Reaction products formed in e-liquids between flavor aldehydes and solvent chemicals have differential toxicological properties from their parent flavor aldehydes and may contribute to the health effects of e-cigarette aerosol in the respiratory system of e-cigarette users.

**Implications:** With no inhalation toxicity studies available for acetals, data from this study will provide a basis for further toxicological studies using *in vitro* and *in vivo* models. This study suggests that manufacturers' disclosure of e-liquid ingredients at time of production may be insufficient to

inform a comprehensive risk assessment of e-liquids and electronic nicotine delivery systems use, due to the chemical instability of e-liquids over time and the formation of new compounds.

## Introduction

In the past decade, the United States has seen a rapid increase in the use of electronic cigarettes (e-cigarettes), both by smokers and individuals who have never smoked, especially among youth (middle and high school children) and young adults. As per 2019 National Youth Tobacco Survey (NYTS) data, one in three current youth users were frequent (>20 days use) users and majority of them reported to use JUUL e-cigarettes.<sup>1</sup> Among e-cigarette users, and especially among youths, there is a widespread misperception of harm resulting from e-cigarette use and their ingredients, which is influencing higher initiation rates.<sup>1</sup> Though e-cigarettes aerosol contains lower amounts of the harmful chemicals in combustible cigarette smoke, users are still exposed to significant amounts of toxicants, especially reactive carbonyl free radicals, heavy metals, nitrosamines, and particulate matter.<sup>2-7</sup> Chronic inhalation and exposure of e-cigarette aerosol in animal and humans led to malfunction of innate immune and inflammatory responses to pulmonary pathogenic infections, impaired airway mucociliary clearance and cardiovascular function, DNA damage and mutational susceptibility in lung and bladder cells.<sup>8-19</sup>

A major factor that is influencing a rapid increase in e-cigarette initiation and use is the availability of several thousands of unique appealing flavors.<sup>20</sup> Amounts of the flavor chemicals added has a wide range, up to molar concentrations, with flavor amounts as high as ~34% being reported in a cinnamon-flavored e-liquid.<sup>20-22</sup> *In vitro* exposure of lung epithelial cells and immune cells to flavored e-liquid or aerosols has been demonstrated to lead to cytotoxicity.<sup>21,23-28</sup> Further, flavors can exacerbate inflammatory responses and changes in lung function mechanics in mice and humans following e-cigarette aerosol exposure.<sup>29-31</sup>

While many of the flavorants added to e-cigarette liquids have GRAS (generally recognized as safe) status for addition to foods, this designation does not apply to the inhalation of these compounds.<sup>32</sup> In fact, some flavor chemicals such as benzaldehyde, diacetyl, or cinnamaldehyde are known respiratory health hazards, with permissible exposure levels issued by the U.S. Occupational Safety and Health Administration (OSHA).<sup>33</sup> More importantly, for the majority of the flavorants in e-liquids, no inhalation toxicology data are available.

A frequently used class of e-cigarette flavor chemicals is aldehydes, which include compounds such as vanillin, ethyl vanillin, benzaldehyde, cinnamaldehyde, and their derivatives.<sup>21,34</sup> In addition to acting as odorants, these flavor aldehydes are also sensory and respiratory irritants, activating the respiratory irritant receptor TRPA1 expressed in the sensory nerves innervating the upper and lower airways.<sup>35-38</sup> Aldehydes are generally reactive and can form adducts through covalent bonding with cellular proteins, nucleic acids, and other biomolecules, causing cellular and metabolic toxicities.<sup>35,39</sup> Flavor aldehydes such as vanillin, ethyl vanillin, and cinnamaldehyde were demonstrated to be cytotoxic in bronchial epithelial cells, to suppress respiratory immune responses, and to disrupt airway ciliary motility and mitochondrial function.<sup>9,21,23,25,26,40-43</sup>

We recently demonstrated that several flavor aldehydes including vanillin, ethyl vanillin, and benzaldehyde form chemical adducts with the major e-liquid solvents, propylene glycol (PG), and vegetable

glycerol (VG), under storage conditions and at room temperature, generating stable flavor aldehyde PG/VG acetals.<sup>37</sup> Chemical analysis of flavor aldehyde containing e-liquids and aerosols, including JUUL products, revealed the presence of PG/VG acetals of several flavor aldehydes.<sup>20-22,34,37,43,44</sup> Analyzing 277 commercial e-liquids for their total flavor content, Omaiye et al., found benzaldehyde PG acetal in 118 e-liquids, indicating widespread presence in commercial e-liquids.<sup>21</sup> Vanillin and ethyl vanillin are among the top 10 most frequently used e-liquid flavorings, suggesting the formation and widespread presence of corresponding acetals in these e-liquids.<sup>21,22,37</sup> We recently demonstrated that benzaldehyde and vanillin PG acetal activate sensory irritant receptors more strongly and potently than the parent aldehydes, suggesting they might be respiratory irritants.<sup>37</sup>

Because of their widespread presence in popular e-liquids and their increased irritancy, we hypothesized that flavor aldehyde PG acetals may be more toxic in the respiratory system than their parent aldehydes. To address this we examined and compared the effects of major flavor aldehydes and their corresponding PG acetals on several respiratory cell types, BEAS-2B cells, a human bronchial epithelial cell line; HNEpC, primary human nasal epithelial cells isolated from normal human nasal mucosa; and, A549 cells, a human alveolar epithelial cell line, using (1) a metabolic toxicity assay to monitor changes in oxygen consumption rates (OCRs) and assess changes in key parameters of mitochondrial respiration (basal, respiration, adenosine triphosphate (ATP) production, and respiratory capacities) for benzaldehyde, vanillin, ethyl vanillin, and their acetals, (2) the fluorescent LIVE/DEAD cell assay (benzaldehyde, vanillin, and their acetals), and (3) Click-iT EDU cell proliferation assay (benzaldehyde, vanillin, and their acetals).

## Materials and Methods

### Chemicals

Benzaldehyde ( $\geq 99.5\%$  purity), benzaldehyde PG acetal ( $\geq 95\%$ ), vanillin (99%), ethyl vanillin ( $\geq 98\%$ ), and cinnamaldehyde (99%) were all purchased from Sigma-Aldrich (Saint Louis, MO). Vanillin PG acetal (97%) and ethyl vanillin PG acetal (98%) were obtained from Bedoukian Research (Danbury, CT).

### Bronchial Epithelial Cell Culture (BEAS-2B)

Experiments in this study were conducted using BEAS-2B cells (RRID: CVCL\_0168), A549 cells (RRID: CVCL\_0023), and HNEpC. These are respiratory epithelial cell types widely used as *in vitro* cell culture models. BEAS-2B and A549 cell lines were purchased from Duke Cell Culture Facility (Source: ATCC, Manassas, VA) and HNEpC from PromoCell GmbH (Heidelberg, Germany). BEAS-2B cells were cultured in BEGM Bronchial Epithelial Cell Growth Medium BulletKit (Lonza, Walkersville, IL) containing BEGM basal medium and SingleQuots supplements and growth factors. Cells were plated onto flasks and plates coated with a mixture of fibronectin (0.01 mg/mL; Sigma-Aldrich, Saint Louis, MO), bovine collagen type I (0.03 mg/mL; Sigma-Aldrich) and bovine serum albumin (0.01 mg/mL; Sigma-Aldrich) dissolved in cell culture medium and overnight incubated at 37°C in a 5% CO<sub>2</sub> and

95% humid atmosphere. A549 cells were cultured in F-12K medium supplemented with 10% fetal bovine serum and 100 units/mL penicillin and 0.1 mg/mL streptomycin. HNEpC were cultured in Airway Epithelial Cell Growth Medium (PromoCell) that contained growth supplements, growth factors and antibiotic-antimycotic mix (10 000 units/mL of penicillin, 10 000 µg/mL of streptomycin, and 25 µg/mL of Amphotericin B; ThermoFisher Scientific, Waltham, MA).

### Live-Cell Mitochondrial Respiration Analysis

Live-cell metabolic assay, Seahorse XF Cell Mito Stress Test (Agilent Technologies, Santa Clara, CA), was used to assess the effects of benzaldehyde, vanillin, ethyl vanillin, and their corresponding PG acetals on mitochondrial function of BEAS-2B and A549 cells. The test was conducted by using a Seahorse XFe96 Extracellular Flux Analyzer (Agilent Technologies) as per manufacturer protocol to obtain the effects of tested chemicals on multiple parameters of mitochondrial function, such as basal respiration, ATP production-coupled respiration, maximal respiration, and spare respiration capacity. A549 cells and BEAS-2B cells were plated in XF96 cell culture microplates (~15 000 cells/well for A549; ~25 000 cells/well in pre-coated plates for BEAS-2B) and allowed to grow until they reach near full confluence. For calibration, Xfe96 sensor cartridges used in the assay were hydrated the night before the assay day in SeahorseXF Calibrant solution at 37°C. Just before starting the Seahorse assay, complete growth medium for the cells in the plate was replaced with XF assay media. Plates were subsequently incubated at 37°C without CO<sub>2</sub> for ~1 hour. Working solutions of drugs that are injected to study key parameters of mitochondrial respiration were freshly prepared just before the assay by diluting stock solutions in XF assay media. These included (1) oligomycin for ATP production and proton-leak; (2) FCCP (carbonyl cyanide-4-phenylhydrazone) for maximal respiration and spare respiratory capacity (SRC); and (3) rotenone and antimycin A for non-mitochondrial respiration. The assay protocol was set up to inject chemicals in the following order (Figure 1; Supplementary Figure S1A). (1) Either vehicle control (assay media, dimethyl sulfoxide, PG) or positive control (3 mM cinnamaldehyde) or flavor aldehydes (1, 3, and 5 mM) or their corresponding PG acetals (1, 3, and 5 mM); (2) 1 µM oligomycin; (3) FCCP (0.5 µM for BEAS-2B and 1 µM for A549 cells); and (4) 0.75 µM rotenone and 1.5 µM antimycin A. Data were normalized to basal OCR (average of first three readings before injection A). As per manufacturer's recommendations, changes in OCR were calculated to measure the various parameters of mitochondrial function. Graphs representing these changes ( $\Delta$ OCR) for various parameters and associated statistical analysis (Student's *t* tests between flavor aldehyde and corresponding PG acetal) were generated using GraphPad Prism7 (La Jolla).

### Live/Dead Cell Staining Assay

Cellular cytotoxicity of benzaldehyde, vanillin, and their corresponding PG acetals on BEAS-2B cells and HNEpC was determined using the LIVE/DEAD Viability/Cytotoxicity Kit (ThermoFisher Scientific). LIVE/DEAD cell assay reagents Calcein AM and Ethidium homodimer (EthD-1) were used to assess the live and dead cell populations, respectively. Indicator concentrations of 2 µM Calcein AM and 4 µM of EthD-1 were used for fluorescence microscopy, as suggested in the manufacturer's protocol. BEAS-2B cells and HNEpC were plated onto 96-well clear bottomed, black-well culture plates (coated plates for BEAS-2B) at

a density of 18 000 cells/well. After cells reached acceptable cell densities (60%–70% confluence), cells were exposed to various concentrations (1–10 mM) of either flavor aldehydes (benzaldehyde and vanillin) or their corresponding PG acetals for 24 hours. Chemical solutions were freshly prepared just before the addition to cells. After 24 hours of exposure, cells were gently washed twice with phosphate-buffered saline (100 µL/well/wash) and 50 µL of the dye mixture was added and subsequently incubated with the dye solutions for 30–45 minutes at room temperature. After the incubation period, fluorescence images of the experimentally treated and untreated control cells were taken with an EVOS FL Auto microscope (ThermoFisher) using green fluorescent protein (470/22 Ex; 510/42 Em) and RFP (531/40 Ex; 593/40 Em) light cubes for imaging Calcein AM (live) and EthD-1 (dead) positive cells, respectively. ImageJ (NIH, Bethesda, MD; RRID: SCR\_003070) was used to quantify number of live, dead, and total cells in each image and cytotoxicity was measured by calculating the percentage of dead cells. To assess and compare the influence of the tested compounds on cell growth, these live/dead cell assay images were utilized and the covered cellular surface area (% cellular surface area) was calculated using ImageJ (NIH). Graphs representing cellular cytotoxicity (% dead cells and % cellular surface area) and associated statistical analysis (Student's *t* tests) were generated using GraphPad Prism7 (La Jolla, CA; RRID: SCR\_000306).

### Click-iT Edu Cell Proliferation Assay

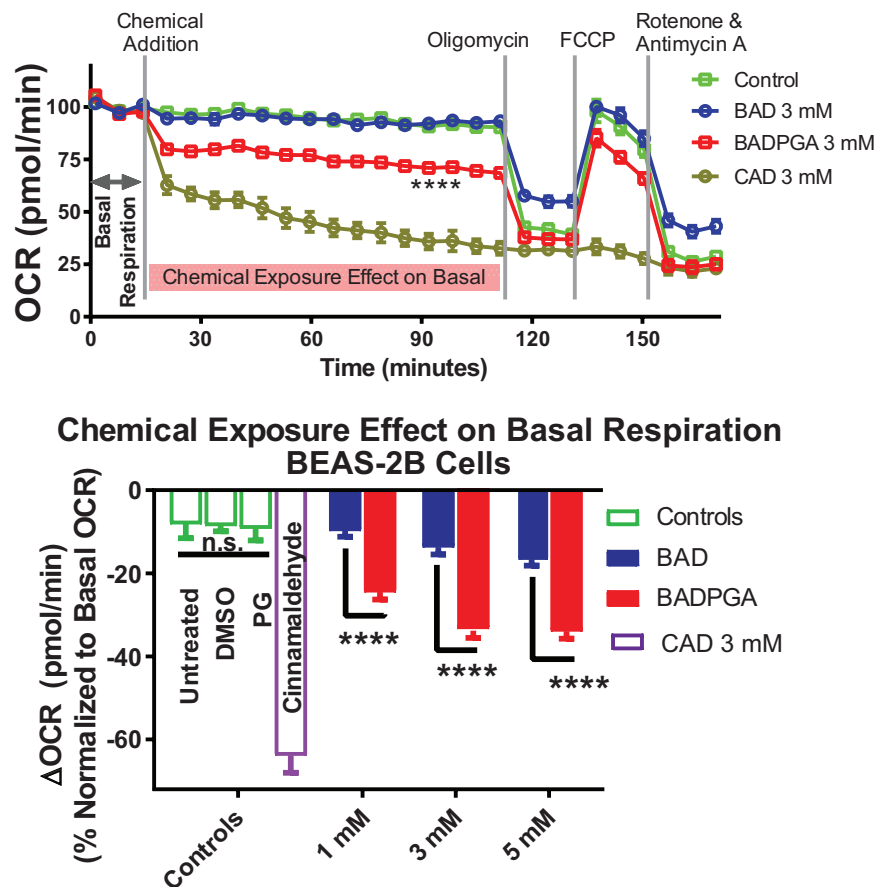
Impact of vanillin and vanillin PG acetal on cell proliferation was determined in BEAS-2B cells using the Click-iT Edu HCS cell proliferation assay (ThermoFisher Scientific). This assay detects newly synthesized DNA, where EdU (5-ethynyl-2'-deoxyuridine), a nucleoside analog of thymidine is incorporated into DNA during active DNA synthesis and can be detected by a alkyne-azide click reaction between Edu's alkyne and an azide containing Alexa Fluor dye. Cells were labeled with an optimized Edu concentration of 10 µM for fluorescence microscopy, as suggested in the manufacturer's protocol. BEAS-2B cells were plated in 96-well plates as described above in the Live/Dead cell assay and grown to 50%–60% confluency. Cells were then exposed to various concentrations (0.3–10 mM) of vanillin and vanillin PG acetals for 24 hours in the presence of 10 µM Edu. After 24 hours of exposure, cells were gently washed twice with phosphate-buffered saline (100 µL/well/wash), fixed with 3.7% paraformaldehyde for 15 minutes and subsequently permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 15 minutes. For Edu detection, Alexa Fluor 488 azide containing Click-iT reaction cocktail (prepared as per manufacturer's protocol) is added to each well and incubated for 30 minutes. Subsequently, cells were rinsed with rinse buffer and counterstained by a HCS NuclearMask Blue stain for 30 minutes to register for the total cells. Click-iT Alexa Fluor 488 labeled Edu nuclear intensity was quantified using a FlexStation III benchtop scanning fluorometer chamber (Ex: 495; Em: 519 nm) (Molecular Devices, San Jose, CA). Next, fluorescence images of the experimentally treated and untreated control cells were taken with an EVOS FL Auto microscope (ThermoFisher) using green fluorescent protein (470/22 Ex; 510/42 Em) and DAPI (4',6-diamidino-2-phenylindole; Ex: 357/44; Em: 447/60) light cubes for detecting Edu (DNA synthesis/cell proliferation) and HCS NuclearMask Blue stain (total cells) positive cells, respectively. Graphs representing cell proliferation and associated statistical analysis (Student's *t* tests) were generated using GraphPad Prism7 (La Jolla, CA; RRID: SCR\_000306).

## Results

### Effects of Flavor Aldehyde PG Acetals on Mitochondrial OCR in Lung Epithelial Cells

Flavor aldehydes such as benzaldehyde, cinnamaldehyde, and ethyl vanillin were shown to compromise mitochondrial function in human airway epithelial cells and immune cells.<sup>9,41,42</sup> To investigate whether the PG adducts of these aldehydes would also affect mitochondrial respiration and other cellular metabolic mechanisms, we compared the acute effects of flavor aldehyde PG acetals and their parent aldehydes on OCRs of BEAS-2B and A549 cells, using the Seahorse XF Cell Mito Stress Test assay (Supplementary Figure S1A). Exposure of BEAS-2B and A549 cells to various concentrations (1, 3, and 5 mM) of benzaldehyde tested did not affect basal respiration, with no significant change in OCR compared with untreated (basal) cells, except at the highest tested concentrations (5 mM) in A549 cells (Figure 1; Supplementary Figure S2; Supplementary Tables S1 and S2). In contrast, exposure of BEAS-2B cells to benzaldehyde PG acetal at the same concentrations (1, 3, and 5 mM) reduced OCR in a concentration-dependent manner (1 mM:  $-9.8 \pm 1.5$  vs.  $-24.5 \pm$

$1.8^{***}$ ; 3 mM:  $-13.7 \pm 1.7$  vs.  $-33.8 \pm 1.7^{***}$ ; 5 mM:  $-16.7 \pm 1.5$  vs.  $-33.8 \pm 1.7^{***}$ ) (Figure 1; Supplementary Table S1). In A549 cells, benzaldehyde PG acetal also reduced OCR (1 mM:  $0.36 \pm 2.6$  vs.  $-0.83 \pm 1.9^{ns}$ ; 3 mM:  $-1.4 \pm 2.4$  vs.  $-8.2 \pm 2.3^{**}$ ; 5 mM:  $-5.7 \pm 2.9$  vs.  $-19.4 \pm 1.3^{***}$ ) (Supplementary Figure S2; Supplementary Table S2). Similar experiments were performed for vanillin, ethyl vanillin, and their corresponding acetals (Supplementary Figures S1 and S2; Supplementary Table S1 and S2). Exposure of BEAS-2B and A549 cells to various concentrations (1, 3, and 5 mM) of vanillin reduced OCR in a concentration-dependent manner (Supplementary Figures S1B, D and S2; Supplementary Table S1 and S2). However, exposure of the cells to vanillin PG acetal reduced OCR more robustly than vanillin (BEAS-2B, 1 mM:  $-16.7\% \pm 1.6$  vs.  $-27.5\% \pm 3.1^{**}$ ; 3 mM:  $-29.9\% \pm 2.6$  vs.  $-46.7\% \pm 2.7^{***}$ ; 5 mM:  $-44.5\% \pm 1.9$  vs.  $-57.7\% \pm 1.3^{***}$ ) (A549: 1 mM:  $-3.0\% \pm 1.8$  vs.  $-6.3\% \pm 1.5^{ns}$ ; 3 mM:  $-16.9\% \pm 1.5$  vs.  $-26.8\% \pm 1.8^{**}$ ; 5 mM:  $-30.5\% \pm 1.9$  vs.  $-53.7\% \pm 2.3^{***}$ ), in a concentration-dependent manner (Supplementary Figures S1B, D and S2; Supplementary Tables S1 and S2). Ethyl vanillin also caused a decline in basal OCR in a concentration-dependent manner, with ethyl vanillin PG acetal



**Figure 1.** Effects of benzaldehyde and benzaldehyde PG acetal on mitochondrial basal respiration in BEAS-2B cells. Seahorse XF Cell Mito Stress Test assay was performed to measure changes in mitochondrial function. Following a 15-minute baseline measurement of oxygen consumption rate (OCR), bronchial epithelial cells were exposed to either benzaldehyde or benzaldehyde PG acetal for the next 90 minutes to determine the effect of chemical exposure on basal respiration. (Top) Representative time–response recordings of OCR for BEAS-2B cells exposed to 3 mM concentrations of either benzaldehyde or benzaldehyde PG acetal. Cinnamaldehyde (CAD) (3 mM) was employed as positive control. Data were normalized to basal OCR (timepoints before addition of any chemical). (Bottom) Maximal change in basal OCR ( $\Delta$ OCR) after 90-minute exposure was compared between responses to benzaldehyde or benzaldehyde PG acetal, tested at three different concentrations (1, 3, and 5 mM). The Seahorse XF Cell Mito Stress Test assay was repeated at least four times ( $n = 4$  or  $5$ ) and each assay had 3–8 replicates for each chemical exposure. Data represent mean  $\pm$  SEM of normalized  $\Delta$ OCR. Stars indicate statistical significance as compared with corresponding flavor aldehyde and determined by unpaired  $t$  test ( $****p < .0001$ ). PG = propylene glycol.

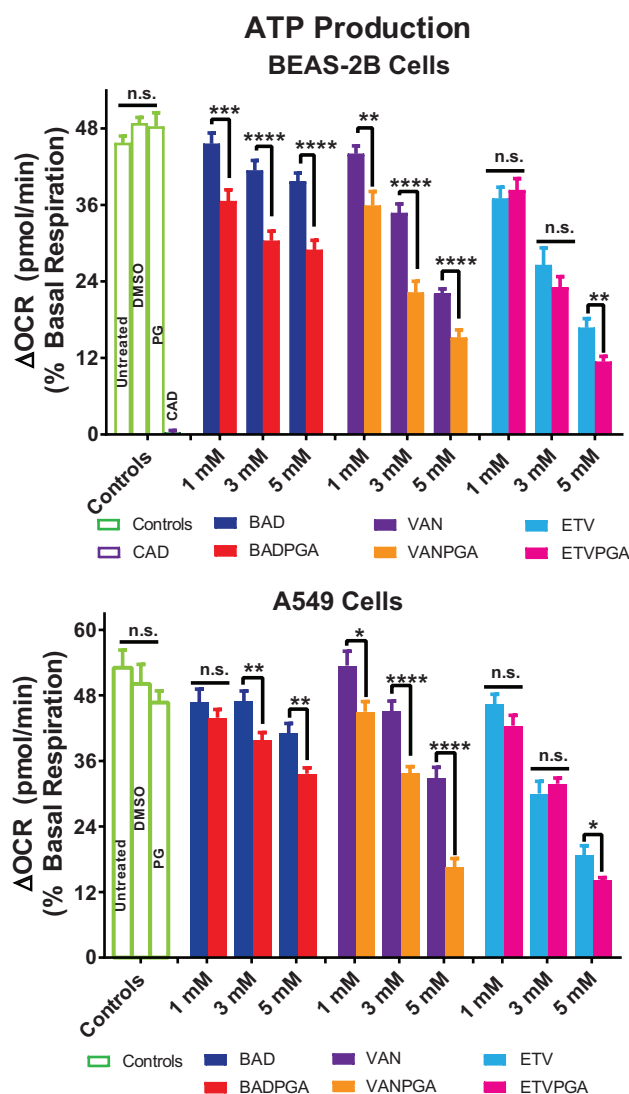
causing a further reduction at the two highest concentrations tested (3 and 5 mM) for BEAS-2B cells (1 mM:  $-20.4\% \pm 2.6$  vs.  $-22.8\% \pm 2.1$ ; 3 mM:  $-38.0\% \pm 3.4$  vs.  $-47.2\% \pm 2.7^*$ ; 5 mM:  $-49.8\% \pm 2.2$  vs.  $-57.3\% \pm 2.1^*$ ) (Supplementary Figure S1C and D; Supplementary Table S1) and at 5 mM for A549 cells (1 mM:  $-14.1\% \pm 0.7$  vs.  $-9.9\% \pm 1.9$ ; 3 mM:  $-32.1\% \pm 1.7$  vs.  $-28.5\% \pm 2.4$ ; 5 mM:  $-42.5\% \pm 2.1$  vs.  $-57.5\% \pm 1.3^{***}$ ) (Supplementary Figure S2; Supplementary Table S2). These data clearly demonstrate that flavorant PG acetals have differential effects on mitochondrial OCR during basal respiration, compared with their parent aldehydes, with all tested acetals reducing mitochondrial function more strongly.

### Differential Effects of Flavor Aldehyde PG Acetals Exposure on ATP Production in Bronchial Epithelial Cells

Mitochondrial production of ATP is critical for a wide range of cellular processes in lung epithelial cells, especially for ciliary beating and surfactant production. Diminished ATP production capabilities in lung epithelial cells will increase defenses against reactive oxygen species and cause cellular stress and cytotoxicity. We compared the effects of PG acetals exposure on ATP production in BEAS-2B and A549 cells with those of the parent aldehydes. Benzaldehyde and vanillin (3 and 5 mM), and ethyl vanillin (1, 3, and 5 mM) significantly reduced ATP production in BEAS-2B cells (Figure 2; Supplementary Table S1). Benzaldehyde and vanillin (5 mM), and ethyl vanillin (3 and 5 mM) also significantly reduced ATP production in A549 cells (Figure 2; Supplementary Table S2). In comparison, for both BEAS-2B cells and A549, the corresponding PG acetals of benzaldehyde and vanillin reduced ATP production, more potently and in a concentration-dependent manner, than parent aldehydes, namely benzaldehyde and vanillin, respectively (Figure 2; Supplementary Tables S1 and S2). Ethyl vanillin PG acetal exposure also reduced ATP production more strongly than ethyl vanillin, albeit only at the highest concentration tested (Figure 2; Supplementary Tables S1 and S2). Taken together, these results demonstrate that though flavor aldehydes reduced mitochondrial ATP synthesis in bronchial epithelial cells, their corresponding PG acetals compromised the ATP synthesis more strongly than their parent aldehydes.

### Effects of Flavor Aldehyde PG Acetals on Maximal and Spare Respiratory Capacities in BEAS-2B Cells

A decrease in the respiratory capacity of cells is a critical indicator for mitochondrial dysfunction. Spare respiratory capacity (SRC) is calculated as the difference in maximal respiration and basal respiration and indicates the ability of a cell to respond to increased energy demand, such as necessary for cell division and differentiation, cellular stress responses, and cell fate determination. We examined the effects of flavor aldehydes and their PG acetals on maximal respiration and SRC of BEAS-2B and A549 cells. Treatment with various concentrations of benzaldehyde did not change either the maximal respiration or the SRC of BEAS-2B or A549 cells. In contrast, exposure to benzaldehyde PG acetal reduced both maximal respiration and SRC in a concentration-dependent manner in BEAS-2B cells, but not in A549 cells (Figure 3A and B; Supplementary Figure S3A and B; Supplementary Tables S1 and S2). Treatment with 3 and 5 mM vanillin and ethyl vanillin reduced maximal respiration and SRC in BEAS-2B cells and treatment with 5 mM vanillin and 3 and 5



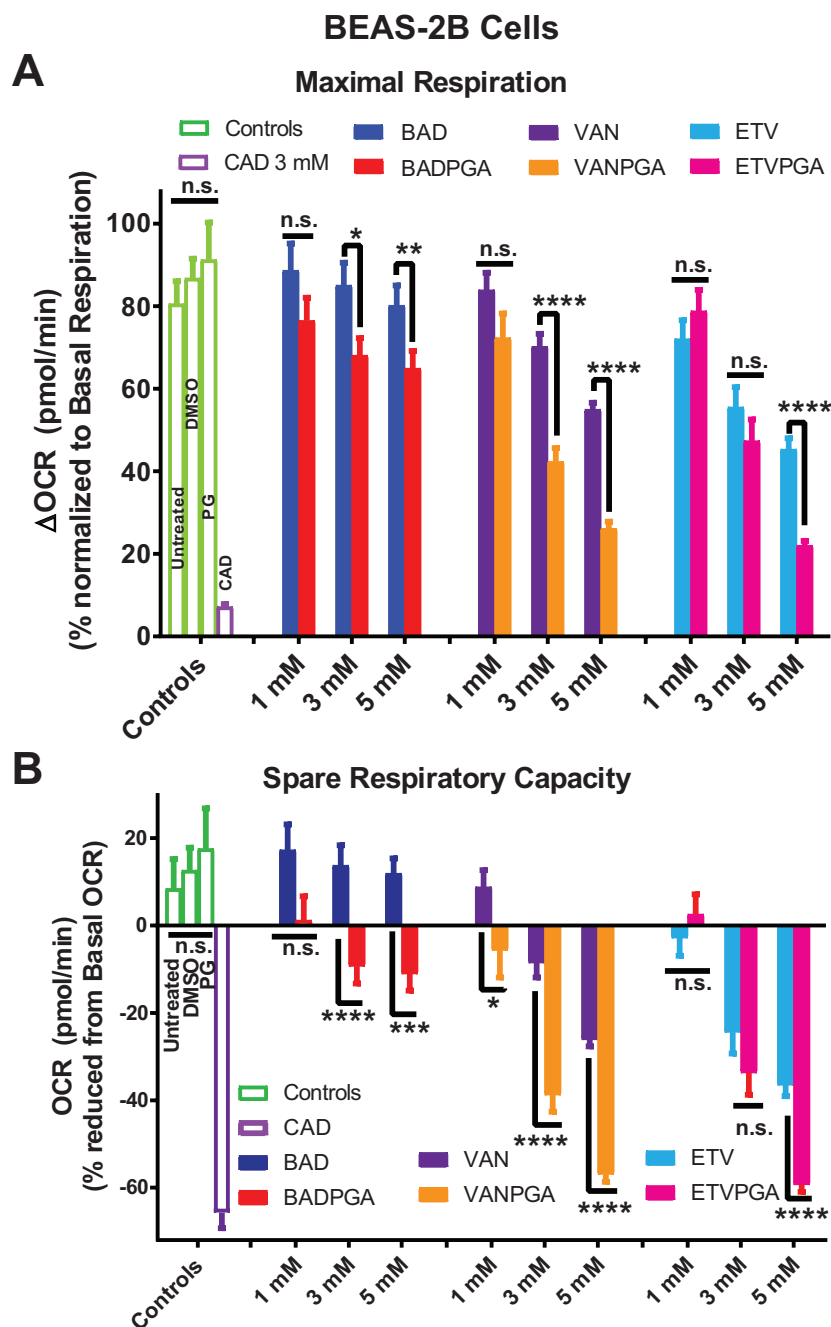
**Figure 2.** Effects of flavor aldehyde PG acetals and parent flavor aldehydes on mitochondrial ATP production in human pulmonary epithelial cells. BEAS-2B (top) and A549 (bottom) cells were exposed to various concentrations of flavor aldehydes (BAD), vanillin (VAN), or ethyl vanillin (ETV), and their corresponding acetals (BADPGA, VANPGA, and ETVPGA) (1, 3, and 5 mM) with subsequent addition of 1  $\mu$ M oligomycin to determine the effect on ATP production. Flavor aldehydes included. The Seahorse XF Cell Mito Stress Test assay was repeated at least four times ( $n = 4$  or 5), with each chemical exposure run in quadruplicates in each assay. Data represent mean  $\pm$  SEM of normalized  $\Delta$ OCR.  $\Delta$ OCR is the change in oxygen consumption rate (OCR) from basal respiration after addition of 1  $\mu$ M oligomycin. Stars indicate statistical significance as compared with corresponding flavor aldehyde and determined by unpaired  $t$  test (\*\*\*\* $p < .0001$ , \*\*\* $p < .001$ , \*\* $p < .01$ , \* $p < .05$ ). PG = propylene glycol.

mM ethyl vanillin reduced maximal respiration and SRC in A549 cells. Exposure to 3 or 5 mM vanillin PG acetal or 5 mM ethyl vanillin PG acetal had a more robust effect on maximal respiration and SRC compared with their corresponding flavor aldehydes in both BEAS-2B and A549 cells (Figure 3A and B; Supplementary Figure S3A and B; Supplementary Tables S1 and S2). These results suggest that flavor aldehyde PG acetals more strongly affect the abilities of respiratory epithelial cells to respond to bioenergetic challenges than their parent flavor aldehydes.

### Cytotoxic Effects of Flavor Aldehyde PG Acetals

With exposure of respiratory epithelial cells to flavor aldehyde PG acetals more strongly influencing cellular respiration and potentially their abilities to cellular growth and division, we determined their cytotoxic and cytostatic effects in comparison with their parent flavor aldehydes. Fluorescent LIVE/DEAD cell assays were conducted in BEAS-2B cells and HNEpC to compare the potential

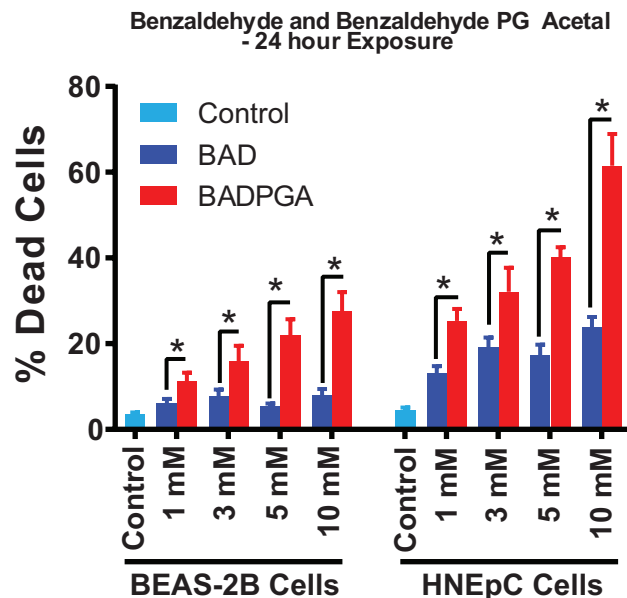
cytotoxic effects of exposure (24 hours) to benzaldehyde and benzaldehyde PG acetal. While exposure to benzaldehyde caused moderate cytotoxicity in HNEpC or no significant cytotoxicity in BEAS-2B cells, incubation with benzaldehyde PG acetal induced significant cell mortality in both cell types (Figure 4; Supplementary Figure S4A), in a concentration-dependent manner and at all concentrations tested. The primary HNEpC were more sensitive to the acetal



**Figure 3.** Maximal and spare mitochondrial respiration capacities of human bronchial epithelial cells exposed to flavor aldehyde or corresponding PG acetals. (A) Maximal respiration of BEAS-2B cells exposed to various concentrations of flavor aldehydes (benzaldehyde, BAD; vanillin, VAN; ethyl vanillin, ETV) or their corresponding acetals (BADPGA, VANPGA, and ETVPGA) (1, 3, and 5 mM) with subsequent addition of 0.5  $\mu$ M FCCP to determine the effect. (B) Spare respiratory capacity in the same cells as in (A). The Seahorse XF Cell Mito Stress Test assay was repeated at least four times ( $n = 4$  or 5) and each assay had three to eight replicates for each chemical exposure. Data represent mean  $\pm$  SEM of normalized  $\Delta$ OCR.  $\Delta$ OCR is the change in oxygen consumption rate (OCR) from basal respiration after addition of 0.5  $\mu$ M FCCP. Stars indicate statistical significance as compared with corresponding flavor aldehyde and determined by unpaired  $t$  test (\*\*\*\* $p < .0001$ , \*\*\* $p < .001$ , \*\* $p < .01$ , \* $p < .05$ ). FCCP = carbonyl cyanide-4-phenylhydrazone, PG = propylene glycol.

exposure, resulting in a higher percentage of dead cells. Comparison of vanillin and vanillin PG acetal revealed that vanillin PG acetal was more cytotoxic than vanillin in BEAS-2B cells only at the highest concentration tested (10 mM), with no differences at lower concentrations (1, 3, and 5 mM) (Supplementary Figure S5A).

Next, we determined the effects of flavor aldehyde PG acetal exposure on cellular growth. Compared with benzaldehyde, exposure to benzaldehyde PG acetal for 24 hours significantly reduced the surface area of cellular growth at all concentrations tested (Supplementary Figure S5C). Both vanillin and vanillin PG acetal impaired cell growth in a similar concentration-dependent manner (Supplementary Figure S5B). To more accurately quantify the cytostatic and antiproliferative effects of vanillin and vanillin PG acetal on respiratory epithelial cells, we performed the Click-iT Edu assay that measures the amount of newly synthesized DNA. BEAS-2B cells were exposed to various concentrations (0.3–10 mM) of vanillin and vanillin PG acetal for 24 hours in presence of Edu, a nucleoside analog that can be labeled using a reactive fluorescent moiety. Fluorimetric quantification of Edu signal intensity revealed that both vanillin and vanillin PG acetal reduced Edu accumulation in a concentration-dependent manner (Supplementary Figure S6B). This result was further confirmed by fluorescence microscopy, also confirming that exposure to both vanillin and vanillin PG acetal reduced total cell numbers (Supplementary Figure S6A). In summary, these results indicate that benzaldehyde PG acetal has stronger cytotoxic effects in respiratory epithelial cells than benzaldehyde, both for cell viability and cell growth. Vanillin PG acetal and vanillin have comparably potent cytostatic and antiproliferative effects.



**Figure 4.** LIVE/DEAD dual fluorescence assay in BEAS-2B (bronchial epithelial cells) and HNEpC (primary human nasal epithelial cells) comparing effects of benzaldehyde PG acetal (BADPGA) with benzaldehyde (BAD). Quantification of percent dead cells for untreated cells and cells exposed to various concentrations of benzaldehyde and benzaldehyde PG acetal is shown. Data represent mean  $\pm$  SEM. Stars indicate statistical significance as compared with corresponding flavor aldehyde and determined by unpaired *t* test ( $*p \leq .05$ ).  $n = 3$  experiments for BEAS-2B cells and  $n = 2$  for HNEpC, with each experiment having multiple image replicates (each image has several hundreds of cells). PG = propylene glycol.

## Discussion

In the present study, we examined the cytotoxic and metabolic effects of flavor aldehyde solvent adducts present in e-liquids and vapor in respiratory epithelial cells (BEAS-2B cells, A549 cells, and HNEpC). Flavor aldehyde PG acetals evaluated included adducts of popular e-liquid flavorants such as benzaldehyde (berry/fruit), vanillin, and ethyl vanillin (vanilla/candy). Compared with their parent aldehydes, the PG acetals reduced cellular proliferation and viability more strongly. This may be due to their increased metabolic toxicity, as the PG acetals more potently compromised mitochondrial respiration.

Reported concentrations of popular flavor aldehydes such as benzaldehyde, vanillin, ethyl vanillin, and cinnamaldehyde differ widely in commercial e-cigarette liquids, with some liquids containing molar amounts. For example, there are reports of cinnamon e-liquids containing as high as ~34% (343 mg/mL; 2.6 M) cinnamaldehyde.<sup>21</sup> Our prior research has demonstrated that these flavor aldehydes undergo chemical reactions with e-liquid solvents PG to form flavor aldehyde PG acetals,<sup>37,44</sup> which have been widely detected in commercial e-liquids.<sup>20–22,34,37,43,44</sup> These acetals are efficiently carried over into vapor and remain stable at physiological conditions for several days,<sup>37</sup> suggesting that e-cigarette users' airways are exposed to these compounds. In addition, PG acetals can have potential respiratory irritant effects resulting from the more robust activation of sensory irritant receptors (TRPA1 and TRPV1) expressed in sensory nerve endings in the airways and lungs.<sup>37</sup>

In this study, we reveal differential cytotoxic effects of these acetals on respiratory epithelial cells. In contrast to benzaldehyde that did not cause cell death over the range of concentrations tested (1–10 mM), benzaldehyde PG acetal caused cell death at concentrations as low as 1 mM (0.16 mg/mL). Concentrations of benzaldehyde PG acetal exceeded 100 mM (~18 mg/mL) in some fruit-flavored e-liquids, with ~80% vapor carryover reported, suggesting continuous exposure of users of these e-liquid aerosols.<sup>21,37</sup> The effects of vanillin and vanillin PG acetal were more benign, with cytotoxicity observed only at the highest concentration tested (10 mM). Interestingly, Hua et al.<sup>25</sup> showed that vanillin and ethyl vanillin is cytotoxic in the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, starting from as low as 1 mM. Notably, the MTT assay is a measure of cell metabolic activity, which along with cytotoxic effects, measures cytostatic activity (shift from proliferation phase to quiescence phase). In the present study, the cytostatic effects of vanillin and its corresponding PG acetal were noted even at the lowest concentration tested (0.3 mM). These cytotoxic effects of acetals on the lung epithelial cells will be in addition to their potential respiratory irritant effects.<sup>37</sup> Taken together, inhalational exposure effects of e-cigarette liquid constituents, especially from chronic long-term e-cigarette use, may be compounded by acetal toxicity, potentially leading to stronger respiratory toxicological effects.

The toxicological mechanism associated with aldehydes and their derivatives can mostly be explained by the formation of covalent adducts with nucleophilic targets, either with thiolate (eg, cysteine) or amine (eg, lysine) groups of amino acids and other biological macromolecules.<sup>35</sup> The potency of toxicity is proportional to adduct reaction rate, that is primarily driven by the electrophilicity and presence of  $\alpha,\beta$ -unsaturated aldehyde moiety.<sup>39</sup> The adducts can impair structural proteins, enzymes, nucleic acids, and other biological molecules, leading to loss of key cellular and metabolic functions and subsequent cell growth inhibition and death.

In previous studies, reactive aldehydes present in cigarette smoke, such as acrolein, formaldehyde, acetaldehyde and crotonaldehyde, impaired either ciliary beat frequency or mitochondrial function or both.<sup>45,46</sup> Aromatic flavor aldehydes added to the e-cigarette liquids, such as benzaldehyde, vanillin, ethyl vanillin, and cinnamaldehyde diminished mitochondrial function in various cell types, including lung epithelial cells and immune cells.<sup>16,41,42,47</sup> Our experiments using the Seahorse mitostress test clearly established that exposure of lung epithelial cells (BEAS-2B and A549) to the corresponding flavor aldehyde acetals more strongly diminished key bioenergetic parameters of mitochondrial respiration.

The potential biochemical mechanisms underlying the differential toxicity of acetals compared with their parent aldehydes are unknown. Acetals are expected to be less reactive than aldehydes and in synthetic chemistry, acetal formation is frequently used to “protect” aldehyde moieties from unwanted reaction. This suggests that mechanisms other than direct reactivity of the compounds are responsible for the observed results. Some of these mechanisms might potentially include specific inhibition of key mitochondrial proteins; modulating glycolytic pathway of cellular respiration; inhibiting NADH oxidase or complex I of the respiratory chain; or reduction in steric hindrances in covalent adduct formation with mitochondrial proteins. PG acetals are more hydrophobic than their corresponding aldehydes due to the addition of additional (unpolar) alkyl groups that “shield” the rather polar C=O aldehyde bond, so it is possible that the resulting difference in solubility may play a role in how the PG acetals behave intracellularly. For example, increased lipophilicity of the acetals over the aldehydes might allow for an efficient penetration of acetals into mitochondria without requiring active transport, and reaching the inner membrane of the mitochondria more freely than parent aldehyde and leading to mitochondrial dysfunction.<sup>48</sup> Also, with mitochondrial inner space being acidic, the acetals could undergo hydrolysis and release the toxic reactive aldehydes. One mechanism that can be excluded is the uncoupling of the electron transport chain, as we did not observe any aldehyde- or acetal-induced increase in oxygen consumption. Acetals also depleted ATP and the ability for ATP production and reduced maximal and SRC. Significant reduction in SRCs was observed in presence of higher concentration of the acetals, which indicates that the cells are not capable to handle cellular stress, do not have the required energy to proliferate or fulfill their normal function.

The concentration of benzaldehyde and its corresponding benzaldehyde PG acetal in some neat e-liquids can be as high as ~225 mM (~23 mg/mL; with ~80% carryover rate into aerosol) and ~110 mM (~18 mg/mL; with ~75% carry over rate), respectively.<sup>37</sup> While these are the concentration in the neat e-liquid, which is diluted with air during the aerosolization step, the efficient carryover indicates that concentrations in the small droplets that make up the aerosol are close. Using these concentrations as a baseline, they are ~22–225 times higher than the concentrations used in the present Seahorse assays (1, 3, and 5 mM). Similarly, the concentrations of vanillin, ethyl vanillin, and their acetals in commercial e-liquids are much higher (~3–60 times) than the concentrations we used in the assay.<sup>21,37</sup> One of the concerns around vanillin is that it is one of the top 2 most popular e-liquid flavor additives, likely due to its familiar sweet-associated odor.<sup>21,22</sup> As a result many vapers are likely exposed to vanillin and its PG acetal regularly, yet the chronic effects of inhaling these compounds remains unclear. Exposures in users will vary significantly, due to differences in device characteristics and

power output, puff topography, and pH of the vaped e-liquids.<sup>49</sup> For example, vanillin PG acetal is present in aerosols of vanilla-flavored JUUL aerosol,<sup>44</sup> a device that delivers high amounts of nicotine in the form of nicotine benzoate salt to users. Earlier work by Omaiye et al. suggested that the primary driver for JUUL cytotoxicity in BEAS-2B cells is the high content of nicotine benzoate in these products. This conclusion was based on correlation analyses of metabolic activity (MTT assay) data and nicotine concentration in both e-liquids and their aerosols.<sup>27</sup> Future studies should address any potential additive or synergistic effects of nicotine, flavor aldehyde, and their corresponding acetals, and examine the cellular signaling networks affected, including those related to senescence, proliferation, apoptosis, necrosis, and stress.

## Conclusions

The data presented in this study clearly demonstrate that solvent adducts of reactive flavor aldehydes are cytotoxic to pulmonary epithelial cells and inhibit their mitochondrial function with often higher efficacies than their parent flavor aldehydes. These results are highly relevant to our understanding of the pulmonary health effects of flavored vape products. With recent studies demonstrating the presence of these secondary reaction products in highly popular JUUL e-liquids, and our data demonstrating cytotoxicity and metabolic toxicity, there is great concern these products contribute to e-cigarette toxicity. These data provide a foundation for further toxicological studies using organ and animal models, and in e-cigarette users, both in the context of normal health and disease conditions. From a tobacco regulatory perspective, this study suggests that regulations requiring manufacturers’ disclosure of e-liquid ingredients at time of production may be insufficient to inform a comprehensive risk assessment of e-liquids and electronic nicotine delivery systems use, due to the chemical instability of e-liquids over time and the formation of new compounds, such as the demonstrated cytotoxic flavor aldehyde acetals.

## Supplementary Material

A Contributorship Form detailing each author’s specific involvement with this content, as well as any supplementary data, are available online at <https://academic.oup.com/ntr>.

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## Declaration of Interests

The funding organization had no role in the design and conduct of the study; the collection, management, analysis, and interpretation of the data; the preparation, review, or approval of the manuscript; nor in the decision to submit the manuscript for publication. The content is solely the responsibility of the authors and does not necessarily represent the views of National Institutes of Health (NIH) or the Food and Drug Administration (FDA). Dr. Jordt reports receiving personal fees from Hydra Biosciences LLC and Sanofi S.A. and nonfinancial support from GlaxoSmithKline Pharmaceuticals outside the submitted work. No other financial disclosures were reported by the authors of this paper.

## Author Contributions

SVJ and SEJ conceptualized and designed the study; SVJ acquired and analyzed the data; AND analyzed the data; SVJ interpreted the data; SVJ and SEJ drafted the manuscript; SVJ carried out statistical analysis; SVJ and SEJ provided supervision; HEC and JBZ critically revised the manuscript for important intellectual content.

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