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RESEARCH ARTICLE | *Electronic Cigarettes: Not All Good News?*

Flavored e-cigarette liquids reduce proliferation and viability in the CALU3 airway epithelial cell line

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Rowell TR, Reeber SL, Lee SL, Harris RA, Nethery RC, Herring AH, Glish GL, Tarran R. Flavored e-cigarette liquids reduce proliferation and viability in the CALU3 airway epithelial cell line. *Am J Physiol Lung Cell Mol Physiol* 313: L52–L66, 2017. First published April 20, 2017; doi:10.1152/ajplung.00392.2016.—E-cigarettes are generally thought of as a safer smoking alternative to traditional cigarettes. However, little is known about the effects of e-cigarette liquids (e-liquids) on the lung. Since over 7,000 unique flavors have been identified for purchase in the United States, our goal was to conduct a screen that would test whether different flavored e-liquids exhibited different toxicant profiles. We tested the effects of 13 different flavored e-liquids [with nicotine and propylene glycol/vegetable glycerin (PG/VG) serving as controls] on a lung epithelial cell line (CALU3). Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as an indicator of cell proliferation/viability, we demonstrated a dose-dependent decrease of MTT metabolism by all flavors tested. However, a group of four flavors consistently showed significantly greater toxicity compared with the PG/VG control, indicating the potential for some flavors to elicit more harmful effects than others. We also tested the aerosolized "vapor" from select e-liquids on cells and found similar dose-dependent trends, suggesting that direct e-liquid exposures are a justifiable first-pass screening approach for determining relative e-liquid toxicity. We then identified individual chemical constituents for all 13 flavors using gas chromatography-mass spectrometry. These data revealed that beyond nicotine and PG/VG, the 13 flavored e-liquids have diverse chemical constituents. Since all of the flavors exhibited some degree of toxicity and a diverse array of chemical constituents with little inhalation toxicity available, we conclude that flavored e-liquids should be extensively tested on a case-by-case basis to determine the potential for toxicity in the lung and elsewhere.

tobacco; COPD; cancer; nicotine; aerosols

E-CIGARETTES (e-cigs) have been growing in popularity since their debut in 2007 and are estimated to become a $50 billion global market by 2025 (37). E-cigs differ from tobacco cigarettes in that they do not contain tobacco, have varied nicotine concentrations (0–36 mg/ml), and produce an inhalable aerosol ("vapor") that is generated without combustion. Instead, an e-cig liquid (e-liquid) is drawn and heated over a battery-operated coil as the user inhales. E-liquids are usually composed of a vehicle with varying ratios of propylene glycol (PG) and vegetable glycerin (VG) that contain nicotine and chemical flavors. Recently, the Food and Drug Administration introduced rules to regulate e-cig products (https://www.fda.gov/TobaccoProducts/Labeling/ProductsIngredientsComponents/ucm456610.htm) (38, 40). Despite this legislation, there continues to be much debate over the safety and efficacy of these products. E-cigs have commonly been marketed as a safer smoking alternative because they lack the carcinogens from tobacco and presumably fewer of the pyrolysis products from combusting tobacco that are associated with smoking-related diseases. However, some e-cig devices are capable of producing pyrolysis products (i.e., reactive aldehydes) and oxidant species similar to traditional cigarettes (27, 43, 48), but the conditions under which users would actually be exposed to disease-causing levels of these products remain a source of controversy.

While the direct health effects of cigarette smoke exposure have been extensively studied with evidence-based links found between tobacco use and both lung cancer and chronic obstructive pulmonary disease, e-cig research is still lagging behind consumer use. A review of all known studies reporting effects of e-cig aerosols and e-liquids on the lung amounted to less than 15 in 2015 (41). Fortunately, the interest concerning lung health effects of e-cigs has led to many more research publications since then. However, there is still much that is unknown about the biological effects of e-cigs. Of particular concern, e-cig use in middle and high school students has tripled in just 3 yr (31) and by 2015, more than a quarter of middle and high students had tried e-cigs (51). Furthermore, the availability of over 7,000 unique flavors in the United States (54) alone may contribute to their popularity in adolescents (3).

It is currently unknown whether or not long-term e-cig use will cause respiratory diseases similar to cigarette smoke, none at all, or something entirely different. For example, bronchiolitis obliterans or "Popcorn Workers' Lung" is scarring of the small airways that can range from mild and reversible to severe and irreversible. Prolonged inhalation of diacetyl, a buttery-flavored chemical used in microwave popcorn manufacturing and elsewhere, can and has caused this disease in some workers at microwave popcorn manufacturing plants (5, 24). Although diacetyl is safe to eat and thus found on the "generally

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recognized as safe" list, it is clearly not safe to inhale. Diacetyl and many other flavorings have only been tested and approved for ingestion and have not been tested for inhalation toxicology. Despite the known link between diacetyl and bronchiolitis obliterans, Allen et al. (2) reported that either diacetyl or 2 other prominent butter-flavored chemicals (2,3-pentanedione and acetoin) were detected in 47 of 51 flavored e-liquid aerosols tested. Thus there is the potential for e-liquid flavors to have as yet unknown and possibly negative effects on the lung, as has recently been discussed (6).

Given the variety of available flavors and devices, information is needed regarding the biological effects of different e-liquids and their individual constituent(s) on the different cell types in the respiratory system. Therefore, we used a high-throughput screening approach to assess the potential effects of 13 different flavored e-liquids and their respective controls on cell proliferation and an array of viability and toxicity markers over a range of doses following direct- and vaped- e-liquid exposure. Although several researchers have investigated the effects of e-liquids or e-cig aerosols on the lung, few studies have focused on *1*) identifying component chemicals and *2*) assessing their biological effects (4, 7, 15, 22, 23, 42, 46, 50). Therefore, we also conducted mass spectrometry analysis on all 13 flavors to pair biological outcomes with chemical constituent(s) identified in each flavor to understand which flavors and individual constituents may alter lung epithelial cell proliferation and/or viability.

METHODS

Flavored e-cig liquids. All flavored e-cig liquids (e-liquids) were purchased from The Vapor Girl (https://www.thevaporgirl.com/). The tested flavors were Captain Black Cigar, Peanut Butter Cookie, T-bone, Popcorn, Black Licorice, Energon (orange energy drink), Vanilla Tobacco, Banana Pudding (Southern Style), Kola, Hot Cinnamon Candies, Menthol Tobacco, and Solid Menthol. All e-liquids were ordered to contain 12 mg/ml nicotine. An additional 0 mg/ml nicotine Captain Black Cigar was purchased as a nicotine-free control. At the time of purchase, the vehicle liquid was advertised as a 70/30 ratio of propylene glycol (PG) to vegetable glycerin (VG). Thus a vehicle control was made in our laboratory using 70 PG/30 VG. For all aerosol experiments, additional Peanut Butter Cookies, Banana Pudding, and Hot Cinnamon Candies e-liquids were purchased from The Vapor Girl. All three additional e-liquids were ordered to contain 12 mg/ml nicotine and a 55/45 ratio of PG/VG. Therefore, we made an additional 55 PG/45 VG control for the aerosol experiments.

Chemicals and reagents. PG, VG, DMSO, probenecid, and methanol were purchased from Sigma-Aldrich. DAPI, calcein (AM), MitoTracker Red (CMXRos), fluo-4 (AM), and the Vybrant MTT Cell Proliferation Assay Kit were purchased from Life Technologies. Nicotine was purchased from Alfa Aesar. The Cytotoxicity Detection Kit^{PLUS} (LDH) was purchased from Roche. DAPI, calcein (AM), MitoTracker Red (CMXRos), and fluo-4 (AM) were reconstituted using DMSO and applied to cells in experiments where the final DMSO concentration was $\leq$0.1%.

Cell culture. CALU3 cells were cultured in MEM alpha with 10% FBS and penicillin/streptomycin (GIBCO) as described (10). For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, MEM alpha without phenol was used per the manufacturer's guidelines. Cells were seeded into 96-well black-walled clear bottom plastic plates (Corning). For e-liquid 24-h studies, cells were seeded at 12,500 or 45,000 per well for 12 h overnight and the 24-h treatment began the next morning. E-liquids were diluted (%vol/vol) in CALU3 media. After 24 h, e-liquid-treated media was removed from cells and assays were performed. For aerosol 24-h studies, cells were seeded at 25,000 per well for 4–8 h and media were changed just before aerosol treatments were performed. After aerosol exposures, cells were incubated for 24 h before aerosol-exposed media were removed and assays were performed.

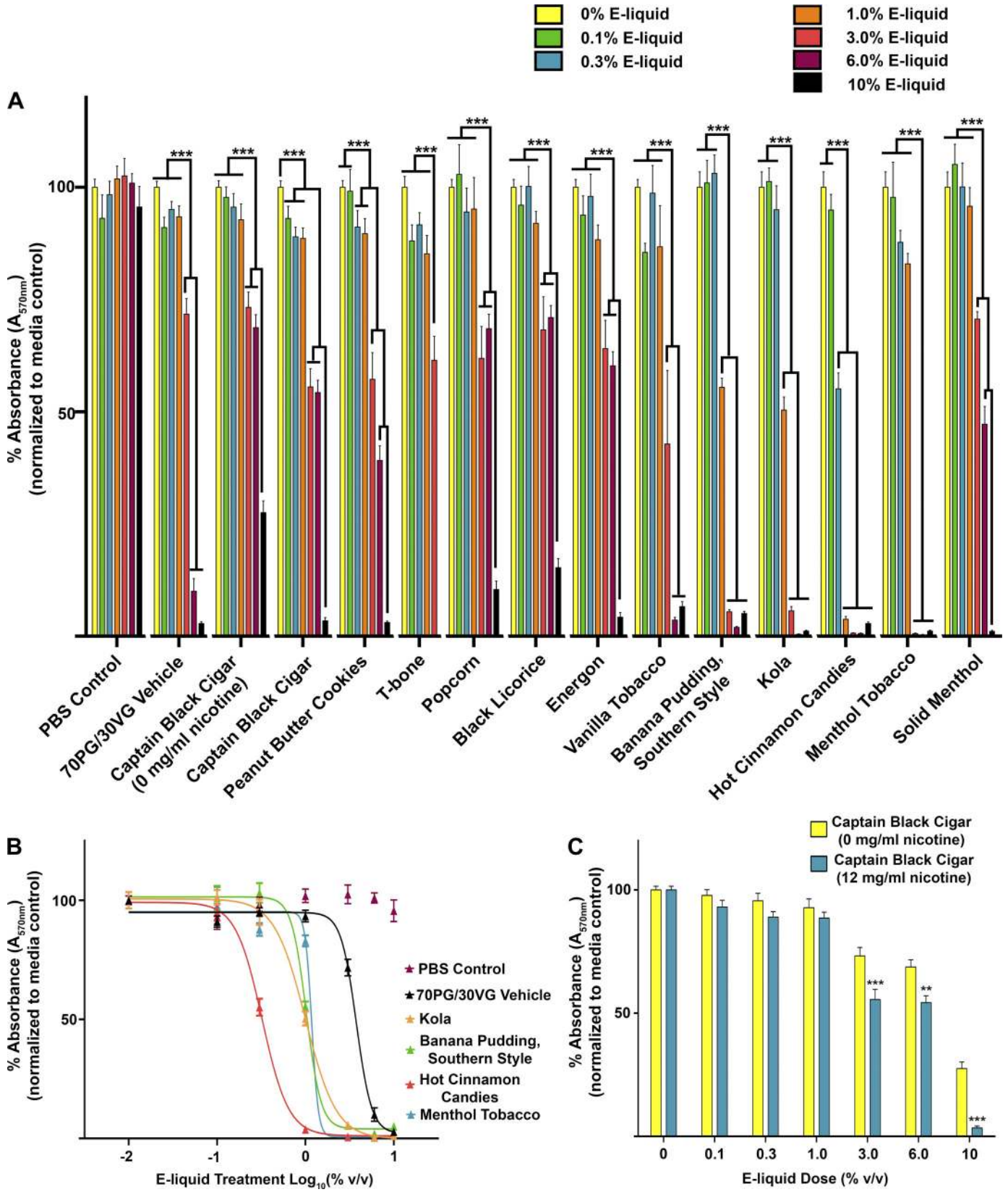
Cell proliferation. The MTT assay was performed as instructed by the manufacturer after cells were treated for 24 h with either PBS, 70 PG/30 VG, nicotine, or flavored e-liquids. Cells were allowed to proliferate for 4 h after removal of the treatments. Data were calculated as percent absorbance of each treatment compared with the average of the 0% e-liquid (media control) treatments in each plate. Nonlinear regression curves were fit to each flavor or nicotine ± PG/VG dose responses in the MTT assays using GraphPad Prism to calculate IC_{50} values where appropriate.

Cell number and viability. Total cell number was measured at the end of the 24-h e-liquid and aerosol exposures. Cultures were rinsed with PBS and fixed with 100% methanol. After fixation, cells were rinsed again with PBS and stained with DAPI for 10 min. Cultures were rinsed following staining, and DAPI fluorescence intensity was measured using the Tecan Infinite Pro plate reader [excitation (ex): 360 ± 5 nm; emission (em): 460 ± 5 nm]. Total cell number was calculated as percent fluorescence of the e-liquid- or aerosol-treated cells compared with the average of the 0% e-liquid or 0 puff (media control) wells in each plate.

Cell/mitochondrial viability was assessed using calcein and MitoTracker Red fluorescent indicators. After 24-h e-liquid or aerosol exposures, treated media were exchanged for fresh media containing either 3 μM calcein or 125 nM MitoTracker Red. Cultures were incubated for 30 min at 37°C. Cultures were then rinsed, media were replaced with a standard Ringer's solution, and fluorescence intensities were read using the Tecan Infinite Pro plate reader for calcein (ex: 495 ± 5 nm; em: 516 ± 5 nm) or MitoTracker Red (ex: 579 ± 5 nm; em: 599 ± 5 nm), respectively. Cell/mitochondrial viability was calculated as a percent fluorescence of e-liquid or aerosol-treated cells compared with the average of the 0% e-liquid or 0 puff (media control) wells in each plate.

Cytotoxicity (membrane permeability – LDH release). Membrane permeability due to e-liquid exposure was measured using the Cytotoxicity Detection Kit^{PLUS}. Media were collected from e-liquid-treated wells after 24-h exposure and the LDH assay was performed per manufacturer's instructions. Data were calculated as percent LDH release compared with a lysed control and reported as %LDH release, where %LDH release = [(experimental value − low control)/(high control − low control)] × 100.

Fig. 1. Flavored e-liquids cause dose-dependent decreases in cell proliferation and viability. CALU3 cells were seeded at 12,500 per well in 96-well plates and were challenged with increasing doses of e-liquid flavors diluted in media (%vol/vol) for 24 h. Cell proliferation/viability was measured at the end of the 24-h treatment using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. *A*: mean %absorbance was plotted for all flavors and controls in all doses. *B*: nonlinear regression curves were fit to calculate the IC_{50} of each flavor. *C*: MTT responses were compared in Captain Black Cigar containing either 0 or 12 mg/ml nicotine to determine the effects of nicotine. Bars and triangles represent average %absorbance measured normalized to 0% e-liquid (media control) treatment per plate ± SE; n = 12–24 wells run in 4–8 independent experiments per treatment. Statistics were calculated using a linear mixed model with pairwise comparisons for doses within flavor (*A*) or between flavors in each dose (*C*). *A*: P values for overall tests of dose within flavor are denoted (***$P < 0.001$), and, where applicable, further pairwise significant differences ($P < 0.05$) are indicated using cluster lines above the graph. *C*: P values for pairwise differences are denoted (**$P < 0.01$, ***$P < 0.001$).



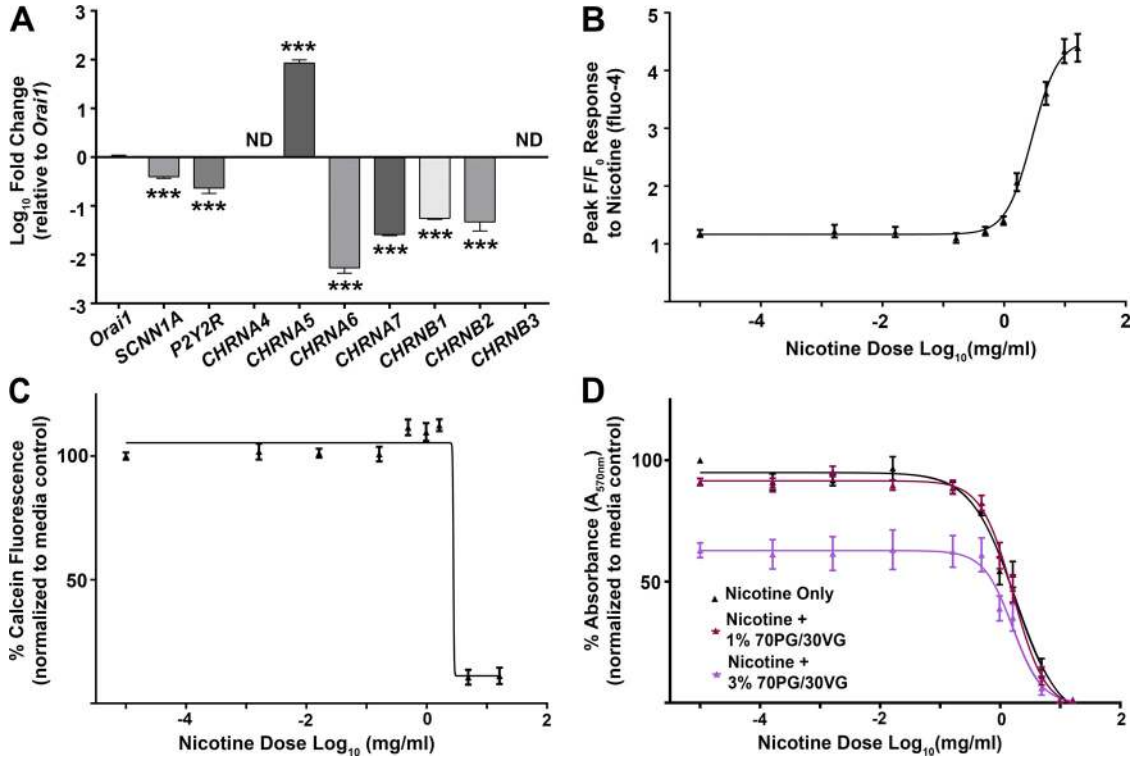


Fig. 2. Nicotine alone decreases cell proliferation and increases cytotoxicity that is independent of nicotinic acetylcholine receptor (nAChR) stimulation. *A*: RNA was isolated from untreated CALU3 cells and mRNA expression of nAChR subunits relative to *Orai1*, a Ca^{2+} channel, were measured ($n = 4$–10 wells per gene). *B* and *C*: CALU3 cells were seeded at 45,000 per well in 96-well plates overnight and were challenged with nicotine doses acutely to measure Ca^{2+} activity (fluo-4) or measure viability (calcein). Peak changes in fluo-4 fluorescence (F/F_0) per nicotine dose were plotted and fit with a nonlinear regression curve to calculate the EC_{50} ($n = 14$–30 wells per treatment). Cell viability was measured 1 h post-nicotine exposure by measuring calcein fluorescence and fitting the dose response with a nonlinear regression curve ($n = 11$–18 wells per treatment). *D*: cells were seeded at 12,500 per well in 96-well plates overnight and were challenged with varying doses of nicotine ± 70 propylene glycol (PG)/30 vegetable glycerin (VG) diluted in media for an additional 24 h. Cell proliferation/viability was measured at the end of the 24-h treatment using the MTT assay. Mean %absorbance was plotted for all treatments and doses and fit with nonlinear regression curves to calculate the IC_{50} of each treatment (Table 1; $n = 12$–15 wells per treatment). Bars represent average gene expression relative to *Orai1* ± SE (*A*). Triangles represent average %fluorescence or %absorbance measured normalized to 0 mg/ml dose (media control) per plate ± SE (*B*–*D*). Statistics were calculated using a linear model of log-transformed outcomes with a fixed effect for gene type and pair-wise comparisons between genes (*A*) (***$P < 0.001$; ND, not determined).

Ca^{2+} signaling. Changes in cytosolic Ca^{2+} concentration were measured using 8 μM fluo-4 dye loaded into cells in the presence of 1 mM probenecid for 40 min at 37°C. Cultures were then rinsed, and media were replaced with a standard Ringer's solution and fluorescence intensities were read every 15 or 30 s using a Tecan Infinite Pro plate reader for fluo-4 (ex: 494 ± 5 nm; em: 516 ± 5 nm). A fluorescent baseline was established before the nicotine doses were added to the wells, and changes in fluorescence were normalized to the baseline (F/F_0). The peak change in F/F_0 was measured for each dose, and a nonlinear regression curve was fit using GraphPad Prism to calculate the EC_{50}.

RNA extraction, cDNA synthesis, and quantitative RT-PCR. RNA was extracted from untreated CALU3 cells using the Qiagen RNeasy kit following the manufacturer's protocol. cDNA was synthesized using the Bio-Rad iScript cDNA synthesis kit following the manufacturer's protocol. Gene expression was measured using Taqman gene expression assays from Applied Biosystems for *Orai1*, *Scnn1A*, *P2Y2R, GAPDH*, *CHRNA4*, *CHRNA5*, *CHRNA6*, *CHRNA7*, *CHRNB1*, *CHRNB2*, and *CHRNB3* using human primers. Genes of interest were normalized to *GAPDH* and fold change was calculated using ΔΔCT method relative to *Orai1*.

Aerosol exposures. E-liquids were heated to generate aerosols using Uwell Crown tanks with 0.25-Ω dual-coils and a Sigelei Fuchai 200W device. The power output was set to either 40 or 100 W, as indicated. Filter pads (GE Healthcare Life Sciences) with a 2-μm pore size were used to collect particulates from the aerosol. The filter pads were weighed before and after 15 or 35 puffs of either 40 W or 100 W, 55 PG/45 VG were passed through them. CALU3 cells seeded in 96-well plates were exposed to a range of puffs (0–35) from either air, 55 PG/45 VG vehicle, 70 PG/30 VG vehicle, Peanut Butter Cookies, Banana Pudding, or Hot Cinnamon Candies. A syringe was used to collect, measure, and apply the aerosol to the wells. A 70-ml puff was applied once every 30 s and was distributed among six wells at once using a three-dimensional printed acrylic six-channel manifold. After aerosol exposures, the cells were incubated for an additional 24 h before measuring total cell number or viability. These were calculated as percent fluorescence of the aerosol-treated cells compared with the average of the 0 puff (media control) wells per plate, which were covered with silicone strips to avoid aerosol exposures.

Mass spectrometry. Samples of e-liquids were diluted 10- or 50-fold in methanol and analyzed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 6890 GC with an Agilent MSD mass spectrometer. One-microliter volumes were introduced by manual injection and were separated on an Agilent DB-5 column with helium carrier gas. The temperature was ramped from 60 to 300°C at a rate of 20°C/min. GC-MS spectra were analyzed using NIST AMDIS software coupled to the NIST 2008 mass spectral database for automated database searching. Constituent profiles of flavors were compared between all 13 e-liquid flavors using peak areas under the curve from GC-MS data that were discretized into a value of 0 (absent) or 1 (present) and compared using R software.

Statistical analyses. Data (see Figs. 1, *A* and *C*; 3, *A–D*; 4, *A* and *B*; and 5, *A* and *B*) were fit using a linear mixed model with main effects for flavor and dose, a flavor by dose interaction, and a random intercept to control for possible plate effect. A similar analysis was performed (see Figs. 5, *C* and *D*), where puff number and wattage were analogous to flavor and dose. Based on these models, subanalyses were performed to investigate if there was an association between dose and response within a flavor. To reduce inflation of type 1 errors, a step-down approach was used in testing. Overall tests for a statistically significant dose and flavor interaction were performed first, and tests for the presence of a dose and response association within each individual flavor were performed only if the overall test was significant. Further tests to determine which doses were significantly different within a flavor were performed only if the previous test confirmed the existence of a dose and response association within that specific flavor. In the nicotine-dosing experiments, responses within each nicotine ± PG/VG treatment were compared with the respective 0 mg/ml. Graphpad Prism software was used to compare the log(IC_{50}) values between dose-response curves for select flavored e-liquids (%vol/vol) and nicotine (mg/ml) ± PG/VG (see Figs. 1*B* and 2*D* and Table 1).

For Fig. 2*A*, we fit a linear model to each log-transformed outcome with a fixed effect for gene type. Log transformations were used to ensure adherence to the modeling assumptions. If an overall test for the effect of gene type was found to be significant, we tested for pair-wise differences between each gene and *Orai1*. In Fig. 5*E* we fit a linear model with main effects for puff number and wattage and a puff number by wattage interaction term. Significant differences in puff numbers within each wattage level and significant differences in wattage levels within each puff number were identified using contrasts. Statistical modeling was performed using R statistical software (49) with the nlme package (36). Statistically significant relationships in all figures were reported (#,*$P \leq 0.05$; ##,**$P \leq 0.01$; ###,***$P \leq 0.001$).

RESULTS

E-liquid exposures inhibit cell proliferation/viability in a dose-dependent manner. To compare the effects of different flavored e-liquids on airway epithelia, we selected a range of flavors that covered not only traditional menthol and tobacco cigarette flavors but a variety of foods and beverages. We also purchased the Captain Black Cigar flavor with and without nicotine to control for the effects of nicotine. Cells were exposed to a range of e-liquid dilutions directly into the culture media over 24 h to assess cell viability and proliferation after treatment, with 70 PG/30 VG serving as the vehicle control. To control for the possible effects of diluting the media, we also provided a PBS control group where we serially diluted media with PBS. We used the MTT assay to indirectly assess the number of viable cells and their ability to proliferate in each treatment. We found dose-dependent decreases in each e-liquid flavor tested, irrespective of nicotine, as well as our 70 PG/30 VG vehicle. These effects were not due to dilution of the growth media since the PBS dilutions had no effect on MTT absorbance (Fig. 1*A*). The T-bone flavored e-liquid was not tested at a 6 or 10% dose because our purchased stock had run out and the vendor discontinued this flavor before these experiments were completed. There were dose-dependent decreases in MTT metabolism for all flavors and the 70 PG/30 VG vehicle at ≥3% dose. Moreover, four flavors [Banana Pudding (Southern Style), Kola, Hot Cinnamon Candies, and Menthol Tobacco] showed significantly greater decreases in MTT metabolism compared with 70 PG/30 VG at 3% ($P \leq 0.001$). When these four flavors were fitted with dose-response curves to calculate their IC_{50}s, these flavors had lower IC_{50}s than 70 PG/30 VG (Fig. 1*B* and Table 1), suggesting that they were more toxic. We also directly compared the responses of cells exposed to the Captain Black Cigar flavor ± nicotine and found that there were more severe effects in the nicotine-containing e-liquid compared with the nicotine-free e-liquid at ≥3% (Fig. 1*C*), suggesting that nicotine exerted additional effects beyond what was seen with the base e-liquid.

Nicotine decreases cell proliferation/viability dose dependently and is cytotoxic. Since we found additional dose-dependent effects of nicotine beyond what was seen with the Captain Black e-liquid alone, we sought to determine whether these negative effects were mediated by nicotinic acetylcholine receptors (nAChRs). We used quantitative PCR to survey nAChR subtype gene expression in CALU3 cells relative to a common membrane Ca^{2+} channel (*Orai1*; Fig. 2*A*). As addi-

Table 1. *$log_{10}(IC_{50})/log(EC_{50})$ and IC_{50}/EC_{50} values for %MTT absorbance e-liquid + nicotine dose responses, Ca^{2+} assay, and %calcein fluorescence for nicotine dose responses*

	Values	Values
	Group 1	
Treatment	$log_{10}(IC_{50})$ ± SE	IC_{50}, %vol/vol
PBS	ND	ND
Vehicle (70 PG/30 VG)	0.5755 ± 0.02	3.763
Captain Black Cigar (0 mg/ml nicotine)	ND	ND
Captain Black Cigar	ND	ND
Peanut Butter Cookies	ND	ND
T-Bone	ND	ND
Popcorn	ND	ND
Black Licorice	ND	ND
Energon	ND	ND
Vanilla Tobacco	0.4730 ± 14.79	2.792
Banana Pudding, Southern Style	0.0094 ± 0.02	1.022
Kola	−0.0005 ± 0.02***	0.999
Hot Cinnamon Candies	−0.4908 ± 0.02***	0.323
Menthol Tobacco	0.0714 ± 14.18	1.179
Solid Menthol	ND	ND
	Group 2	
Treatment	$log_{10}(IC_{50})$ ± SE	IC_{50}, mg/ml
Nicotine only	0.2513 ± 0.07	1.784
Nicotine +1% 70 PG/30 VG	0.2157 ± 0.04	1.643
Nicotine +3% 70 PG/30 VG	0.2048 ± 0.08	1.603
	Group 3	
Treatment	$log_{10}(EC_{50})$ ± SE	EC_{50}, mg/ml
Nicotine only	0.4605 ± 0.05	2.887
	Group 4	
Treatment	$log_{10}(EC_{50})$ ± SE	EC_{50}, mg/ml
Nicotine only	~0.4431 ± ND	ND

List of $log_{10}(IC_{50})/log_{10}(EC_{50})$ and IC_{50}/EC_{50} values for dose-response curves in Figs. 1 and 2. Nonlinear regression curves were fit to the mean %absorbance values of all doses within each flavored e-cigarette liquids (e-liquids) (Fig. 1*B*) and nicotine ± 70 propylene glycol (PG)/30 vegetable glycerin (VG) (Fig. 2*D*). A nonlinear regression curve was fit to mean peak changes in cytosolic Ca^{2+} fluorescence with nicotine dosing (Fig. 2*B*). A nonlinear regression curve was fit to mean %calcein fluorescence with nicotine dosing (Fig. 2*C*). The $log_{10}(IC_{50})$ ± SE is reported where appropriate for all treatments. The $log_{10}(EC_{50})$ ± SE is reported for where appropriate. ND represents "not determined," where curves could not be fit in the range of doses tested. The IC_{50} and EC_{50} are reported, where appropriate, in either %vol/vol for flavored e-liquids or mg/ml for nicotine ± 70 PG/30 VG treatments. Statistics were calculated using Prism software to compare $log_{10}IC_{50}$ values where applicable. %MTT, %3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. ***$P < 0.001$ in the above flavors compared with the 70 PG/30 VG vehicle.

tional controls, we also looked at *Scnn1a* (epithelial sodium channel alpha subtype) and *P2Y2R* (purinergic receptor) expression. Five of 7 nAChR subtypes were detected in CALU3 cells, but only *CHRNA5* was expressed above the levels of *Orai1, Scnn1a, or P2Y2R* levels, while *CHRNA6*, *CHRNA7*, *CHRNB1*, and *CHRNB2* had much lower expression levels. nAChRs are ligand-gated ion channels that are permeable to Ca^{2+} ions, and since there was detectable nAChR subtype expression in CALU3 cells, we tested for functional activity using a fluorescent cytosolic Ca^{2+} indicator (fluo-4). The peak change in fluorescence per nicotine dose was plotted (Fig. 2*B*), and the EC_{50} was calculated (Table 1). The EC_{50} for nicotine was 2.89 mg/ml (17.8 mM) in CALU3 cells. However, the EC_{50} of nicotine for various nAChRs has been reported in the micromolar range (9, 20). Since increases in cytosolic Ca^{2+} can also be caused by cytotoxicity from permeabilized membranes, we measured cell viability and found a decrease in calcein fluorescence in cultures treated with ≥4.9 mg/ml nicotine (Fig. 2*C* and Table 1), suggesting that the effects of Ca^{2+} were due to cytotoxicity rather than being mediated by nAChRs.

Finally, we measured percent MTT absorbance in cells exposed to increasing doses of nicotine ± 1 or 3% 70 PG/30 VG to understand the potential negative role that nicotine ± vehicle might be having on CALU3 proliferation/viability. We chose a range of doses that encompassed the nicotine concentrations in our e-liquid exposures (%vol/vol) from Fig. 1*A*. Irrespective of the presence of PG/VG, we found that there were dose-dependent decreases in percent absorbance with increasing doses of nicotine (Figs. 2*D*). Treating cells

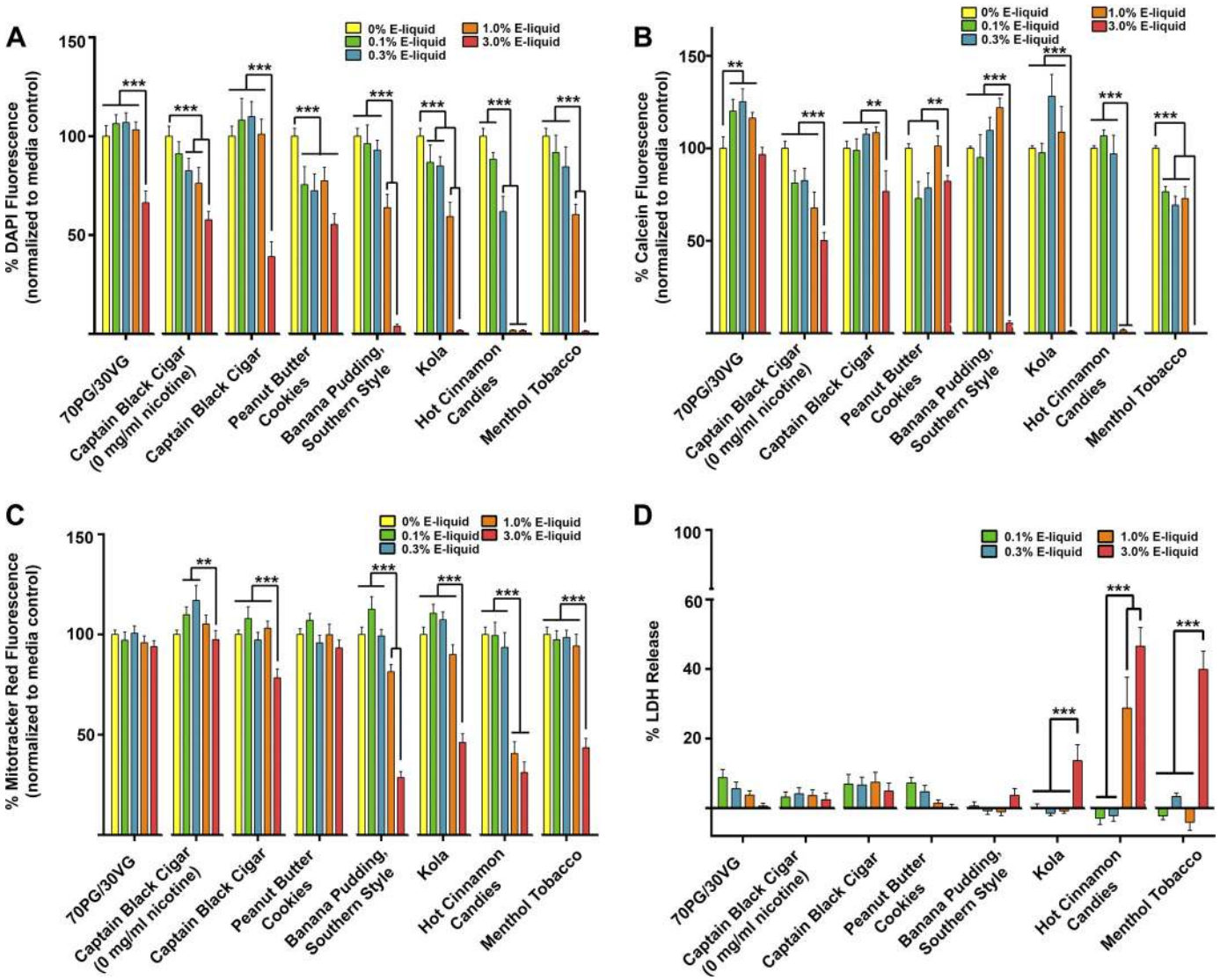


Fig. 3. E-liquids decrease cell number/viability in subconfluent CALU3 cultures. CALU3 cells were seeded at 12,500 per well in 96-well plates for 12 h before e-liquids were diluted in media in a dose-dependent manner (%vol/vol), and cells were challenged for 24 h. *A*: cell number was measured by fixing cells and measuring DAPI fluorescence. Cell/mitochondrial viability was measured using calcein (*B*) and MitoTracker Red (*C*). *D*: cytotoxicity was measured from the cell supernatants collected at 24 h and compared with lysed cell-positive controls. Bars represent average %fluorescence or %absorbance measured normalized to 0% e-liquid (media control) treatment per plate ± SE; $n = 9$–15 wells run in 3–5 independent experiments per treatment. Statistics were calculated using a linear mixed model with pairwise comparisons for doses within flavor (*A*–*D*). *P* values for overall tests of dose within flavor are denoted (**$P < 0.01$, ***$P < 0.001$), and, where applicable, further pairwise significant differences ($P < 0.05$) are indicated using cluster lines above the graph.

with 1% 70 PG/30 VG in combination with nicotine did not have additional effects. However, 3% 70 PG/30 VG decreased the threshold of MTT absorbance alone compared with either 0 mg/ml nicotine (media control) or 1% 70 PG/30 VG ($P \leq 0.001$). There was no difference between $\log_{10}(IC_{50})$ values of each nicotine treatment ±70 PG/30 VG (Table 1), suggesting that adding nicotine to 70 PG/30 VG did not have a synergistic effect on cell proliferation. However, since most flavors caused a significant decrease in MTT absorbance at 3% (Fig. 1*A*), it is likely that 3% 70 PG/30 VG, rather than nicotine, caused the decrease, since 3% e-liquid contains 0.36 mg/ml nicotine, which is insufficient to affect MTT metabolism when combined with 70 PG/30 VG (Fig. 2*D*).

The four flavors of interest decreased cell number/viability in subconfluent CALU3 cultures. The initial screening of the 13 purchased e-liquid flavors on CALU3 cells directed our attention to 4 flavors of interest because of their lower IC_{50}s in the MTT assays compared with 70 PG/30 VG. Therefore, we continued screening the effects of all of the flavors on other measures of cell viability and toxicity but focused on the effects of these four flavors in this paper [i.e., Banana Pudding (Southern Style), Kola, Hot Cinnamon Candies, and Menthol Tobacco]. We also tested Peanut Butter Cookies, a less toxic flavor, as well as Captain Black Cigar ± nicotine to control for the potential effects of nicotine. We performed additional analyses by measuring total cell number using DAPI staining (Fig. 3*A*). We found dose-dependent decreases in cell number 24 h after exposure to 70 PG/30 VG, Captain Black Cigar ± nicotine, Peanut Butter Cookies, as well as our four flavors of interest. We then used the fluorescent dyes calcein (Fig. 3*B*) and MitoTracker Red (Fig. 3*C*) as indicators of viable cells and active mitochondria, respectively (13, 21). Overall, 70 PG/30 VG only exerted effects on total cell number at 3% (Fig. 3*A*). However, the Captain Black Cigar e-liquids, irrespective of nicotine, as well as the four more toxic flavors of interest, showed dose-dependent decreases in all three measures (Fig. 3, *A–C*). Moreover, the four flavors of interest were significantly more toxic than either 70 PG/30 VG or Captain Black Cigar ± nicotine at the 3% dose ($P \leq 0.0001$). Since cell density and viability were reduced, we further investigated the potential for cytotoxicity using LDH release as a marker (26). A 24-h exposure to 70 PG/30 VG, Captain Black Cigar ± nicotine, Peanut Butter Cookies, or Banana Pudding (Southern Style) did not induce LDH release. However, there were significant dose-dependent increases in LDH release following exposure to Kola, Hot Cinnamon Candies, and Menthol Tobacco flavors (Fig. 3*D*).

Hot Cinnamon Candies and Menthol Tobacco 24-h e-liquid exposures show cytotoxicity in confluent CALU3 cultures. Since the previous experiments were performed on subconfluent, proliferating cultures to accommodate the MTT assay (Figs. 1–3), we next assessed the effects of the flavors on confluent, non-proliferating cultures to ascertain whether decreases in cell number/viability were due to cytotoxicity or decreased cell growth. We seeded CALU3 cells into 96-well plates at a higher density where they formed confluent monolayers before conducting the 24-h e-liquid exposures. We found that there were dose-dependent decreases in DAPI fluorescence following exposure to the 70 PG/30 VG vehicle, Peanut Butter Cookies, and the 4 flavors of interest (Fig. 4*A*). However, the decreases in Peanut Butter Cookies, Banana Pudding (Southern Style), and Kola were not significantly greater than that seen with 70 PG/30 VG, while those seen with Hot Cinnamon Candies and Menthol Tobacco were significantly different. Additionally, there were dose-dependent decreases in calcein fluorescence with the 70 PG/30 VG vehicle, Hot Cinnamon Candies, and Menthol Tobacco at 3% (Fig. 4*B*). However, the decreases in 3% Hot Cinnamon Candies and Menthol Tobacco were greater than those seen for 70 PG/30 VG ($P \leq 0.001$).

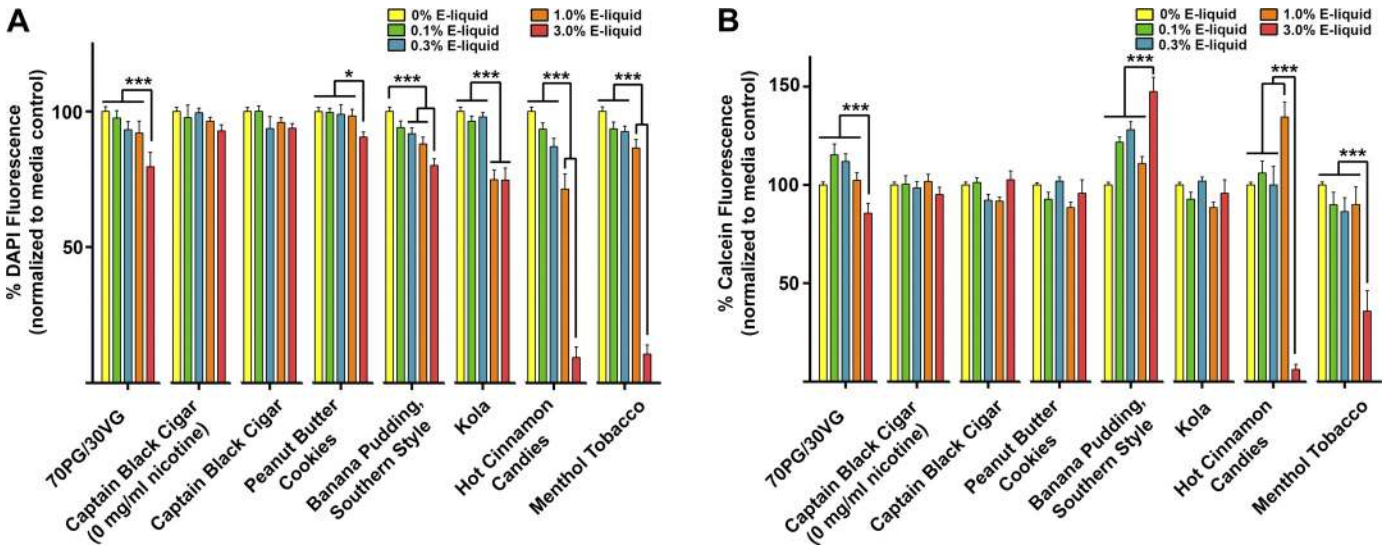
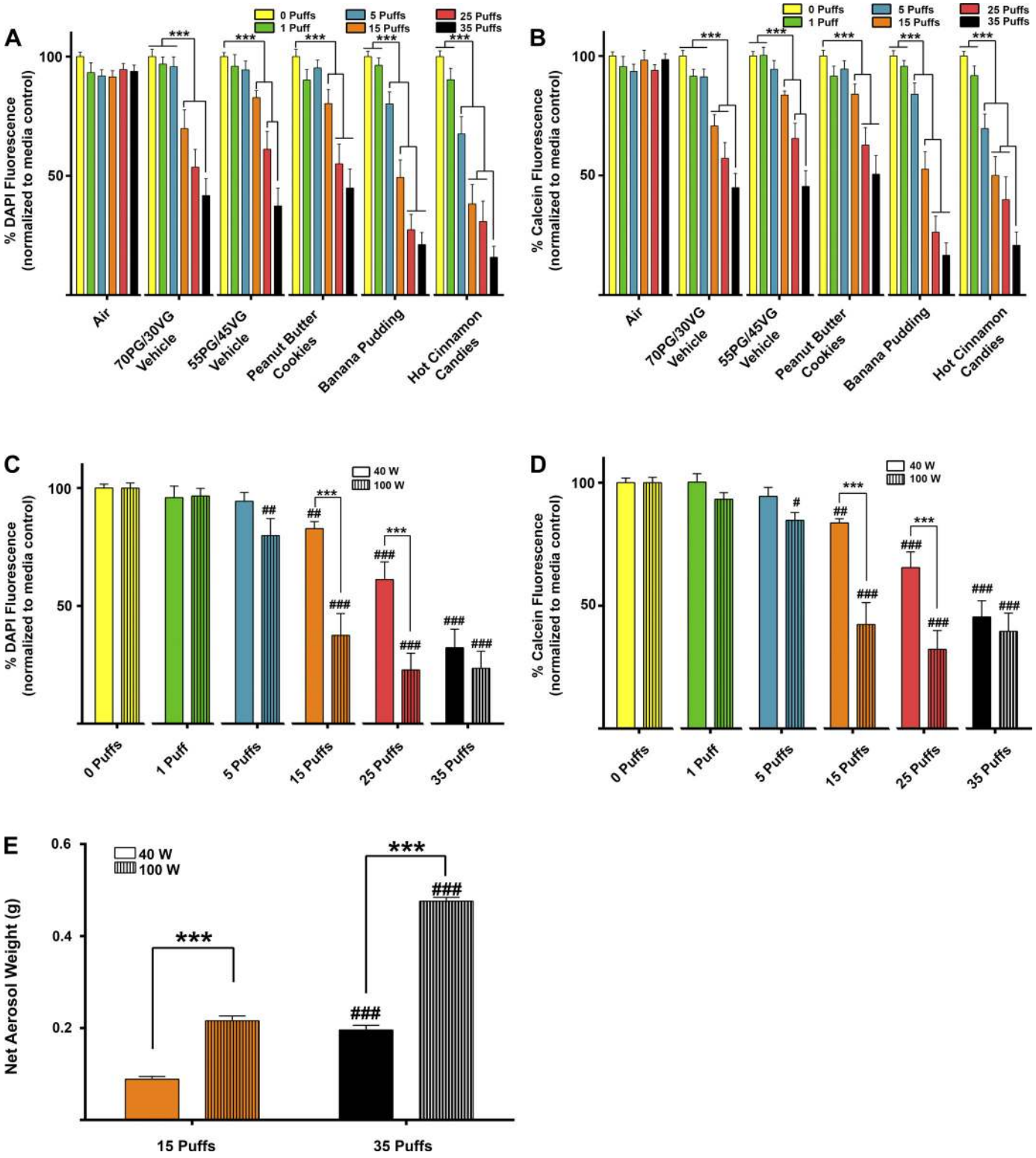


Fig. 4. Confluent CALU3 cultures show cytotoxicity after Hot Cinnamon Candies and Menthol Tobacco flavor exposure. CALU3 cells were seeded at 45,000 per well in 96-well plates for 12 h until confluent monolayers were formed. E-liquids were diluted in media in a dose-dependent manner (%vol/vol), and cells were challenged for 24 h. Cell number was measured by fixing cells and measuring DAPI fluorescence (*A*), and cell viability was measured using calcein (*B*). Bars represent average %fluorescence measured normalized to 0% e-liquid (media control) treatment per plate ± SE; $n = 12$ wells run in 4 independent experiments per treatment. Statistics were calculated using a linear mixed model with pairwise comparisons for doses within flavor (*A* and *B*). *P* values for overall tests of dose within flavor are denoted ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$), and, where applicable, further pairwise significant differences ($P < 0.05$) are indicated using cluster lines above the graph.

Aerosolized e-liquids have similar toxicity profiles as neat e-liquids. We next exposed CALU3 cells to aerosolized e-liquid "vapor" from the PG/VG controls (70 PG/30 VG and 55 PG/45 VG), Peanut Butter Cookies (less harmful flavor), and two of our more toxic flavors (Banana Pudding and Hot Cinnamon Candies). E-liquids were loaded into a tank attached to an e-cig device, and a syringe was used to collect and measure out 4 s/70 ml puffs that were then manually administered to cells at 30-s intervals. Cells were given 100 μl new media before exposure and then left in the aerosol-exposed media for 24 h before total cell number (%DAPI fluorescence) and cell viability (%calcein fluorescence) were measured. A



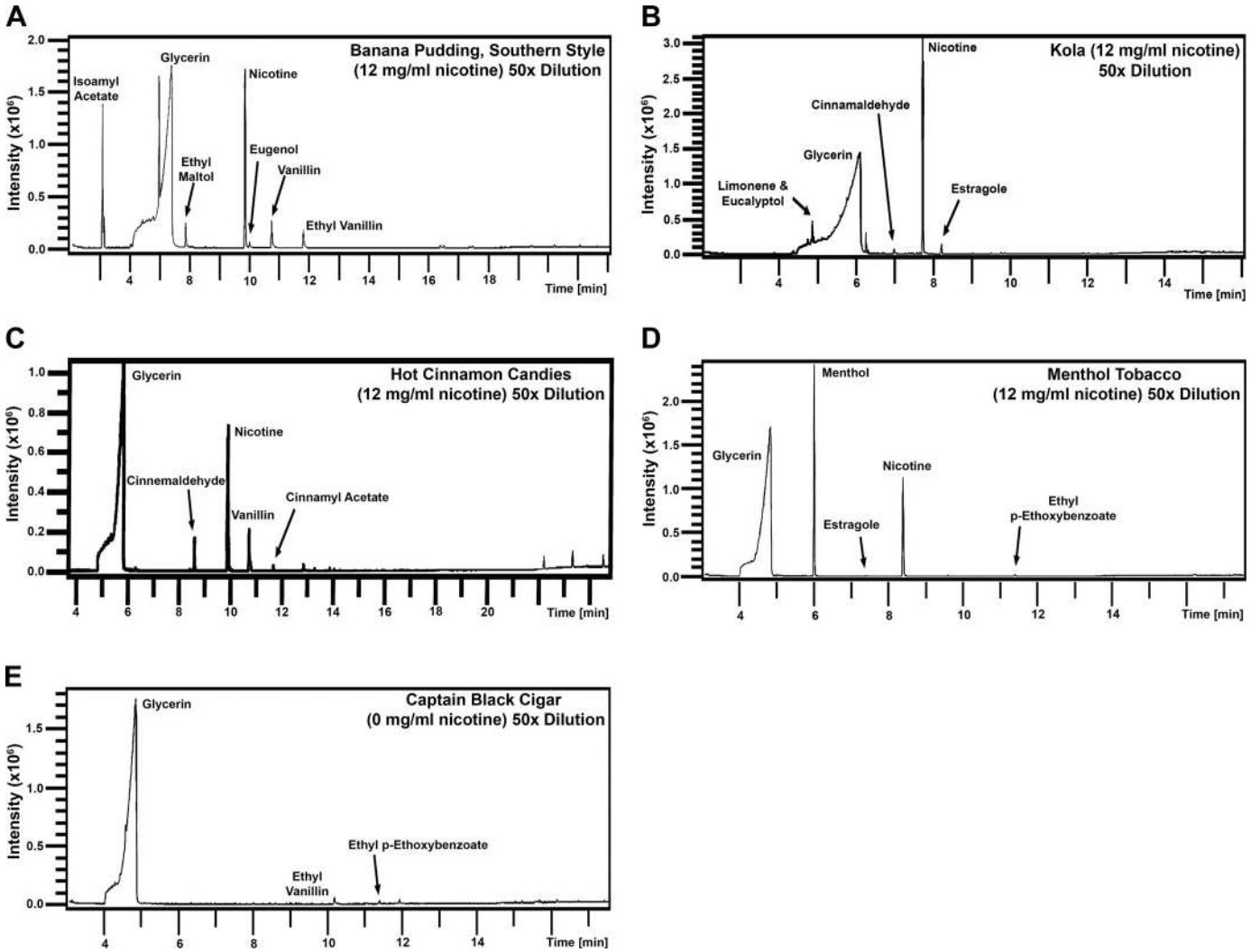


Fig. 6. Gas chromatography-mass spectrometry (GC-MS) identified individual chemical constituents in the 13 different e-liquids. GC-MS was used to detect between 9 and 25 individual chemical constituents for individual e-liquid flavors across all 13 flavors tested. Annotated gas chromatograms of Banana Pudding (Southern Style) (*A*), Kola (*B*), Hot Cinnamon Candies (*C*), Menthol Tobacco (*D*), and Captain Black Cigar (0 mg/ml nicotine) (*E*) depict examples of peaks derived for individual constituent identification in e-liquids diluted 50 times.

media control group (0 puffs) was run in every plate, and wells were covered with fitted silicone strips during the exposure to ensure no unwanted exposures. Control groups with equal numbers of air puffs were also run. A 55 PG/45 VG vehicle group was added because the vendor had shifted from a 70 PG/30 VG to 55 PG/45 VG ratio and additional Peanut Butter Cookies, Banana Pudding, and Hot Cinnamon Candies e-liquids were required to conduct our experiments.

We found that all of the flavors and the PG/VG vehicle controls caused dose-dependent decreases in cell number (Fig. 5*A*). However, there was no effect of our air control group. We also found that both Banana Pudding and Hot Cinnamon

Fig. 5. E-cig aerosols dose dependently decrease cell number/viability in subconfluent CALU3 cultures. CALU3 cells were seeded at 25,000 per well in 96-well plates for 4–8 h before aerosol exposure. Aerosols were generated at 40 or 100 W and each 70 ml puff was distributed among 6 wells using a multichannel manifold at a rate of 1 puff/30 s. Media were not changed for 24 h following aerosol exposure. *A–D*: dose-dependent outcomes of cell number (DAPI) or cell viability (calcein) between flavors or PG/VG controls were measured (n = 18–54 wells per treatment). *C* and *D*: effects of wattage (40 and 100 W) were compared in the 55 PG/45 VG treatment where %DAPI and calcein fluorescence were measured (n = 18–48 wells per treatment). Aerosol phase particles were captured from 55 PG/45 VG at either the 40 or 100 W settings for 15 and 35 puffs using Cambridge filter pads. *E*: aerosol was collected on preweighed filter pads, and the net weight (g) of aerosol-phase particles were plotted (n = 7 per treatment). Bars represent average %fluorescence measured normalized to 0 puff (media control) treatment per plate ± SE (*A–D*) or average net weight of filter pads per treatment ± SE (*E*). Statistics were calculated using a linear mixed model with pairwise comparisons for doses within exposure treatment (*A–D*). A linear model was used to obtain the statistical results in *E*. *A–E*: P values for overall test of dose within flavor are denoted (***$P < 0.001$) with further pairwise significant differences ($P < 0.05$) indicated using cluster lines above the graph. Differences shown without brackets were compared with either the 0 puff control for respective treatments (*C* and *D*) or between puff numbers within a wattage setting (*E*) (#$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$).

Candies were more toxic than either Peanut Butter Cookies or the PG/VG groups after aerosol exposure at greater or equal to five puffs (Fig. 5*A*; $P < 0.001$). We also found again that all flavors and PG/VG vehicle controls caused dose-dependent decreases in cell viability (Fig. 5*B*), and again, there was no effect of air exposure. Both Banana Pudding and Hot Cinnamon Candies were more toxic than either Peanut Butter Cookies or the PG/VG groups after aerosol exposure at greater or equal to five puffs (Fig. 5*B*; $P < 0.001$). Importantly, the same order of toxicity demonstrated after aerosol "vape" exposure was also seen after e-liquid exposures, suggesting that direct e-liquid exposure is valid for determining relative toxicity.

Since many e-cig devices have adjustable power settings, we decided to investigate the impact of this parameter of aerosol output on cell number and viability as well. The aerosol data in Fig. 5, *A* and *B*, were generated at 40 W. We next compared the effects of the 55 PG/45 VG vehicle produced at 40 vs. 100 W. We found that aerosol generated at 100 W exerted significant biological effects after 5 puffs, while the threshold for the 40 W setting was 15 puffs (Fig. 5, *C* and *D*). We also observed significant differences in the DAPI and calcein outcomes for 40 vs. 100 W settings at 15 and 25 puffs. However, wattage no longer had any effect at 35 puffs. We then passed either 15 or 35 puffs of the 55 PG/45 VG vehicle at either 40 or 100 W through preweighed Cambridge filter pads (2-μm pores) to collect aerosol particles. We observed a significant increase in weight for both wattage settings, indicating that the filter pads were collecting aerosolized e-liquid. We also detected a significantly greater weight when aerosol was generated at 100 W than at 40 W per puff number. Taken together, these data suggest that either increasing the number of puffs or the wattage increases the amount of aerosol that cells are exposed to. There was no significant difference in the weight of filter pads exposed to fifteen, 100-W puffs or 35, 40-W puffs 55 PG/45 VG (Fig. 5*E*), suggesting that cells were exposed to a similar toxic burden with either setting (Fig. 5, *C* and *D*).

GC spectra identify a range of chemical constituents per flavor, displaying the variety of unique flavor profiles in commercially available e-liquids. GC-MS was conducted on all 13 e-liquids flavors. Annotated gas chromatograms for representative flavors are shown in Fig. 6, *A–E*. Between 9 and 25 individual chemical constituents were identified in each e-liq-

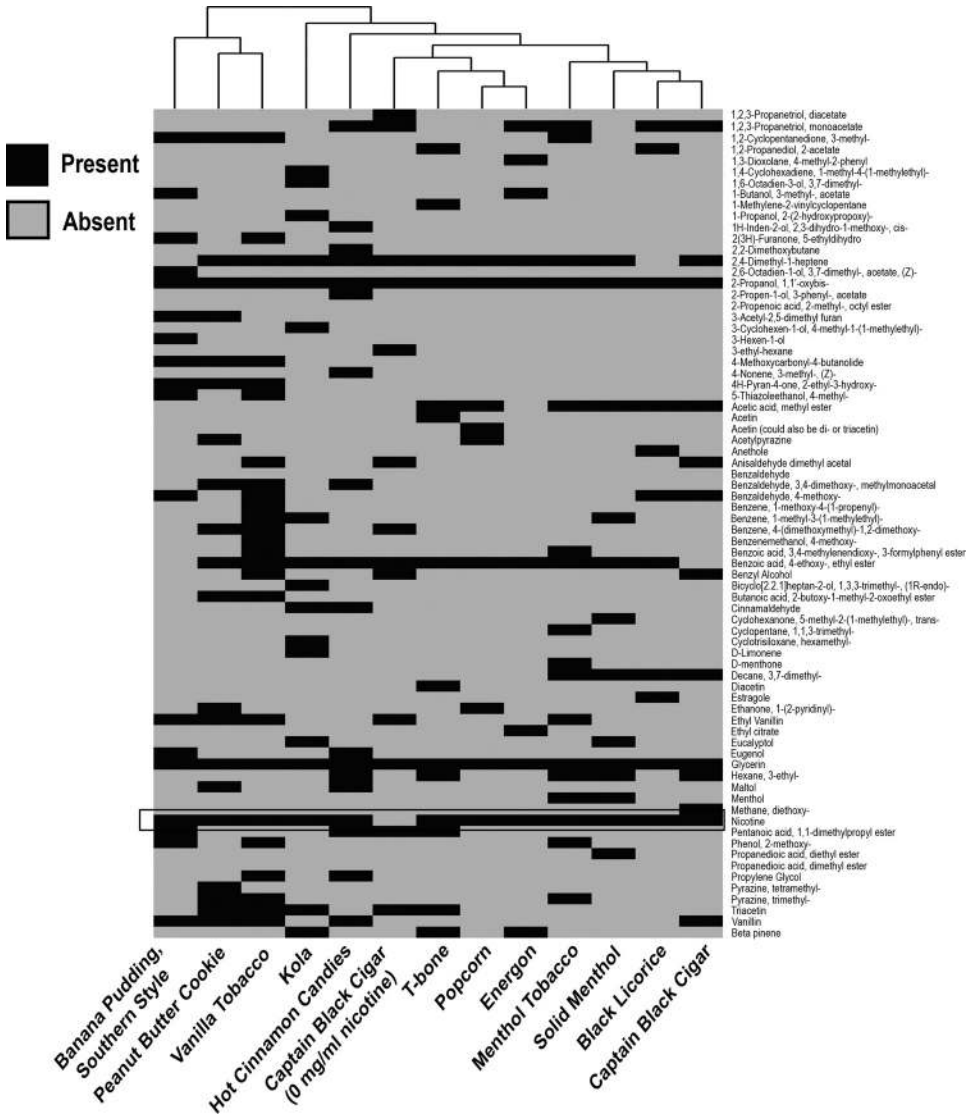


Fig. 7. Heat map of individual chemical constituents from 13 different e-liquid flavors tested. All e-liquid flavors were compared for constituent flavor profile using a heat map showing presence (black) or absence (gray) of a single constituent detected, respectively. The black box indicates the presence or absence of nicotine in each flavor as an example. All identified constituents are listed on the right and brackets group flavors by "likeness" using hierarchical clustering.

uid flavor that we investigated. Flavors in this screen were grouped using hierarchical clustering based on the presence or absence of all constituents detected (Fig. 7). For example, our analysis demonstrated that Banana Pudding (Southern Style) is least similar to Captain Black Cigar (12 mg/ml nicotine) and most similar to Peanut Butter Cookies and Vanilla Tobacco based on their constituent profiles.

Comparing flavor profiles in our four flavors of interest to identify potential shared or unique constituents that could contribute to cytotoxicity or inhibition of cell proliferation. Since we reported that Banana Pudding (Southern Style), Kola, Hot Cinnamon Candies, and Menthol Tobacco had the most negative effects on cell proliferation and viability, we chose to focus on comparing their constituent profiles to potentially target unique or shared constituents that could be causing cytotoxicity or inhibiting cell proliferation in CALU3 cells for future studies. Hot Cinnamon Candies and Menthol Tobacco showed cytotoxicity in confluent CALU3 cultures and when compared, their flavor profiles shared 8 constituents and have 9 and 11 unique constituents, respectively (Fig. 8*A*). Since Banana Pudding (Southern Style) and Kola inhibited cell proliferation, we compared these 2 flavors and found 3 shared constituents and 14 and 15 unique constituents, respectively (Fig. 8*B*). A detailed list of the constituents identified in these comparisons can be found in Table 2. When all four of these flavors were compared, they only shared three constituents (Fig. 8*C*). However, there were 9, 6, 7, and 11 unique constituents in Banana Pudding (Southern Style), Menthol Tobacco, Hot Cinnamon Candies, and Kola, respectively (Table 3).

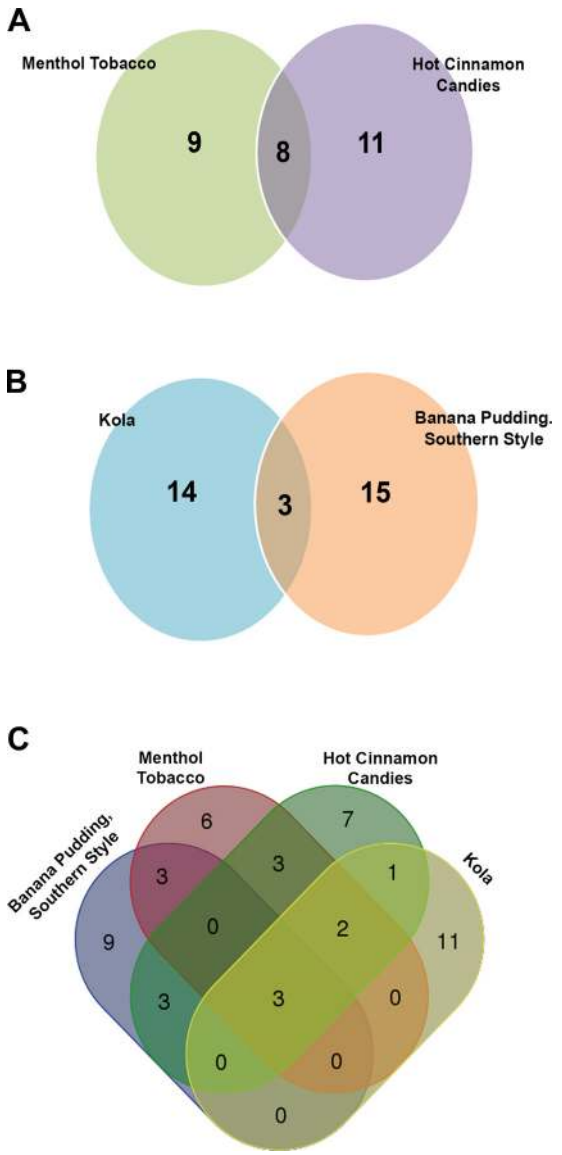


Fig. 8. Flavor profiles for the 4 flavors of interest compared with identify potential constituents responsible for either cytotoxicity or cell proliferation inhibition. Comparing between Menthol Tobacco and Hot Cinnamon Candies e-liquid flavor profiles, only 8 constituents are shared (*A*) while Kola and Banana Pudding (Southern Style) only share 3 constituents (*B*). When all 4 are compared against each other, Banana Pudding (Southern Style), Menthol Tobacco, Hot Cinnamon Candies, and Kola have 9, 6, 7, and 11 unique constituents, respectively (*C*).

DISCUSSION

In this study, we found that all 13 flavors of e-liquids, as well as the PG/VG vehicle, caused dose-dependent decreases in MTT absorbance in CALU3 cells, indicating that all e-liquids negatively affected cell proliferation. These effects were not due to the dilution of growth media since comparable dilutions with PBS were without effect (Fig. 1, *A* and *B*). Using this process, we also identified flavors of interest [Banana Pudding (Southern Style), Kola, Hot Cinnamon Candies, and Menthol Tobacco] that were significantly more toxic than the 70 PG/30 VG vehicle, indicating that some flavors are more harmful than others (Table 1). Similar dose-dependent effects have previously been reported. For instance, Bahl et al. (4) tested the effects of 40 flavored e-liquids and categorized them as "non-cytotoxic," "moderately cytotoxic," and "highly cytotoxic" based on their effects on human embryonic stem cells, mouse neural stem cells, and human pulmonary fibroblasts. They also found that cytotoxicity was caused by certain chemical constituents found in these e-liquids rather than by nicotine. Behar et al. (7) also tested the effects of 10 cinnamon flavored e-liquids on human embryonic stem cells and pulmonary fibroblasts, using the MTT assay, and found that all flavors exhibited cytotoxicity with stem cells being more sensitive than fibroblasts. Sherwood and Boitano (46) screened specific flavored constituents on immortalized human bronchial epithelial cells (16HBE14o-) for toxicity thresholds and found cytotoxic threshold for five of the seven chemicals tested. Taken together, both the data reported here and the published data all suggest a broad heterogeneity of responses that is e-liquid dependent.

We also characterized the effects of flavored e-liquids using other well-established markers of exposure including cell number (DAPI), cell viability (calcein, MitoTracker Red), and cytotoxicity (LDH release). We also chose to measure total cell number and cell viability in both subconfluent (Fig. 3, *A–D*) and confluent (Fig. 4, *A* and *B*) cultures to assess whether these flavored e-liquids inhibited proliferation or elicited a cytotoxic response, respectively. Using these complementary techniques, we again found that Banana Pudding (Southern Style), Kola, Hot Cinnamon Candies, and Menthol Tobacco caused dose-dependent decreases in DAPI, calcein, and MitoTracker Red fluorescence that were greater than what was induced by the 70 PG/30 VG vehicle (Figs. 3, *A–C*). However, only Hot Cinna-

Table 2. *List of chemical constituents used in Fig. 8, A and B, comparisons of Menthol Tobacco and Hot Cinnamon Candies or Kola and Banana Pudding (Southern Style)*

Constituent	*A*: Menthol Tobacco Only	*B*: Hot Cinnamon Candies Only	*C*: Both Menthol Tobacco & Hot Cinnamon Candies	*D*: Kola Only	*E*: Banana Pudding (Southern Style) Only	*F*: Kola & Banana Pudding (Southern Style)
1	1,2-Cyclopentanedione, 3-methyl-	1H-inden-2-ol, 2,3-dihydro-1-methoxy-, *cis*-	1,2,3-Propanetriol, monoacetate	1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	1,2-Cyclopentanedione, 3-methyl-	2-Propanol, 1,1 oxybis-
2	Benzoic acid, 3,4-methylenedioxy-, 3-formylphenyl ester	2,2-Dimethoxybutane	2,4-Dimethyl-1-heptene	1,6-Octadien-3-ol, 3,7-dimethyl-	1-Butanol, 3-methyl-, acetate	Glycerin
3	Cyclopentane, 1,1,3-trimethyl-	2-Propen-1-ol, 3-phenyl-, acetate	2-Propanol, 1,1′-oxybis-	1-Propanol, 2-(2-hydroxypropoxy)-	2(3H)-Furanone, 5-ethyldihydro-	Nicotine
4	D-menthone	4-Nonene, 3-methyl-, (Z)-	Acetic acid, methyl ester	2,4-Dimethyl-1-heptene	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-	
5	Decane, 3,7-dimethyl-	Benzaldehyde, 3,4-dimethoxy-, methylmonoacetal	Benzoic acid, 4-ethoxy-, ethyl ester	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	3-Acetyl-2,5-dimethyl furan	
6	Ethyl vanillin	Cinnamaldehyde	Glycerin	Benzene, 1-methyl-3-(1-methethyl)-	3-Hexen-1-ol	
7	Menthol	Eugenol	Hexane, 3-ethyl-	Benzoic acid, 4-ethoxy-, ethyl ester	4-Methoxycarbonyl-4-butanolide	
8	Phenol, 2-methoxy-	Maltol	Nicotine	Bicyclo[2.2.1]heptan-2-ol, 1,3,3-trimethyl-, (1R-endo)-	4H-pyran-4-one, 2-ethyl-3-hydroxy-	
9	Pyrazine, trimethyl-	Pentanoic acid, 1,1-dimethylpropyl ester		Cinnamaldehyde	5-Thiazoleethanol, 4-methyl-	
10		Propylene Glycol		Cyclotrisiloxane, hexamethyl-	Benzaldehyde, 4-methoxy-	
11		Vanillin		D-limonene	Ethyl vanillin	
12				Eucalyptol	Eugenol	
13				Triacetin	Pentanoic acid, 1,1-dimethylpropyl ester	
14				Beta pinene	Phenol, 2-methoxy-	
15					Vanillin	

List of chemical constituents identified and compared using Venn diagrams in Fig. 8, *A* and *B*. Chemical constituents identified from gas chromatography-mass spectrometry (GC-MS) of e-liquids in 4 flavors of interest. Data were compared using R software, and lists were generated of unique and shared constituents between *group 1* (Menthol Tobacco and Hot Cinnamon Candies; *A–C*) or *group 2* [Kola and Banana Pudding (Southern Style); *D-F*].

mon Candies and Menthol Tobacco exerted effects on confluent cultures (Fig. 4, *A* and *B*). These data suggest that some flavors (i.e., Banana Pudding Southern Style and Kola) tend to inhibit cell proliferation, while other flavors (i.e., Hot Cinnamon Candies and Menthol Tobacco) are more cytotoxic. Importantly, our data indicate that the growth phase must be taken into consideration when measuring the effects of e-liquids and when comparing different studies.

E-liquids are typically heated to temperatures (100–250°C) at which glycerin can decompose (53) and form reactive aldehydes. However, whether or not e-liquids undergo significant chemical transformation, including pyrolysis and degradation, following the heating required for aerosol formation is controversial. Thus, to test the effects of heating/aerosolization, we "vaped" e-liquids using a common third generation tank-style e-cig device using 4 s/70 ml puffs, based on existing topography (14, 16, 39). Using DAPI and calcein staining in subconfluent cultures as our markers of exposure, we found that all except the air treatment were sensitive to aerosolized e-liquid exposure. Moreover, the Peanut Butter Cookies flavor had similar dose responses to the PG/VG vehicle treatments, while Banana Pudding and Hot Cinnamon Candies exposures were more toxic (Fig. 5, *A* and *B*). Importantly, we observed the same trends in decreasing cell number and viability with aerosol exposure (Fig. 5, *A* and *B*) as seen with direct e-liquid exposure (Figs. 1–4). That is, Banana Pudding and Hot Cinnamon Candies flavors were more toxic than Peanut Butter Cookies and the PG/VG vehicle both after neat e-liquid exposure and after "vaping."

We also investigated the effect of wattage on aerosol output and subsequent cellular toxicity. When we compared the effects of multiple 70 ml puffs of 55 PG/45 VG on cells generated at either 40 or 100 W, we found that the 100-W setting left-shifted the dose-dependent effects, compared with the 40-W setting (Fig. 5, *C* and *D*). Indeed, when we passed 55 PG/45 VG through a filter pad to collect aerosol-phase particles, an increase in weight, as a proxy for aerosol output, could be achieved by both increasing the puff number and by increasing the wattage (Fig. 5*E*). Thus the effects that we saw are likely due to an increase in aerosol produced by increasing the wattage (power). These observations also follow online e-cig forums on subohm "vaping" that describe 40 W as being on the cooler side with less aerosol produced, while 100 W is on the hotter side with more aerosol produced using the UWell Crown tanks [http://vaping360.com/crown-sub-ohm-tank-review-top-filling-sub-ohm-tank-uwell/, http://ecigarettereviewed.com/uwell-crown-review/]. Our data are similar to other studies that have exposed cells to either e-cig aerosols or aerosol that has been condensed back to a liquid, where both e-cig aerosol or condensate exposures result in measurable toxicity and/or ox-

Table 3. *List of unique chemical constituents identified for Fig. 8C comparisons*

Constituents	Banana Pudding (Southern Style)	Kola	Hot Cinnamon Candies	Menthol Tobacco
1	4H-pyran-4-one 2-ethyl-3-hydroxy-	1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	1H-inden-2-ol, 2,3-dihydro-1-methoxy-, *cis*-	Pyrazine, trimethyl-
2	1-Butanol, 3-methyl-, acetate	Beta pinene	2,2-Dimethoxybutane	Cyclopentane, 1,1,3-trimethyl-
3	3-Hexen-1-ol	Eucalyptol	Maltol	D-menthone
4	4-Methoxycarbonyl-4-butanolide	1,6-Octadien-3-ol, 3,7-dimethyl-	Benzaldehyde, 3,4-dimethoxy-, methylmonoacetal	Decane, 3,7-dimethyl-
5	2(3H)-furanone, 5-ethyldihydro-	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	Propylene Glycol	Menthol
6	Benzaldehyde, 4-methoxy-	Bicyclo[2.2.1]heptan-2-ol, 1,3,3-trimethyl-, (1R-endo)-	4-Nonene, 3-methyl-, (Z)-	Benzoic acid, 3,4-methylenedioxy-, 3-formylphenyl ester
7	3-Acetyl-2,5-dimethyl furan	Benzene, 1-methyl-3-(1-methylethyl)-	2-Propen-1-ol, 3-phenyl-, acetate	
8	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-	Cyclotrisiloxane, hexamethyl-		
9	5-Thiazoleethanol, 4-methyl-	D-Limonene		
10		1-Propanol, 2-(2-hydroxypropoxy)-		
11		Triacetin		

List of unique chemical constituents identified from Venn diagram comparisons in Fig. 8*C*. Chemical constituents identified from GC-MS of e-liquids in 4 flavors of interest. Data were compared using R software, and lists were generated of unique and shared constituents between all 4 flavors of interest Banana Pudding (Southern Style), Kola, Hot Cinnamon Candies, and Menthol Tobacco.

idative stress (22, 27, 35, 42, 43, 45). However, ours is one of the only studies to investigate and compare the effects of both neat e-liquids and their respective aerosols in a variety of flavors.

Nicotine is the addictive substance in tobacco smoke and e-liquids that drives addiction and maintenance of use (8). Nicotine exerts its physiological effects through nAChRs, which are ligand-gated ion channels that are expressed both in the nervous systems and the lung (1, 11, 30, 34, 55). We found that CALU3 cells expressed a number of nAChR subunits, with α5 being the most abundant (Fig. 2*A*). Stimulation of nAChR with nicotine elicits an increase in cytoplasmic Ca^{2+} levels, with an EC_{50} in the low micromolar range (9, 20). We tested the effects of nicotine on cytoplasmic Ca^{2+} homeostasis by measuring the change in fluo-4 fluorescence and found the EC_{50} to be 2.89 mg/ml nicotine (17.8 mM) with nicotine levels greater than ~2 mg/ml causing acute cytotoxicity (Fig. 2, *C* and *D* and Table 1). Since we found a difference in the Captain Black Cigar flavor ± nicotine on MTT metabolism (Fig. 1*C*), we then investigated the effects of nicotine on our cells. We tested the effects of nicotine alone and in combination with 1 or 3% PG/VG using the MTT assay (Fig. 2*D*). We found a dose-dependent decrease in percent absorbance with an IC_{50} of ~1.7 mg/ml (10 mM; Table 1). Although adding 3% PG/VG with nicotine decreased the magnitude of the response, the IC_{50}s were not different (Fig. 2*D* and Table 1). Thus, although nicotine and 3% PG/VG individually reduced MTT metabolism, together, they were not synergistic. Furthermore, since 3% e-liquid contains ~0.36 mg/ml nicotine (2.2 mM), at this dose, it is likely that PG/VG rather than nicotine caused the decrease in cell proliferation since this value was below the thresholds at which nicotine increased cytoplasmic Ca^{2+} and induced cytotoxicity (Fig. 2, *B* and *C*). We included concentrations of nicotine used in our dilutions (i.e., 0–1.2 mg/ml nicotine) as well as those reported in the literature. For example, e-liquids with up to 36 mg/ml are commercially available and nicotine delivery can vary depending on the device itself (16–18, 44). Similarly, Schweitzer et al. (45) found dose-dependent decreases in cell proliferation in lung endothelial cells exposed to 1–20 mM nicotine, which falls within our dose-response range. Garcia-Acros et al. (19) administered aerosol containing PG/VG ± 36 mg/ml nicotine to bronchial epithelia and found that nicotine alone reduced ciliary beat frequency and reduced cystic fibrosis transmembrane conductance regulator activity, suggesting a failure of mucus clearance and impaired host defense against pathogens. However, Lam et al. (25) found that ~100 nM nicotine increased gene expression of nAChRs, while West et al. (52) found an increase in cell number after 1 nM–10 mM of nicotine exposure in human bronchial epithelia. It is possible that increased proliferation could occur via nAChRs with lower more physiological levels of nicotine (1 nM–1 mM), while our results, and those of Garcia-Acros et al. (19), may have been nonspecific cytotoxic effects from the extremely high (i.e., mM) nicotine levels seen in e-liquids. Further studies will be required to differentiate between receptor-mediated and nonspecific effects of nicotine, and to understand the contribution of nicotine to the potential toxicity of e-liquids.

In our study, we conducted GC-MS analysis on all 13 flavors (Fig. 6) and performed hierarchical clustering (Fig. 7) before focusing on the individual constituents found in our 4 more toxic flavors of interest (Fig. 8). In this approach, we found that Banana Pudding (Southern Style) was more similar to Peanut Butter Cookies than to Captain Black Cigar. Further comparisons of just the four more toxic flavors of interest identified flavoring constituents such as cinnamaldehyde and vanillin, which were shown to have potentially cytotoxic properties elsewhere (7, 23, 27, 46, 50). We also found that benzene derivatives were identified in several e-liquids (Fig. 7). Of note, benzene has been directly linked with the induction of cancer (32). We also identified 9, 11, 7, and 6 unique constituents, respectively (Table 3), most of which have no available

toxicity data. Importantly, our data indicate the list of constituents for each e-liquid should be made available to the consumers to better make informed choices.

In conclusion, our study provides biological data from direct and aerosol based exposures to a diverse range of e-liquid flavors, PG/VG, and nicotine. While we do not yet know the concentrations of e-liquid in the lungs after inhalation, recent studies have found that particle size from e-cig aerosols is similar (33) or slightly smaller (29), than cigarette smoke, suggesting that it may deposit in the same fashion (28). Moreover, based on aerosol particle size, the predicted deposition of e-cig aerosol in the lungs is 15–45% (47), which is similar to the reported range of some jet nebulizers (~13–25%) (12). Thus, if 10 ml of e-liquid is "vaped," and given that the airway surface liquid volume in the lung is ~3 ml, this could lead to a dilution of 5–15%, suggesting that our dosing range of ≤10% e-liquid (vol/vol) is appropriate. However, additional experiments will be needed to directly measure e-liquid deposition patterns in the lung. Since our biological results were paired to analytical data, this has enabled the identification of a wide range of e-liquid constituents. We also provided evidence that direct e-liquid exposures are comparable to the more realistic, but more time-consuming, aerosol exposures. Thus we are providing a data set that informs as to the basic toxicological parameters of flavored e-liquids on a lung epithelial cell line that could potentially harm the lung of flavored e-cigs users.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.R.R. and R.T. conceived and designed research; T.R.R., S.L.R., and R.A.H. performed experiments; T.R.R., S.L.R., S.L.L., R.C.N., and A.H.H. analyzed data; T.R.R., S.L.R., S.L.L., R.C.N., A.H.H., G.L.G., and R.T. interpreted results of experiments; T.R.R. and S.L.R. prepared figures; T.R.R. and R.T. drafted manuscript; T.R.R., S.L.L., R.C.N., A.H.H., and R.T. edited and revised manuscript; T.R.R., S.L.R., S.L.L., R.A.H., R.C.N., A.H.H., G.L.G., and R.T. approved final version of manuscript.

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