10-2022

Chemical Interactions and Cytotoxicity of Terpene and Diluent Vaping Ingredients

Yanira Baldovinos
Baylor University

Alexandra Archer
Portland State University

James C. Salamanca
Portland State University

Robert Strongin
Portland State University, strongin@pdx.edu

Christie Sayes
Baylor University

Citation Details

Baldovinos, Y., Archer, A., Salamanca, J., Strongin, R. M., & Sayes, C. M. (2022). Chemical Interactions and Cytotoxicity of Terpene and Diluent Vaping Ingredients. Chemical Research in Toxicology. https://doi.org/10.1021/acs.chemrestox.2c00218

Chemical Research in Toxicology

pubs.acs.org/crt

Article

Chemical Interactions and Cytotoxicity of Terpene and Diluent Vaping Ingredients

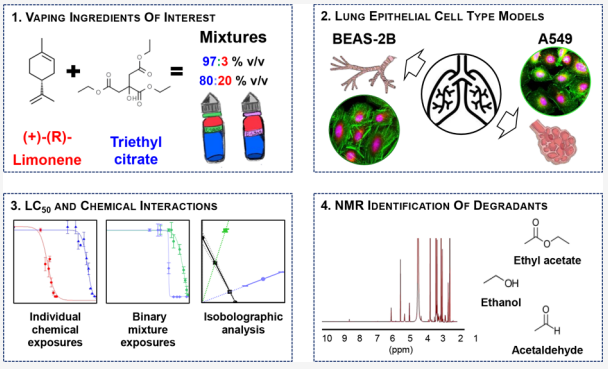
Yanira Baldovinos, Alexandra Archer, James Salamanca, Robert M. Strongin, and Christie M. Sayes*

Cite This: https://doi.org/10.1021/acs.chemrestox.2c00218

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Vaping devices have risen in popularity since their inception in 2007. The practice involves using a variety of commercially available devices. Internal heating systems in devices aerosolize e-liquid formulations of complex mixtures including an active ingredient (e.g., THC, CBD, and nicotine), diluents (or cutting agents), solvents, and flavoring agents (e.g., terpenes and aldehydes). The vaping toxicology literature consists of cytotoxicity studies of individual chemicals and commercial formulas. Because of the variation of e-liquid composition, there is a limited understanding of the toxicity of ingredient combinations. This study analyzed the cytotoxic effects after exposure to individual and binary mixtures of a representative terpene (+-*R*-limonene) and diluent (triethyl citrate) on human lung cell models. Data were analyzed to determine the effects of 97:3 and 80:20% v/v (triethyl citrate/limonene) binary mixtures. BEAS-2B cells, a bronchial epithelial cell, and A549 cells, a type II alveolar epithelial cell, served as models for comparison. LC_{50} values were calculated and isobolograms were used to assess chemical interactions. Results show that limonene was more cytotoxic than triethyl citrate. Isobolographic analyses confirmed that the 97:3% v/v mixture resulted in an antagonistic chemical interaction. The 80:20% v/v mixture resulted in a similar result. Further testing of different ratios of binary mixtures is needed for chemical interaction screening to inform safety assessments.



INTRODUCTION

Vaping has risen in popularity among experienced smokers and young adults since its introduction as an alternative to traditional tobacco cigarettes in 2007. Over 2 million U.S. middle and high school students have vaped cannabis with an e-cigarette, which is one of many devices used for vaping, with reports of prior 30-day usage continuing to rise.[1,2] Vaping nicotine and cannabis has gained popularity among recreational users, particularly young adults and teens, likely because of the novelty and capability of the personalization of vaping products, which differentiate them from traditional tobacco products.[3] Surveys show that the allure to vaping products in young adults is the low cost, the ability to engage in visual tricks, concealability of new generation vapes, and attractive flavorings.[4,5]

Vaping devices are not singular in shape and heating power, contributing to the presence of various iterations in the market that range from vape pens, box mods, and e-hookahs.[6] Portable vaping devices, also known as e-cigarettes, are designed to include a battery, e-liquid reservoir, vaporizing chamber with a heating element, and different settings of voltage, power, and temperature for heat transformation of e-liquids into aerosols.[7,8] e-Liquids are liquid-filled cartridges containing a mixture of chemicals that are heated into aerosols comprised of gas and particulates.[6,8] The chemical composition of e-liquids may include an active ingredient such as the following: tetrahydrocannabinol, cannabidiol, or nicotine; diluents or cutting agents such as triethyl citrate; solvents such as glycerol; and flavoring agents such as terpenes, alcohols, esters, and aldehydes.[9] Active ingredients like THC can result in disruption at the CB_1 receptor, contributing to neuronal disruption.[10] Flavoring agents are used to enhance the taste of vaping aerosols. Liquid diluents serve to dilute and change the viscosity of the e-liquid mixture, aiding in solubilization of cannabis-containing vapes.[11] Different ratios of these ingredients are used, and in some cases e-liquids are free of the active ingredient, resulting in e-liquid formulations with higher ratios of diluents and flavorings.[1]

According to Zhu et al., an Internet survey of the online vaping market reported 466 brands selling about 7764 unique flavors.[12] This is indicative of the present demand for e-liquids with flavoring agents. Although different models of vaping devices use similar approaches in aerosolizing e-liquids, the e-liquid composition dictates the heating parameters of the

Special Issue: Tobacco: Chemistry, Mechanisms, Biomarkers and Disease Prevention VSI

Received: July 8, 2022

device due to the different physical properties of ingredients that require higher or lower temperatures to form aerosols.[7,8] For example, vaping ingredients like propylene glycol and vegetable glycerin are commonly used separately or in combination as a diluent and have differing properties, allowing propylene glycol to aerosolize more readily than vegetable glycerin.[7] Differences in physical properties between diluents, flavoring agents, and other vaping ingredients lead to faster consumption during heating. As a result, thermal decomposition of the e-liquid mixture leads to the formation and abundance of a wide range of toxic degradants like aldehydes.[13] The addition of flavoring agents have been found to be linked to the presence of aldehydes like acrolein and formaldehyde after heating.[13] From cigarette smoke studies, formaldehydes were reported to be more easily retained in the respiratory tract, and acrolein was more cytotoxic with increasing dose of exposure in hamster ovary cells.[14]

Because of the complexity of vaping devices (i.e., design, power, thermal capacity, etc.), e-liquid starting composition, and products after thermal decomposition, there is a need to determine methods that isolate and efficiently characterize the chemical composition of popular vaping ingredients and resulting toxicity effects. This need has become urgent because of the recent outbreak in 2019 when the CDC designated an increasing number of hospitalizations with patients presenting adverse respiratory symptoms and injuries as EVALI patients as a result of their common history of vaping.[15] *In vitro* studies have found toxicity links after exposures to vaping ingredients like triethyl citrate with increasing concentration and rapid lung damage after exposures to propylene glycol in mice studies, but there is still uncertainty concerning the specific biological mechanisms contributing to the presentation of lung injury in EVALI patients.[11,16] More information is needed concerning single constituents and mixtures of vaping ingredients to proceed in defining their potential hazard index. The purpose of this study was to use chemical characterization in conjunction with lung *in vitro* cytotoxicity data to understand the potential changes that occur between single and mixture exposures of a terpene (limonene) and diluent (triethyl citrate). Further analysis using NMR allowed for chemical analysis of a vaped binary mixture to aid in isolating the concentration of degradant presentation to be used for future toxicity analysis.

MATERIALS AND METHODS

Chemicals and Reagents. All reagents were purchased at the highest purity available. Triethyl citrate (TEC, 99%), (*R*)-(+)-limonene (limonene, 97%), and Triton X-100 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (Burlington, MA, USA). Dulbecco's modified Eagle's medium (DMEM) nutrient mixture, RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin solution, and Dulbecco's phosphate buffered saline (PBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Cell Culture. BEAS-2B and A549 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The BEAS-2B cells are a bronchial epithelial cell line derived from normal tissue that is SV-40 immortalized. A549 cells are type II alveolar epithelial cells sourced from human lung carcinoma epithelial cells.[17] Both lung cell models were cultured as a monolayer in culture conditions of 37 °C and 5% CO_2. BEAS-2B and A549 cells were used for inoculation exposure assessment for LC_{50} determination and chemical interaction analysis. BEAS-2B cells were grown in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin solution in a T75 flask. A549 cells were passaged using the same techniques using RPMI media supplemented with 10% FBS and 1% penicillin-streptomycin solution. At 85% confluency, cultures were plated into 24-well plates at 40 000 cells/cm^2 and grown until 90% confluent (recommended at 120 000 cells/cm^2).[18] Importantly, A549 and BEAS-2B cell types have distinct characteristics that could result in differential observed toxicological effects. BEAS-2B is an epithelial cell line isolated from noncancerous human bronchial epithelium, whereas A549 cells are adenocarcinomic human alveolar basal epithelial cells.[18]

Cultures were treated to different liquid solutions of triethyl citrate or D-limonene as a single constituent or as a mixture for 24 h. A total of three replicates were used for each test. Stock solutions (1.5% v/v) of each chemical were formulated by diluting chemical standards in a 1:99% v/v solution of DMSO and cell culture medium. This was repeated to formulate seven additional, but diluted, concentrations per constituent type. The 97:3 and 80:20% v/v mixtures (triethyl citrate/D-limonene) stock solution was comprised of 97 and 80% v/v triethyl citrate chemical standard, 3 and 20% v/v of D-limonene chemical standard, and diluted with a solution of cell medium and 1% v/v of DMSO for lower concentrations. Lower concentrations of the 1.5% v/v mixture stock solutions were diluted further with 1:99% v/v DMSO and cell medium solution. Then 1% v/v DMSO in cell medium, untreated cell medium, and 0.1% v/v Triton X-100 served as controls for the study. An untreated cell medium served as a negative control, and exposure to 0.1% v/v of Triton X-100 was used as a positive control for cytotoxicity.

Dose−Response Relationships and LC_{50} Calculations. Cytotoxicity assessments were carried out on the BEAS-2B and A549 cells that had been previously cultured in 24-well plates with the toxicants for a 24 h exposure to single and two binary mixtures of limonene and triethyl citrate. A 97:3% v/v mixture was used for initial testing as it is an equipotent mixture based on the individual LC_{50} results from individual exposures of limonene and triethyl citrate. An 80:20% v/v mixture was used as a model of reported e-liquid solutions that have increased concentrations of flavoring agents compared to that of other e-liquid components.[9] Mixtures were formulated as described above in the cell culture methods. The assay used to assess cytotoxicity was the methyltetrazolium salt (MTS) assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA). MTS is a cell proliferation assay that measures mitochondrial metabolic activity.[19] Because of the ease of use and accuracy of MTS, it is commonly used as an *in vitro* cytotoxicity assay.[20] MTS absorbance was used to calculate the LC_{50} values for single and mixed exposures.

Viability data was normalized to untreated values and fitted to the Hill equation using Sigma Plot 14.0 (Systat Software, San Jose, CA, USA). After an appropriate R^2 value was achieved, a final dose−response curve was plotted. In Sigma Plot, the linear extrapolation and bootstrap method were used in combination to calculate the LC_{50} % v/v of exposures to limonene only, triethyl citrate only, and 97:3 and 80:20% v/v (triethyl citrate/limonene) solutions.

Isobolographic Analysis and Statistical Analysis. Isobologram plots were prepared in MS Excel for exposures to both cell types. Additivity lines were drawn using the LC_{50} values for individual exposures to triethyl citrate and limonene. For the two binary mixtures, 97:3 and 80:20% v/v of triethyl citrate/limonene were used on the basis of single-component LC_{50} values (assuming additivity) and literature analysis of reported flavoring agent concentrations in characterized e-liquids. A trend line was plotted for each binary mixture. The intersection between the additivity line and the mixture trend lines was used to calculate the predicted LC_{50}, which falls on the additivity line based on a prediction of an additive chemical interaction.[19] An interaction index was calculated on the basis of the ratio of the predicted and measured LC_{50} values. A Student's *t*-test was run to determine the significance between the predicted and measured LC_{50} values of the mixture.[19] Significantly different tests are indicative of a difference in chemical interaction of the predicted LC_{50}, which is based on the assumption of an additive interaction.[19]

https://doi.org/10.1021/acs.chemrestox.2c00218
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

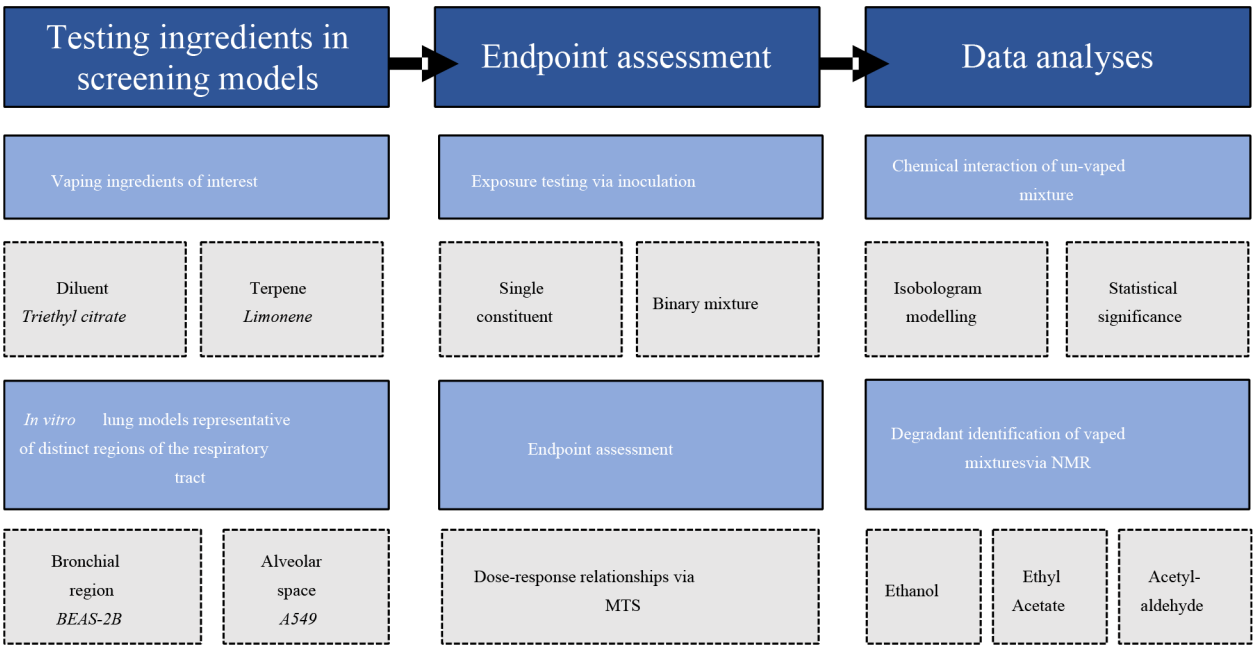


Figure 1. Flow chart of experimental design used to screen for cytotoxicity via dose−response relationships for single constituents and binary mixtures of vaping ingredients. For this study, a terpene (e.g., limonene) and a diluent (e.g., triethyl citrate) were used. Dose−response relationships were assessed in BEAS-2B and A549 cells. The experimental design includes chemical interaction analyses for unvaped mixtures as well as NMR analysis of the vaped mixtures.

Characterization of Vaped Mixtures via Nuclear Magnetic Resonance Spectroscopy. Reaction products occurring during the aerosolization of triethyl citrate and limonene in 97:3 and 80:20 molar ratios were analyzed via ^{1}H NMR. Aerosol was drawn using a single cigarette smoking machine (SCSM-STEP, CH technologies) set at a flow rate of 1.1 L/min. Puff topography used was a modified CORESTA standard puffing regime with a 55 mL puff volume for 3 at 30 s intervals, for a total of 30 puffs per trial. A CCELL cartridge was weighed before and after a 0.4 mL triethyl citrate/limonene mixture (or control pure triethyl citrate or limonene standards) was added to the 0.5 mL capacity cartridge attached to a 510-threaded battery. The lithium battery was set for 3.5 V.

Aerosols were generated and analyzed as follows. Particulate matter was collected on a Cambridge filter pad connected in series between the vaping cartridge and impinger. The filter was weighed prior to and after vaping. The gas-phase compounds were collected by bubbling through the impinger containing 1 mL of DMSO-d_6 + 0.05% v/v tetramethyl silane. After vaping, 490 μL of the DMSO-d_6 solution was transferred to a nuclear magnetic resonance (NMR) tube via syringe. As an internal standard, 10 μL of a 10 mmol 2,3,5,6-tetrachloro nitrobenzene (TCNB) stock solution was also added. The sample was analyzed using a Bruker 600 MHz NMR spectrophotometer (512 scans, 3 s relaxation delay). The qNMR method reported and a validated study by Salamanca et al. was used to quantify the gaseous fraction of products produced during vaping.[21]

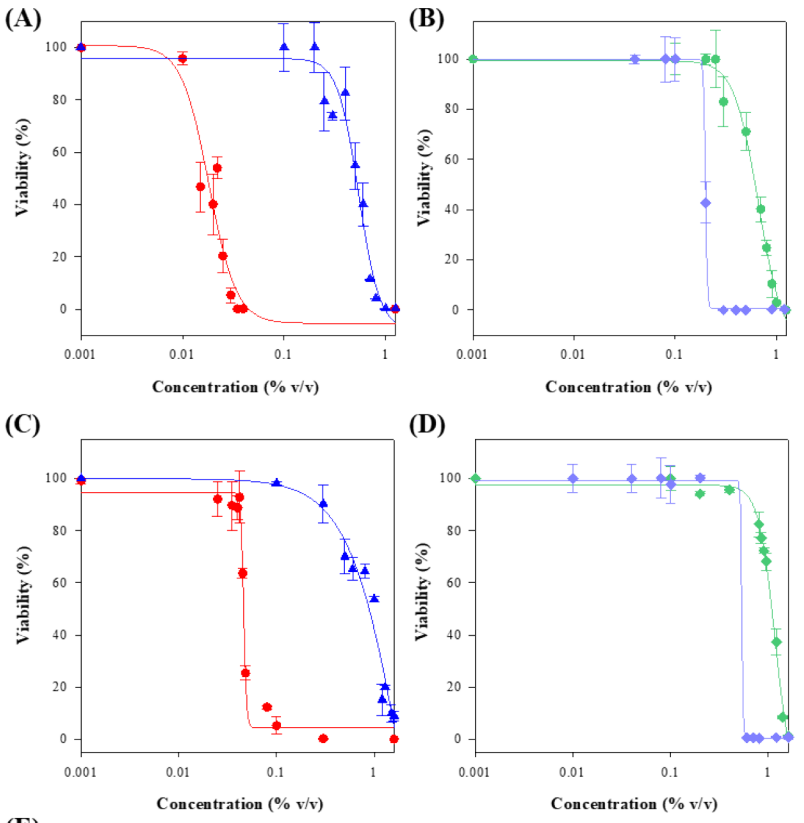
RESULTS

For this study, a stepwise approach was used to analyze two binary mixtures (i.e., 97:3 and 80:20% v/v) using toxicity data to analyze the chemical interaction of the unvaped form of the mixture and nuclear magnetic resonance to characterize degradants of the vaped mixture as shown in Figure 1. Specific to the ratio of 97:3 and 80:20% v/v (triethyl citrate/limonene) solution, the cytotoxicity resulted in an antagonistic reaction, indicating a less toxic interaction compared to the toxicity of single exposures to triethyl citrate and limonene (Figure 1 and Figure 2). Nuclear magnetic resonance analyses showed the chemical transformation in *ex vivo* vaped conditions of the 97:3 mixture (Figure 4).

Figure 2 shows the resulting LC_{50} dose−response curves after 24 h exposures to unvaped limonene or triethyl citrate on BEAS-2B and A549 human lung cells. In response to increasing concentrations of limonene or triethyl citrate, there was a decrease in cell viability, resulting in a dose−response relationship for cytotoxicity analysis. After single exposures of each chemical, limonene was found to be more cytotoxic than triethyl citrate [LC_{50} values for limonene were 0.016 v/v % (1.0 ± 0.025 mM) in BEAS-2B cells and 0.046 v/v % (2.8 ± 0.0062 mM) in A549 cells, compared to those of triethyl citrate at 0.53 v/v % (22 ± 1.9 mM) for BEAS-2B and 0.940 v/v % (40 ± 0.26 mM) for A549 (Figure 2A,C].

Two binary mixtures were tested on both cell models to test for the resulting toxicity in the middle (represented by BEAS-2B epithelial monolayers) and lower (represented by A549 epithelial monolayers) regions of the respiratory tract.[9] A ratio of 97% v/v triethyl citrate and 3% v/v limonene was determined as the equipotent ratio from the LC_{50} values calculated after single exposures. A second binary mixture was tested at 80% v/v triethyl citrate and 20% v/v limonene to test for changes in cytotoxicity due to increased terpene concentration. Higher concentrations of starting stock solutions were utilized to observe close to 0% cell viability for mixture solutions as compared to those of single chemical solutions. For the equipotent mixture, the resulting LC_{50} for BEAS-2B cells was 0.62% v/v (26 ± 0.84 mM) and 1.1% v/v (43 ± 0.84 mM) for A549 cells (Figure 2B,D). In contrast, the 80:20% v/v mixture resulted in an LC_{50} for BEAS-2B cells calculated at 0.18% v/v (7.9 ± 1.9 mM) and 0.43% v/v (19 ± 5.0 mM) for A549 cells, indicating that the increased concentration of limonene resulted in increased cytotoxicity as compared to that of the equipotent mixture (Figure 2B,D). The shift in cytotoxicity due to limonene is similar to the

https://doi.org/10.1021/acs.chemrestox.2c00218
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX



(E)

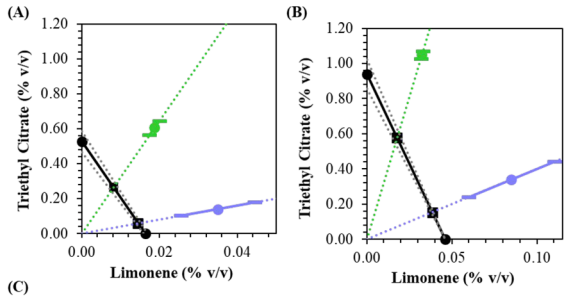
Cell type	Treatment	LC_{50} ± STDEV (% v/v)
BEAS-2B	Limonene	0.016 ± 0.00040
	Triethyl citrate	0.53 ± 0.047
	97:3 % v/v mixture	0.62 ± 0.036
	80:20 % v/v mixture	0.18 ± 0.042
A549	Limonene	0.046 ± 0.00010
	Triethyl citrate	0.94 ± 0.064
	97:3 % v/v mixture	1.1 ± 0.020
	80:20 % v/v mixture	0.43 ± 0.11

Figure 2. LC_{50} dose−response results in unvaped single and two binary mixture exposures of limonene and triethyl citrate tested against two human lung cell models. Concentrations along the *x*-axis reported in % v/v concentrations of single constituents and mixtures diluted in 1:99% v/v DMSO and cell medium solution. (A) Dose−response results after exposure to limonene (red) and triethyl citrate (blue) on BEAS-2B cells, a human bronchial epithelial lung cell. (B) Dose−response results after 97:3 (green) and 80:20% v/v (purple) binary mixture exposure on BEAS-2B cells. (C) Dose−response results after exposure to limonene (red) and triethyl citrate (blue) on A549 cells, a human type II alveolar epithelial lung cell. (D) Dose−response results after binary mixture 97:3 (green) and 80:20% v/v (purple) binary mixture exposure on A549 cells. (E) Table with calculated average LC_{50} % v/v values (±STDEV).

dose−response results measured in the single-constituent exposures where limonene was shown to be more cytotoxic (Figure 2A,C).

LC_{50} values were used to create an isobologram model to determine the chemical interaction of the binary mixtures tested in Figure 2B,D against each human lung cell. Individual LC_{50} values were plotted on the *x* and *y* axes in Figure 3 symbolized by black circle points. These points were connected by a black trend line known as the additivity line. The 95% confidence intervals of the associated error for each LC_{50} are indicated by dotted lines and dictate the additivity zone. The three chemical interactions of antagonism, synergism, and additivity are represented by three zones above, below, and within the additivity range, respectively. The green and purple trend lines running perpendicular to the additivity line are representative of the 97:3 and 80:20% v/v ratio lines. Each point of intersection per mixture of the additivity line and the mixture ratio line is denoted by a black square. The point of intersection of the additivity line and each trend line was used to determine the ratio of limonene and

https://doi.org/10.1021/acs.chemrestox.2c00218
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX



(C)

Cell type	Tested fraction of chemicals (% v/v)		LC_{50} (95% confidence limit) (% v/v)		Interaction index
	Limonene	Triethyl citrate	Predicted	Measured	
BEAS-2B	0.019	0.60	0.27 (0.25-0.29)	0.62 (0.58-0.66)	2.3
	0.035	0.14	0.073 (0.070-0.076)	0.18 (0.13-0.22)	2.4
A549	0.032	1.0	0.59 (0.56-0.63)	1.1 (1.1-1.1)	1.8
	0.085	0.34	0.19 (0.19-0.20)	0.43 (0.30-0.55)	2.2

Figure 3. LC_{50} isobologram models of a 97:3 and 80:20% v/v mixtures of triethyl citrate and limonene tested against two human lung cell models. (A) Antagonistic interaction resulting after 97:3% v/v (green; Student's *t*-test: p = <0.001) and 80:20% v/v (purple; Student's *t*-test: p = 0.014) mixture exposure on BEAS-2B cells. (B) Antagonistic interaction resulting after 97:3% v/v (green; Student's *t*-test: p = <0.001) and 80:20% v/v (purple; Student's *t*-test: p = 0.022) mixture exposure on A549 cells. (C) Predicted and measured LC_{50} values and interaction indices via isobolographic analysis based on % v/v. Predicted measurements were calculated from the intersections of the additivity line (black) and mixture trend lines (green and purple) from Figure3A,B, resulting in predicted LC_{50} values assuming an interaction of additivity.

triethyl citrate, which were then combined to calculate the average predicted LC_{50} of each mixture. On the basis of the isobolographic model the predicted LC_{50} values indicate an assumption of additivity as it falls on the additivity line. The point of intersection of the additivity line and the mixture ratio line is denoted by a black square. The ratio of limonene and triethyl citrate is calculated from the point of intersection to find the average predicted LC_{50} of each mixture that would theoretically induce an additive effect. The green and purple circle data points with the error represented by the error bars symbolize the LC_{50} values calculated for each mixture as shown in the table of Figure 2.

Figure 3A shows the resulting chemical interaction after exposure to 97:3 and 80:20% v/v binary mixtures against BEAS-2B cells are antagonistic. To confirm that the measured LC_{50} resulted in an antagonistic effect, the interaction index was calculated using a ratio of the predicted and measured LC_{50} values, resulting in 2.287 for the 97:3% v/v mixture and 2.401 for the 80:20% v/v mixture. Figure 3B shows the resulting chemical interaction after the exposure to the same mixtures tested in Figure 3A against A549 cells. The chemical interaction for both mixtures tested on the A549 cells resulted in an antagonistic interaction with indices calculated at 1.8 for the 97:3% v/v mixture and 2.2 for the 80:20% v/v mixture. Under the Loewe additivity model, interaction indices of the mixture treated with either cell model is indicative of an antagonistic chemical interaction.

A Student's *t*-test was run on Sigma Plot to test for significance between the measured and predicted LC_{50} values for both cell models. For the 97:3% v/v mixture tested on BEAS-2B and A549 cells, the resulting *p*-value was less than 0.001, indicating a significant difference between the measured and predicted values. The Student's *t*-test for the 80:20% v/v mixture tested on BEAS-2B (p = 0.014) and A549 (p = 0.022) cells indicated statistical significance between the predicted and measured LC_{50} values.

Figure 4 shows the resulting NMR analysis of vaped 97:3% v/v (triethyl citrate/limonene) mixture. The emission products of the 97:3 mixture were not different from those of the 80:20 mixture based on the ^{1}H NMR measurements. Figure 4B represents the full spectra of the components measured in a vaped mixture of 97% triethyl citrate and 3% limonene. On the basis of peak formation and ppm position, three main degradants were isolated in the vaped mixture. Ethyl acetate is one degradant formed as shown in Figure 3C where four peaks are centered around 4 ppm. The second degradant measured was ethanol, as represented by a single peak centered between 4.4 and 4.3 ppm (Figure 3D). Acetaldehyde resulted in three main peaks measured around 9.7 ppm, as shown in Figure 3E.

DISCUSSION

The use of vaping devices has increased exponentially since its introduction into the tobacco market with around 6.9 million reported U.S. adult smokers in 2017.[22] Popularity of e-cigarettes has increased because of the introduction of flavoring agents in e-liquid solutions, resulting in a market of thousands of flavored e-liquids.[12] As a result of demand, vaping devices have evolved in heating properties to induce aerosolization of e-liquid chemicals with varying physical properties. The rapid innovation of e-cigarette devices and e-liquid formulations has resulted in under-regulation and misuse of chemicals that are GRAS designated.[23] GRAS designations for vaping diluents and flavoring agents like triethyl citrate or limonene have been misinterpreted by vaping producers and users as safe for vaping activities. GRAS-approved chemicals are designated safe for ingestion not inhalation, thus contributing to the need for more toxicity data of single vaping ingredients and mixtures.[23]

Vaping studies have started to isolate common vaping ingredients with a focus on diluents and terpenes, which are commonly used in cannabis-containing products. The focus on diluents and terpenes separate from and in combination with the active ingredients can be linked to the presentation of vitamin E acetate (VEA) in BALF samples from EVALI patients across 16 states in the U.S.[24] Although VEA is commonly used in consumer products that are ingested or dermally applied, the presentation of VEA in BALF samples led to a re-evaluation of only investigating the effects of the active ingredients in EVALI patients.[24] Blount et al. also detected other compounds like THC, nicotine, and limonene BALF samples, albeit in low concentrations. The presence of both active ingredients is important to note as nicotine-containing vapes can be a precursor to vaping of e-liquids containing cannabis in users, especially in young adults.[25] The presentation of diluents and terpenes in the samples indicates that the GRAS designation of these common liquids may not be applicable when heat-transformed or exposed to the increasingly sensitive regions of the respiratory tract, leading to more questions of their hazards.[26] The presence of diluents,

https://doi.org/10.1021/acs.chemrestox.2c00218
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

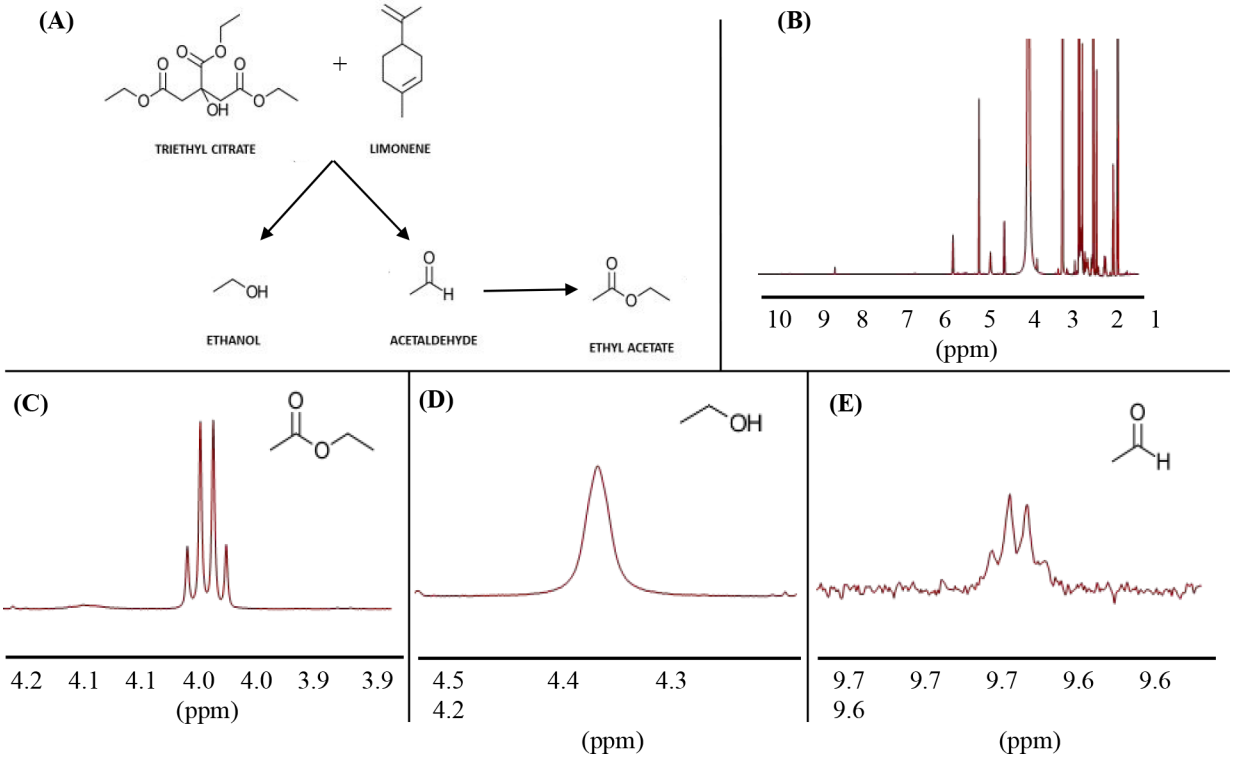


Figure 4. ^{1}H NMR of 97:3 mol/mol triethyl citrate/limonene mixture vaped. (A) Compounds ethanol, ethyl acetate, and acetaldehyde were major products identifiable in the ^{1}H NMR of triethyl citrate/limonene 97:3 condensate mixture collected after vaping. Similar spectra were collected for the 80:20 mixture. (B) Full ^{1}H NMR spectrum in DMSO-d_6. (C) Expanded ethyl acetate peak. (D) Expanded ethanol peak. (E) Expanded acetaldehyde peak.

active ingredients, and terpenes in BALF samples provides evidence to support studies that look at multiple zones of the respiratory tract, specifically the mid and lower regions.

For this study, the use of cells representative of the bronchiolar (BEAS-2B) and alveolar respiratory regions (A549) was important in detecting changes in cytotoxicity in the middle and distal regions of the respiratory tract. Bronchial and epithelial cells are common models used for lung injury studies as these are common regions where xenobiotic metabolism is induced after inhalation exposure.[27] Potential changes in toxicity and chemical interaction in response to exposure between each cell type can aid in the development of high-throughput targeted assessments specific to perturbation to epithelial cells in the bronchial and alveolar regions of the respiratory system. For example, BEAS-2B and A549 cells have been used in respiratory syncytial virus (RSV) infection studies as both cell models have been found to respond differently when infected with RSV.[28] In this study, BEAS-2B cells expressed genes that restricted infection, whereas infected A549 cells activated genes responsible for pro-inflammatory response.[28] This indicates the importance of the application of different cell models for inhalation studies to improve the understanding of downstream effects of inhaled aerosols and particles as they move into more sensitive regions. There must be more testing with different *in vitro* models to find the common pathways that could be linked to potential lung injury.

Because of the variance in e-liquid composition between manufactured or at-home solutions, the isolation of a set of chemicals used in vaping products is difficult. Recently, vaping focused studies have isolated analytical methods to isolate common chemicals used in market e-liquids. This has aided in focusing biological analysis of vaping compounds on commonly reported vaping ingredients. In one study, Guo et al. used gas chromatography−mass spectrometry (GC-MS) to screen cannabis vaping cartridges and found that many of the common flavoring agents used were caryophyllene, bisabolol, myrcene, and limonene, most of which did not correspond to the listed ingredients on the package or the name of the e-liquid.[29] Limonene was found in 8 of the 12 samples tested and was used in conjunction with myrcene in one product type called Blue Dream.[5] Even though products like Blue Dream are marketed for the sedative properties of myrcene, there are also other common terpenes like limonene used in addition that may not be listed.[29] The presentation of limonene in BALF samples and in several vape cartridges indicates attractive properties of limonene that can be used in both nicotine- and cannabis-containing vapes, supporting toxicity assessment conducted in our study.[24,29] A literature assessment of vaping constituent analysis was essential in isolating a common terpene and diluent for toxicological assessment.

The resulting toxicities after single exposures of unvaped triethyl citrate and limonene were investigated to provide a foundational understanding of the starting toxicity of the parent form of the e-liquid ingredients before thermal transformation. Inoculation exposures can also serve as a high-throughput approach for initial toxicity screening of multiple vaping ingredients or mixtures. As a result, the LC_{50} values of triethyl citrate was higher than limonene in both A549 cells and BEAS-2B cells. In the binary mixtures of both triethyl citrate and limonene, the increased ratio of limonene in the 80:20% v/v mixture resulted in higher toxicity than that observed in the equipotent mixture, indicating that the ratio of terpene use in e-liquid mixtures should be limited as toxicity

https://doi.org/10.1021/acs.chemrestox.2c00218
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

can greatly vary between constituents (Figure 2E). This is similar to results seen in solutions tested in Marescotti et al.'s study where mixtures containing flavoring agents with high Tox-Scores were reported to be potential contributors to increased toxicity.[30]

Jiang et al. used a bronchial cell model to test for changes in cellular toxicity and cell membrane integrity after exposure to unvaped and vaped e-liquid ingredients.[11] Similar cellular toxicity results of 100% viability were measured with solvent control tests of 1% DMSO dissolved in media between this study and Jiang et al.'s study, allowing for use of DMSO as the solvent for inoculation exposure testing. Jiang et al. reported a decrease in cell viability as unvaped triethyl citrate was tested at a range from 0.01 to 1% v/v concentrations. Similarly, for this study BEAS-2B cells were used to test for cellular toxicity in the bronchial region of the respiratory tract, and exposures to triethyl citrate alone resulted in viability trends similar to those reported by Jiang et al. The team also found that unvaped and vaped TEC showed dose-dependent cell death with little significance between the liquid and aerosolized forms.[11] Although important to test vaped forms of the ingredients, the findings of Jiang et al.'s study comparing unvaped and vaped triethyl citrate contribute to the possibility of using inoculation studies for quick toxicity assessments of multiple vaping ingredients.

The next phase of this study was to investigate the resulting chemical interaction of an equipotent and 80:20% v/v binary mixtures of triethyl citrate and limonene. Isobolographic analysis was used in this study to assess and define the chemical interaction of the two binary mixture solutions made using chemical standards of triethyl citrate and limonene.[19,31] Chemical interactions could result as synergistic, additive, or antagonistic when compared to the initial single exposures of each chemical, as prescribed by the Loewe additivity model.[32] Essentially, this model is a mass conservation law where the ratio of the binary mixture predicted and measured LC_{50} values provide an interaction index. If the interaction index is less than 1, the effect of the combination is considered synergistic. If the index is equal to 1, then the effect is considered additive, and if the interaction index is greater than 1, then the effect is defined as antagonistic.[19] The point of intersection provides the predicted LC_{50} that would fall on the additive line and serves as a marker of comparison for the measured LC_{50}. For this study, Liu et al.'s isobolographic modeling used for chemical interaction of binary mixtures of water disinfection byproducts was used for the analysis of binary vaping mixtures tested on human lung cells. Our model defined each binary mixture chemical interaction as antagonistic, which was supported by interaction indices reported greater than 1.

Our reported chemical interactions differ from the synergistic interactions reported in Marescotti et al.'s study. This is due to the difference in mixture composition in our study as we focused on a binary mixture of a diluent and terpene, whereas in Marescotti et al.'s study they used flavored mixtures compared to baseline mixtures containing two diluents (propylene glycol (PG) and vegetable glycerol (VG)) and 0.6% nicotine.[30] The addition of an active ingredient and multiple diluents may have had effects on the resulting synergistic interaction reported in this study. A second study looked at equimolar ratios of nicotine-free vaping solutions mixed with the 10 flavoring agents that were tested for toxicity against monocytic cells.[33] This study discovered increased cytotoxicity in equimolar ratios. Our study also served to isolate the chemical interaction specifically for diluent and terpene mixtures as a beginning assessment of the direct effects of these chemcials without introducing an active ingredient.

The use of NMR analysis is helpful in isolating degradation products after heating of the 97:3 mixture. Similar spectra were collected for the 80:20 mixture. The reported degradants that may be more well understood because of previous studies on tobacco products can be helpful in selecting which diluents and terpenes should be further investigated at different ratios for specific biomarkers like ROS production and the presence of inflammatory cytokines. A limitation of this study is the cytotoxicity analysis of only unvaped TEC and limonene. The use of NMR analysis was conducted to provide preliminary data measuring the main degradant byproducts after heating of the equipotent mixture tested for cytotoxicity and chemical interaction analysis (Figures 2 and 3). Figure 4B shows the full ^{1}H NMR spectrum of the vaped mixture running through the 600 MHz NMR (512 scans, 3 s relaxation delay). The identification of each product was confirmed by running standards of each under similar NMR settings. Previously, Jiang et al. had characterized emissions from vaping TEC.[11] They reported "smaller esters" such as diethyl ester propanedioic acid, malonic acid diisopropyl ester, diethyl ester propanedioic acid,and *o*-acetylcitric acid triethyl ester. During the investigation herein, we analyzed products formed upon vaping TEC by ^{1}H NMR. In contrast to the prior study, we found that TEC vaping results in relatively prominent emissions of ethanol and ethyl acetate (Figure 4 and Figure S3), and also acetaldehyde. The main degradants measured via NMR serve as an indication of the main degradants of concern in both binary mixtures tested via inoculation exposure (Figure 2). The degradants can then be used for further toxicity analysis using the same inoculation exposure setup used for dose−response assessment in Figure 2. The existing literature of degradant products can then be used to guide future biological end point assessments.[6,11,26]

CONCLUSION

Although this study fulfilled toxicity and chemical interaction analysis of two binary vaping mixtures, we have only touched the surface of the application of dose−response assessments and isobolographic analysis for vaping solutions. The complexity of e-liquid formulations has led to difficulties in isolating the main toxicants of concern contributing to the presentation of lung illnesses in vaping users of different ages and vaping history backgrounds. e-Liquids can contain up to several diluents, flavoring agents (terpenes, aldehydes, etc.), and an active ingredient contributing to an overwhelming task of defining and categorizing these chemicals before isolating the biological effects after inhalation of these chemicals. Therefore, for this study the focus was on collecting toxicity data of a binary mixture consisting of a diluent (triethyl citrate) and a terpene (limonene) to provide a streamlined approach for future vaping toxicity studies.

Through the application of easy-to-use lung cell models like A549 and BEAS-2B cells, the assessment of toxicity effects of the middle and distal regions of the respiratory tract were able to be used for dose−response curve construction. Easy-to-use cell types aided in providing representative lung cell models for dose−response analysis. Dose−response assessments are essential for hazard risk assessment studies by providing quantitative data that defines the responses to increasing

https://doi.org/10.1021/acs.chemrestox.2c00218
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

concentrations of the toxicant of concern. For this study, the dose−response curves were used to calculate LC_{50} values for single constituent and binary mixture exposures. LC_{50} values showed that the terpene (i.e., flavoring agent) had increased cytotoxicity in both lung cell types. Cytotoxicity was also affected by terpene concentration in the binary mixtures tested, resulting in increased toxicity for the 80:20% v/v, which contained 17% v/v more limonene than the equipotent ratio. This provides a model of how to proceed in the testing of vaping mixtures. Marescotti et al. also found that initial cytotoxicity assessments aided in determining which flavoring agents would contribute to increased cytotoxicity when combined with other flavoring agents and vaping ingredients.[30] The conclusions provided by dose−response analysis provides information to support hazard identification of tested vaping chemicals.

Even though the approach used for this study to isolate and define the chemical interaction effect of two binary mixtures of a diluent and terpene resulted in an antagonistic interaction, this has provided information and support to look at the addition of a third vaping ingredient for future mixture analysis. Two studies have indicated potential synergistic interactions of vaping solutions containing more than two compounds, including and not including the active ingredient in more sensitive lung models like primary cells and monocytes.[30,33] These studies coupled with our results show the importance of testing vaping mixtures in different lung *in vitro* models and the introduction of more than two constituents in mixtures. Lung model type and mixture composition can be factors contributing to the presentation of synergisic responses in these studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.2c00218.

> Figure S1 presents the same data shown in Figure 2 with the main difference in the shifting of the *x*-axis to the left in panels B and D for the presentation of centered dose−response curves; Figure S2 provides the molarity concentrations of the individual chemicals used to formulate each binary mixture depending on cell type; Figure S3 shows the levels of ethanol, ethyl acetate, and acetaldehyde observed by ^{1}H NMR spectroscopy upon vaping triethyl citrate (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Christie M. Sayes − *Department of Environmental Science, Baylor University, Waco, Texas 76706, United States;* orcid.org/0000-0002-5529-4101; Phone: 254-710-3469; Email: Christie_sayes@baylor.edu; Fax: 254-710-3409

Authors

Yanira Baldovinos − *Department of Environmental Science, Baylor University, Waco, Texas 76706, United States*

Alexandra Archer − *Department of Chemistry, Portland State University, Portland, Oregon 97201, United States*

James Salamanca − *Department of Chemistry, Portland State University, Portland, Oregon 97201, United States*

Robert M. Strongin − *Department of Chemistry, Portland State University, Portland, Oregon 97201, United States;* orcid.org/0000-0003-3777-8492

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.chemrestox.2c00218

Author Contributions

Yanira Baldovinos: Conceptualization, methodology, investigation, writing of original draft. Alexandra Archer: Methodology, investigation, writing of original draft. James Salamanca: Methodology and investigation. Robert M. Strongin: Methodology, writing/review, and editing. Christie M. Sayes: Conceptualization, methodology, writing of original draft, writing/review, and editing.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported by the C. Gus Glasscock, Jr. Endowed Fund for Excellence in Environmental Sciences in the College of Arts and Sciences at Baylor University, USA.

■ REFERENCES

(1) Kritikos, A. F.; Johnson, J. K.; Hodgkin, D. Past 30−day marijuana vaping: prevalence and predictors of use in a nationally representative study of US youth. *American journal of preventive medicine* **2021**, *60* (2), 258−266.

(2) Trivers, K. F.; Phillips, E.; Gentzke, A. S.; Tynan, M. A.; Neff, L. J. Prevalence of cannabis use in electronic cigarettes among US youth. *JAMA pediatrics* **2018**, *172* (11), 1097−1099.

(3) Meier, M. H.; Docherty, M.; Leischow, S. J.; Grimm, K. J.; Pardini, D. Cannabis concentrate use in adolescents. *Pediatrics* **2019**, *144* (3), e20190338.

(4) King, B. A.; Jones, C. M.; Baldwin, G. T.; Briss, P. A. The EVALI and youth vaping epidemics—implications for public health. *New England Journal of Medicine* **2020**, *382* (8), 689−691.

(5) Al-Hamdani, M.; Hopkins, D. B.; Hardardottir, A.; Davidson, M. Perceptions and experiences of vaping among youth and young adult e-cigarette users: Considering age, gender, and tobacco use. *Journal of Adolescent Health* **2021**, *68* (4), 787−793.

(6) Cao, Y.; Wu, D.; Ma, Y.; Ma, X.; Wang, S.; Li, F.; Li, M.; Zhang, T. Toxicity of electronic cigarettes: A general review of the origins, health hazards, and toxicity mechanisms. *Science of The Total Environment* **2021**, *772*, 145475.

(7) Cherian, S. V.; Kumar, A.; Estrada-Y-Martin, R. M. E-cigarette or vaping product-associated lung injury: a review. *American journal of medicine* **2020**, *133* (6), 657−663.

(8) Li, Y.; Burns, A. E.; Tran, L. N.; Abellar, K. A.; Poindexter, M.; Li, X.; Madl, A. K.; Pinkerton, K. E.; Nguyen, T. B. Impact of e-liquid composition, coil temperature, and puff topography on the aerosol chemistry of electronic cigarettes. *Chemical research in toxicology* **2021**, *34* (6), 1640−1654.

(9) Omaiye, E. E.; McWhirter, K. J.; Luo, W.; Tierney, P. A.; Pankow, J. F.; Talbot, P. High concentrations of flavor chemicals are present in electronic cigarette refill fluids. *Sci. Rep.* **2019**, *9* (1), 2468.

(10) Iversen, L. Cannabis and the brain. *Brain* **2003**, *126* (6), 1252−1270.

(11) Jiang, H.; Ahmed, C. M. S.; Martin, T. J.; Canchola, A.; Oswald, I. W. H.; Garcia, J. A.; Chen, J. Y.; Koby, K. A.; Buchanan, A. J.; Zhao, Z.; Zhang, H.; Chen, K.; Lin, Y.-H. Chemical and toxicological characterization of vaping emission products from commonly used vape juice diluents. *Chem. Res. Toxicol.* **2020**, *33* (8), 2157−2163.

(12) Zhu, S.-H.; Sun, J. Y.; Bonnevie, E.; Cummins, S. E.; Gamst, A.; Yin, L.; Lee, M. Four hundred and sixty brands of e-cigarettes and counting: implications for product regulation. *Tobacco control* **2014**, *23* (suppl 3), iii3−iii9.

(13) Khlystov, A.; Samburova, V. Flavoring compounds dominate toxic aldehyde production during e-cigarette vaping. *Environ. Sci. Technol.* **2016**, *50* (23), 13080−13085.
(14) (a) Benowitz, N. L. Cigarette smoking and nicotine addiction. *Medical Clinics of North America* **1992**, *76* (2), 415−437. (b) Tang, M. s.; Wang, H. t.; Hu, Y.; Chen, W. S.; Akao, M.; Feng, Z.; Hu, W. Acrolein induced DNA damage, mutagenicity and effect on DNA repair. *Molecular nutrition & food research* **2011**, *55* (9), 1291−1300. (c) Samburova, V.; Bhattarai, C.; Strickland, M.; Darrow, L.; Angermann, J.; Son, Y.; Khlystov, A. Aldehydes in exhaled breath during e-cigarette vaping: pilot study results. *Toxics* **2018**, *6* (3), 46.
(15) Hage, R.; Fretz, V.; Schuurmans, M. Electronic cigarettes and vaping associated pulmonary illness (VAPI): A narrative review. *Pulmonology* **2020**, *26* (5), 291−303.
(16) Wang, Q.; Ahmad Khan, N.; Muthumalage, T.; Lawyer, G. R.; McDonough, S. R.; Chuang, T.-D.; Gong, M.; Sundar, I. K.; Rehan, V. K.; Rahman, I. Dysregulated repair and inflammatory responses by e-cigarette-derived inhaled nicotine and humectant propylene glycol in a sex-dependent manner in mouse lung. *FASEB Bioadvances* **2019**, *1* (10), 609.
(17) Chang, Y.; Yang, S.-T.; Liu, J.-H.; Dong, E.; Wang, Y.; Cao, A.; Liu, Y.; Wang, H. In vitro toxicity evaluation of graphene oxide on A549 cells. *Toxicology letters* **2011**, *200* (3), 201−210.
(18) Lujan, H.; Criscitiello, M. F.; Hering, A. S.; Sayes, C. M. Refining in vitro toxicity models: comparing baseline characteristics of lung cell types. *Toxicol. Sci.* **2019**, *168* (2), 302−314.
(19) Liu, J.; Gibb, M.; Pradhan, S. H.; Sayes, C. M. Synergistic cytotoxicity of bromoacetic acid and three emerging bromophenolic disinfection byproducts against human intestinal and neuronal cells. *Chemosphere* **2022**, *287*, 131794.
(20) Malich, G.; Markovic, B.; Winder, C. The sensitivity and specificity of the MTS tetrazolium assay for detecting the in vitro cytotoxicity of 20 chemicals using human cell lines. *Toxicology* **1997**, *124* (3), 179−192.
(21) Salamanca, J. C.; Munhenzva, I.; Escobedo, J. O.; Jensen, R. P.; Shaw, A.; Campbell, R.; Luo, W.; Peyton, D. H.; Strongin, R. M. Formaldehyde hemiacetal sampling, recovery, and quantification from electronic cigarette aerosols. *Sci. Rep.* **2017**, *7* (1), 11044.
(22) Gotts, J. E.; Jordt, S.-E.; McConnell, R.; Tarran, R. What are the respiratory effects of e-cigarettes? *Bmj* **2019**, *366*, l5275.
(23) Aldy, K.; Cao, D. J.; Weaver, M. M.; Rao, D.; Feng, S. Y. E-cigarette or vaping product use-associated lung injury (EVALI) features and recognition in the emergency department. *Journal of the American College of Emergency Physicians Open* **2020**, *1* (5), 1090−1096.
(24) Blount, B. C.; Karwowski, M. P.; Shields, P. G.; Morel-Espinosa, M.; Valentin-Blasini, L.; Gardner, M.; Braselton, M.; Brosius, C. R.; Caron, K. T.; Chambers, D.; Corstvet, J.; Cowan, E.; De Jesus, V. R.; Espinosa, P.; Fernandez, C.; Holder, C.; Kuklenyik, Z.; Kusovschi, J. D.; Newman, C.; Reis, G. B.; Rees, J.; Reese, C.; Silva, L.; Seyler, T.; Song, M.-A.; Sosnoff, C.; Spitzer, C. R.; Tevis, D.; Wang, L.; Watson, C.; Wewers, M. D.; Xia, B.; Heitkemper, D. T.; Ghinai, I.; Layden, J.; Briss, P.; King, B. A.; Delaney, L. J.; Jones, C. M.; Baldwin, G. T.; Patel, A.; Meaney-Delman, D.; Rose, D.; Krishnasamy, V.; Barr, J. R.; Thomas, J.; Pirkle, J. L. Vitamin E acetate in bronchoalveolar-lavage fluid associated with EVALI. *New England Journal of Medicine* **2020**, *382* (8), 697−705.
(25) Smith, D. M.; Miller, C.; O'Connor, R. J.; Kozlowski, L. T.; Wadsworth, E.; Fix, B. V.; Collins, R. L.; Wei, B.; Goniewicz, M. L.; Hyland, A. J.; Hammond, D. Modes of delivery in concurrent nicotine and cannabis use ("co-use") among youth: Findings from the International Tobacco Control (ITC) Survey. *Substance abuse* **2021**, *42* (3), 339−347.
(26) Jabba, S. V.; Diaz, A. N.; Erythropel, H. C.; Zimmerman, J. B.; Jordt, S.-E. Chemical adducts of reactive flavor aldehydes formed in e-cigarette liquids are cytotoxic and inhibit mitochondrial function in respiratory epithelial cells. *Nicotine and Tobacco Research* **2020**, *22* (Supplement_1), S25−S34.
(27) Lee, J.-Y. J.; Miller, J. A.; Basu, S.; Kee, T.-Z. V.; Loo, L.-H. Building predictive in vitro pulmonary toxicity assays using high-throughput imaging and artificial intelligence. *Archives of toxicology* **2018**, *92* (6), 2055−2075.
(28) Hillyer, P.; Shepard, R.; Uehling, M.; Krenz, M.; Sheikh, F.; Thayer, K. R.; Huang, L.; Yan, L.; Panda, D.; Luongo, C.; Buchholz, U. J.; Collins, P. L.; Donnelly, R. P.; Rabin, R. L. Differential responses by human respiratory epithelial cell lines to respiratory syncytial virus reflect distinct patterns of infection control. *Journal of virology* **2018**, *92* (15), e02202.
(29) Guo, W.; Vrdoljak, G.; Liao, V.-C.; Moezzi, B. Major constituents of cannabis vape oil liquid, vapor and aerosol in california vape oil cartridge samples. *Frontiers in Chemistry* **2021**, *9*. DOI: 10.3389/fchem.2021.694905
(30) Marescotti, D.; Mathis, C.; Belcastro, V.; Leroy, P.; Acali, S.; Martin, F.; Dulize, R.; Bornand, D.; Peric, D.; Guedj, E.; Ortega Torres, L.; Biasioli, M.; Fuhrimann, M.; Fernandes, E.; Frauendorfer, F.; Gonzalez Suarez, I.; Sciuscio, D.; Ivanov, N. V.; Peitsch, M. C.; Hoeng, J. Systems toxicology assessment of a representative e-liquid formulation using human primary bronchial epithelial cells. *Toxicology reports* **2020**, *7*, 67−80.
(31) Gessner, P. K. Isobolographic analysis of interactions: an update on applications and utility. *Toxicology* **1995**, *105* (2−3), 161−179.
(32) Goldoni, M.; Johansson, C. A mathematical approach to study combined effects of toxicants in vitro: evaluation of the Bliss independence criterion and the Loewe additivity model. *Toxicology in vitro* **2007**, *21* (5), 759−769. Dawidowicz, A. L.; Olszowy-Tomczyk, M.; Typek, R. Synergistic and antagonistic antioxidant effects in the binary cannabinoids mixtures. *Fitoterapia* **2021**, *153*, 104992.
(33) Muthumalage, T.; Prinz, M.; Ansah, K. O.; Gerloff, J.; Sundar, I. K.; Rahman, I. Inflammatory and oxidative responses induced by exposure to commonly used e-cigarette flavoring chemicals and flavored e-liquids without nicotine. *Frontiers in physiology* **2018**, *8*, 1130.

https://doi.org/10.1021/acs.chemrestox.2c00218
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX