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Effects of E-Cigarette Flavoring Chemicals on Human Macrophages and Bronchial Epithelial Cells

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Effects of E-Cigarette Flavoring Chemicals on Human Macrophages and Bronchial Epithelial Cells

Anna M. Morris

Thesis submitted
to Health Sciences Center at West Virginia University
in partial fulfillment of the requirements for the degree of
Master of Science in Biomedical Sciences

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Morgantown, West Virginia

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ABSTRACT

Effects of E-Cigarette Flavoring Chemicals on Human Macrophages and Bronchial Epithelial Cells

Anna M. Morris

E-cigarettes and vapes are relatively new devices which are popular among young adults and teens. These devices vaporize an “e-liquid” for the user to inhale, which typically contains a mixture of propylene glycol (PG), vegetable glycerin (VG), varying concentrations of nicotine, and flavoring chemicals. Due to these flavoring chemicals being intended for ingestion as a food additive, their respiratory health effects are not well understood. Thousands of e-cigarette flavors are available on the market, many of which have the potential for toxicity. The majority of e-liquid flavoring chemicals (ELFCs) have not been tested for inhalation safety. In this study, we used pulmonary-associated cell lines to assess the *in vitro* effects of thirty ELFCs to determine their potential cytotoxicity at various concentrations. The ELFC vehicles PG and VG were tested individually and as mixtures that mirrored common ratios found in e-liquids (50/50 and 30/70 PG/VG, respectively). Cultured human monocytes (THP-1) were differentiated into naïve and activated macrophage phenotypes before treatment. Both cultured human bronchial epithelial cells (BEAS-2B) and differentiated macrophages were treated with 10, 100, and 1000 μ M of ELFC and analyzed for cytotoxicity and inflammatory markers, including changes in viability, cell membrane damage, reactive oxygen species (ROS) production, and inflammatory cytokine release. The ELFCs which appear to affect viability the most fall into the categories of aldehydes with large carbon chains attached (decanal, hexanal, and nonanal), compounds containing benzene rings (cinnamaldehyde, eugenol, and vanillin), and chemicals classified as monoterpenes, which contain two isoprene groups (alpha-pinene, eugenol, and limonene). Cell membrane damage, as measured by lactate dehydrogenase release, was elevated in both cell lines after treatment with alpha-pinene, decanal, and nonanal. The THP-1 cells were also more sensitive to ethyl maltol and eugenol. Vanillin, ethyl maltol, and the diketones (2,3-pentanedione, 2,3-heptanedione, and 2,3-hexanedione) elicited high amounts of ROS from both cell lines. The BEAS-2B cells did not produce large amounts of inflammatory cytokines. Naïve THP-1 cells produced high levels of IL-1 β , IL-8, and TNF- α when exposed to ethyl maltol and hexanal. Activated THP-1 cells also had increased IL-1 β and TNF- α when exposed to ethyl maltol, but interestingly many ELFCs had a suppressive effect on inflammatory cytokines produced by activated macrophages. These findings provide insight into the potential lung pathology that e-cigarette and vape users are at risk for and provide a basis for future experiments with ELFC exposures.

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INTRODUCTION

E-cigarettes and vapes, collectively known as electronic nicotine delivery systems (ENDS), have exponentially gained popularity since their introduction to the U.S. market about a decade ago (Rom et al., 2015). They were initially marketed as a supposedly safer alternative to tobacco cigarettes, but many young adults and teens are experimenting with ENDS regardless of tobacco cigarette use (Zhong et al., 2016). These battery powered devices contain a chamber filled with e-liquid, which is heated to produce a vapor which the user then inhales. E-liquid is typically a mixture of propylene glycol (PG), vegetable glycerin (VG), nicotine ranging from 0 to 5% by volume (Eaton et al., 2018), and flavoring chemicals. The majority of these flavoring chemicals are considered “Generally Recognized As Safe” (GRAS) to ingest by the U.S Food and Drug Administration, through evaluations by the Flavor Extracts Manufacturers Association (FEMA). Similarly, the World Health Organization Joint Expert Committee on Food Additives and Contaminants (JECFA) has carried out safety evaluations of food flavoring chemicals. Because these food additives have undergone safety evaluations by these organizations, e-liquid flavoring manufacturers thus far have been able to claim that the food flavorings used in e-liquids are safe, however they have not been assessed as safe for exposure to lung tissue. An additional concern is that the cumulative dose of each flavoring chemical may be much higher for a moderate to heavy e-cigarette user than was anticipated to come from the same food flavoring in the diet. One study testing for systemic toxicity estimates a safe exposure level of 170 to 980 µg/day for flavorings in e-cigarette aerosols, however it did not assess acute respiratory effects (Costigan and Meredith, 2015).

A number of studies are now available providing evidence that e-liquids and e-cigarette aerosols are cytotoxic and can cause respiratory irritation and inflammation (Higham et al., 2016; Scheffler et al., 2015). The e-liquid vehicles PG and VG alone have

been found to be toxic to primary bronchial epithelial cells exposed to amounts higher than in 1% (Gonzalez-Suarez et al., 2017). Some flavoring chemicals are known or likely to be respiratory toxicants. For instance, the commonly used butter flavoring diacetyl was found to cause severe inflammation and scarring of the bronchioles, resulting in bronchiolitis obliterans (Hubbs et al., 2008). A number of common flavoring chemicals (diacetyl, cinnamaldehyde, acetoin, pentanedione, vanillin, maltol and coumarin) induce oxidative stress and inflammation (Gerloff et al., 2017; Muthumalage et al., 2018). Additionally, the flavoring chemicals dipentene, ethyl maltol, citral, linalool, and piperonal were found to generate free radicals when vaporized which can be damaging to cells (Bitzer et al., 2018). Many flavoring chemicals are classified as aldehydes, which are officially recognized as 'primary irritants' of mucosal tissue of the respiratory tract. (Tierney et al., 2015). More recently, certain flavoring chemicals have been found to inhibit immune responses, specifically cinnamaldehyde and ethyl vanillin decreasing neutrophil oxidative burst, and benzaldehyde impairing phagocytosis (Hickman et al., 2019). Disrupting the balance of inflammatory processes in the lung carries health implications. Chronic inflammation from repeated exposures can lead to tissue damage, asthma, and obstructive pulmonary disease, while suppression of normal immune cell actions may increase susceptibility to infection (Moldoveanu et al., 2009). Only a small portion of flavoring chemicals that comprise the thousands of e-liquid flavors available on the market have been assessed for toxicity at this time. Some of the chemicals tested are found to be cytotoxic to respiratory cells, and they appear to exhibit differential effects (either suppressive or inducive) on immune responses and inflammation (Ween et al., 2017; Higham et al., 2016; Lerner et al., 2015). Thus, determining the relative toxicity of e-liquid flavorings necessitates individual, case by case assessment of the specific chemical flavoring, and how it interacts with the other components of the e-liquid to affect the respiratory epithelium and immune system.

Our study assessed cell death, ROS production, and inflammatory markers to better understand their mechanisms of toxicity. Our approach was to test various e-liquid components on two cell lines associated with the lung – human bronchial epithelial cells (BEAS-2B) and human monocytes (THP-1) – and analyze cytotoxicity and inflammatory markers. THP-1 monocytes are commonly substituted for alveolar macrophages in e-cigarette studies (Scott et al., 2018; Stanley et al., 2016; Ween et al., 2017). The THP-1 cells were transformed into both naïve and activated macrophages in order to assess differential responses. Because of the insolubility of several flavoring chemicals in water, different mixtures of PG and VG of various ratios and concentrations found in commercial flavorings were tested on the cell lines to determine a sub-toxic dose of PG/VG so that it could be used as a vehicle for the flavoring chemicals and also to develop a baseline for subsequent experiments. Thirty flavoring chemicals were then dissolved in the PG/VG vehicle and cells were treated at three dosages (10 μ M, 100 μ M, and 1000 μ M) to incorporate a wide range in order to determine the upper and lower thresholds of toxicity. The dosages chosen were based on previous literature (Gerloff et al., 2017; Muthumalage et al., 2018) and calculations of average flavoring chemical concentration a moderate e-cigarette user's lungs would be exposed to in a day, based on the U.S. EPA Exposure Factors Handbook (2011) (Supplementary Table 1).

Due to the vast amount of flavoring chemicals on the market yet to be assessed for respiratory toxicity, we utilized methods of cluster analysis and data modeling in order to predict relative toxicity as a secondary aim. Clustering is a machine learning technique that involves the grouping of data points based on shared properties (Kriegel et al., 2011; Färber et al., 2010). We analyzed our accrued toxicity data in order to potentially predict the toxicity of untested chemicals based on their physiochemical properties. We analyzed the chemicals and grouped them based on properties such as solubility, density, molecular

weight, listed SDS hazards, functional groups, and canonical SMILES. The simplified molecular-input line-entry system (SMILES) is a way of describing the structure of a molecule as a single line of text. (e.g. vanillin: COC1=C[C=CC(=C1)C=O]O).

MATERIALS AND METHODS

Cell Culture

All cells were cultured at 37°C in a 5% CO₂ atmosphere in a Thermo Forma (Thermo Fisher, Waltham, MA). BEAS-2B cells (ATCC, Manassas, VA) were cultured in DMEM:F12 media (ATCC) supplemented with 10% fetal bovine serum and 50 mg/mL penicillin/streptomycin (ATCC). Cells were seeded at a density of 10,000 cells per well in a 96-well plate and grown to around 80% confluency. THP-1 cells (ATCC) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (ATCC), 50 mg/mL penicillin/streptomycin, and 0.004% 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). The THP-1 monocytes were differentiated into naïve macrophages by stimulating them with 150 nM Vitamin D₃ (Sigma-Aldrich, St. Louis, MO) for 48 hours and 10 nM phorbol 12-myristate 13-acetate (PMA) for 12 hours (Sigma-Aldrich). To differentiate the naïve macrophages (M0) into classically activated macrophages (M1), they were stimulated with 10 ng/mL of LPS (Sigma-Aldrich) (Xia et al., 2013). Cells were seeded at a density of 20,000 cells per well in a 96-well plate. Cell line authentication was performed by the Genomics Core Facility at West Virginia University to confirm their identity.

E-liquid Flavoring Selection

An initial master list of 89 chemicals was created by determining the presence of certain flavoring chemicals in published literature or in-house e-cigarette studies (Hutzler et al., 2014; LeBouf et al., 2018; Tierney et al., 2016). The list was winnowed to 30 chemicals based on the relative frequency of detection, diversity of their chemical structures, ones with less coverage in the literature, whether they were on FEMA priority lists, and whether they were listed in a catalog of toxicological literature searches of

“ingredients found in e-liquid and compounds found in e-cigarette emissions” by a company that supports the “nicotine products industry” (Nerudia Ltd.). Exceptions regarding diversity were made for several of the buttery flavor compounds, which were essential to evaluate considering their known associations with severe respiratory disease (Morgan et al., 2016; Hubbs et al., 2008). Additionally, where various versions of the same acid, aldehyde, or alcohol existed, we selected the aldehyde due to its higher reactivity and potential as a respiratory irritant (Tierney et al., 2015). Also taken into consideration was their availability for purchase through Sigma-Aldrich (St. Louis, MO), whether they were available in high purity and “food grade”, and whether their physical characteristics were appropriate for *in vitro* assays (i.e. elected against ones only available in ethanol or methanol).

Preparation of E-liquid solution

Several of the flavoring chemicals were not water soluble and could not be diluted in PBS for treatment purposes. A vehicle solution of propylene glycol (PG) and vegetable glycerin (VG) (Glycerin Supplier, Houston, TX) was used in order to model e-liquid solution and act as a solvent. PG and VG were tested individually and as 30% PG/70% VG and 50% PG /50% VG mixtures, in order to be consistent with common ratios found in e-liquids. The concentrations for testing the vehicle control in media were 1%, 5%, and 10%. A 1% solution of a 50 PG/50 VG mixture was found to be sub-toxic and retained its solvent properties, which was used as the vehicle control for subsequent experiments. The 1% PG/VG vehicle control exhibited no significant difference from the PBS control for all endpoints tested. 100 mM solutions of ELFCs were prepared at room temperature in a 100% 50/50 solution and diluted in PBS to 1% PG/VG. Concentrations of both the liquid and solid flavoring chemicals were calculated by molecular weight and added to the PG/VG vehicle and vortexed until the solution was homogenized at room temperature. The 100 mM ELFC solutions were stored in the 4°C refrigerator for the duration of the study. Cells

were treated with ELFCs using final concentrations of 10, 100 and 1000 μM diluted in PBS. All the flavoring chemicals were classified as “Food Grade” and came from Sigma-Aldrich (St. Louis, MO). PBS and a 1% PG/VG solution were used as negative controls.

Endotoxin

To determine whether any endotoxin was present in the ELFC solutions and interfering with results, a Pierce™ Chromogenic Endotoxin Quantification assay (ThermoFisher, Waltham, MA) was performed according to manufacturer’s instructions and absorbance read at 440 nm. The 100 mM ELFC solution was diluted 1:5 in endotoxin-free water. The absorbance values and calculated concentration values of the ELFCs were not significantly different from the endotoxin-free water blank.

Cellular Viability

Viability was assessed using the AlamarBlue assay (Thermo Scientific, Lenexa, KS). AlamarBlue reports viability by reacting with FMNH₂, FADH₂, NADH, NADPH, and cytochromes to measure the entire reducing potential of the cell. Cells were treated with 10, 100 and 1000 μM of ELFCs and incubated for 4 and 24 hours. 10 μL of AlamarBlue was added to each well and incubated for 4 hours before reading. Fluorescence was measured at 560ex/590em with a Synergy H1 microplate reader (BioTek, Winooski, VT). Values were compared to the 1% PG/VG vehicle control. Hexavalent chromium was used as a positive control.

Lactate dehydrogenase

Membrane damage was assessed with the Homogeneous Membrane Integrity Assay (Promega, Madison, WI). Lactate dehydrogenase (LDH) is released from damaged

cells into the culture medium. The assay utilizes a coupled enzymatic reaction which converts resazurin into resorufin. The fluorescent resorufin signal is directly proportional to the amount of LDH in the media. Cells were treated with 10, 100 and 1000 μ M of the ELFCs and incubated for 4 and 24 hours, after which equal parts of media and reagent were mixed and incubated 10 minutes. Fluorescence was measured at 560ex/590em. Hexavalent chromium was used as a positive control.

Intracellular ROS

Intracellular ROS were measured using the cell permeable dye, 2',7'-Dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is de-esterified intracellularly and turns to highly fluorescent 2',7'-dichlorofluorescein (DCF) upon oxidation. Cells were incubated with the dye for 45 minutes, after which the cells were rinsed with PBS and treated with 10, 100 and 1000 μ M of the ELFCs. The fluorescence intensity was measured at 480ex/530em on the microplate reader to quantify the amount of ROS produced by the cells after chemical exposure for 6 hours. Separate wells of just flavoring chemical and media were included in the plates and subtracted from their respective wells of treated cells to account for any autofluorescence.

Cytokines

For cytokine analysis, cells were grown in 96-well plates as previously indicated and treated with 1000 μ M of ELFC for 4 and 24 hours. 1 μ g/mL of LPS was used as a positive control. The media was collected and frozen at -80° C before assaying. BEAS-2B media was undiluted for the assay, naïve THP-1 media was diluted 1:10, and activated THP-1 media was diluted 1:20 in the kit diluent. The cytokine analysis was conducted

according to manufacturer's instructions using the V-PLEX proinflammatory panel II (Meso Scale Diagnostics, Rockville, MD), which quantifies IL-1 β , IL-6, IL-8, and TNF- α .

Statistics

All experiments had three replicate wells and were repeated 3 times for an n of 3. SigmaPlot 14.0 was used for graphing and statistical analysis. Significance was determined by comparing ELFC treatments to the 1% PG/VG vehicle control. A one-way ANOVA coupled with Dunnett's test was used to determine significance for all assays.

Clustering

Average viability, LDH, intracellular ROS, and cytokine concentration (IL-1 β , IL-6, IL-8, and TNF- α) were calculated for each chemical and stratified by cell type and time point. Groupings of the 30 chemicals plus the PG/VG vehicle control (PBS control and positive control excluded) were determined based on treatment averages from all seven assays using Hierarchical Clustering (Euclidean distance as metric). BEAS-2B cell type dendrograms were cut to form four groups of chemicals with similar assay patterns, and THP-1 (activated and naïve) dendrograms were cut to form three groups of chemicals. Number of groups for each cell type were determined by cutting the dendrogram at the largest height that still resulted in distinct groupings of chemicals based on toxicity. Grouping in this manner allowed the separation of different "classes" of toxicity (e.g. high potency versus medium or low potency) within the chemicals without creating overly specific groupings for investigation of any commonalities amongst the physicochemical properties.

Modeling

Replicates (50, 100, and 150) for each chemical–assay–time point–cell type combination were randomly generated from a Normal distribution with mean and standard deviation equal to the corresponding sample mean and sample standard deviation. For example, the sample mean and sample standard deviation results from each assay from vehicle control treatment of BEAS-2B at the 4 h time point were used as the mean and standard deviation parameters of the Normal distribution to randomly generate 50, 100, and 150 data points per assay. This was repeated for each cell type–time point–chemical treatment combination for all assays. Physicochemical properties including molecular weight (g/mol), density (g/mL), solubility in H₂O (mM), SDS hazard classifications, functional group list, and canonical SMILES were repeated uniformly across replicates within each chemical. Data generation was performed from the sample statistics due the moderately-high dimensionality of the dataset once stratified by cell type and timepoint, which could result in biased or overstated modeling results due to the small size of the training dataset once split for testing versus training. Three replication sizes (50, 100, and 150) were used to test the sensitivity of modeling results based on replication number.

Each set of replicated data was randomly split 80/20 for training/testing, respectively. The physicochemical property data were used in a classification Random Forest (number of trees = 500) to classify materials into groups corresponding to the groupings that were determined from the stratified Hierarchical Clustering of the assay averages. Variable importance was assessed by Mean Decrease in Accuracy and Mean Decrease in Gini Index.

All clustering and modeling analyses were conducted in R version 3.6.0 using randomForest package for modeling (R Core Team 2019; Liaw and Wiener 2002).

RESULTS

Endotoxin analysis

Endotoxin analysis showed negligible amounts present in all prepared concentrations and chemical additives. Measured amounts ranged from <0.055 to 0.074 EU which were not statistically significant compared to endotoxin free control. (*Data not shown*)

Cell Viability

Significant effects on cell viability were not seen at the 10 and 100 μ M concentrations of ELFC exposures in any of the 30 chemicals examined. The 1000 μ M exposure levels did show some significant effects across cell type. A significant decrease in viability was observed in BEAS-2B cells after 4 h with alpha-pinene, eugenol, nonanal, and trans-2-hexen-1-al. Eugenol, nonanal and trans-2-hexen-1-al showed the greatest impact on viability, however alpha-pinene also had a significant effect. After 24 hours significantly decreased viability was seen in alpha-pinene, decanal, eugenol, hexanal, nonanal, and trans-2-hexen-1-al (Table 2, Fig. 1 A). A significant decrease in viability was observed in naïve THP-1 cells after 4 h in alpha-pinene, decanal, eugenol, limonene, and nonanal. Decanal, eugenol, and nonanal were more potent compared to alpha-pinene and limonene. After 24 h, significant difference was also seen in cinnamaldehyde, ethyl maltol, hexanal, and trans-2-hexen-1-al. Again, decanal, eugenol, and nonanal were the most potent (Table 3, Fig. 1 B). A significant decrease in viability was observed in the activated THP-1 cells after 4 h alpha-pinene, decanal, eugenol, and nonanal, but not with limonene as seen in the naïve THP-1 cells. After 24 h, significant differences were also seen in

cinnamaldehyde and hexanal (Table 4, Fig. 1 C). Overall, the THP-1 cells demonstrated more sensitivity to the effects of ELFC than the BEAS-2B cells.

Lactate dehydrogenase

Significant effects on LDH were not seen at the 10 and 100 μ M concentrations of ELFC exposures in any of the 30 chemicals examined. The 1000 μ M exposure levels did show some significant effects across cell type. In the BEAS-2B cells, alpha-pinene, decanal, and nonanal were the only ELFCs to cause any level of significant LDH release at 4 h, however no significant trend was observed after 24 h (Table 2, Fig. 2 A). Naïve THP-1 cells showed no significant trends after 4 h. At 24 h, alpha-pinene, decanal, ethyl maltol, eugenol, hexanal, limonene, and nonanal caused significant LDH release (Table 3, Fig. 2 B). Activated THP-1 cells also showed significant effects after 4 h. At 24 h, ethyl maltol, eugenol, and nonanal caused significant LDH release (Table 4, Fig. 2 C). Again, THP-1 cells appear to be more sensitive than the BEAS-2B cells.

Intracellular ROS

As with the other endpoints, significant effects were not seen in intracellular ROS at the 10 and 100 μ M concentrations of ELFC exposures in any of the 30 chemicals examined. After a 6 h 1000 μ M exposure the BEAS-2B cells produced significant ROS upon exposure to vanillin, ethyl maltol, and diketones (2,3-heptanedione, 2,3-hexanedione, 2,3-pentanedione) but not 2,3-butanedione (diacetyl). Both the naïve and activated THP-1 cells showed the same profile as BEAS-2B cells in ROS generation with vanillin, ethyl maltol, and the diketones (2,3-heptanedione, 2,3-hexanedione, 2,3-pentanedione) but not 2,3-butanedione (diacetyl) eliciting significant amounts of ROS (Tables 2 – 4, Fig. 3 A – C).

Cytokines

Due to a lack of significant effects with the 10 and 100 μ M concentrations, cytokines (IL-1 β , IL-6, IL-8 and TNF- α) were measured after 4 and 24 hour exposures to the 1000 μ M dose only.

IL-1 β

In BEAS-2B cells, alpha-pinene and nonanal elicited IL-1 β at 4 h but no significance was observed at 24 h (Table 2, Fig. 4 A). Naïve THP-1 cells after 4 h demonstrated significant increases in IL-1 β for alpha-pinene, hexanal, and limonene. After 24 h, hexanal continued to elicit IL-1 β , while ethyl maltol induced an even greater amount (Table 3, Fig. 4 B). The activated THP-1 cells exhibited significant effects relating to IL-1 β with significant decreases being observed in cinnamaldehyde, decanal, eugenol, L-carvone, nonanal, and trans-2-hexen-1-al, and significant increases in alpha-pinene and ethyl maltol at 4 h. At 24 h, ethyl maltol induced copious amounts of IL-1 β release, whereas cinnamaldehyde, decanal, eugenol, L-carvone, nonanal, and trans-2-hexen-1-al exhibited significant decreases in IL-1 β . Interestingly significant effects were found to either increase or decrease IL-1 β production depending on the ELFC (Table 4, Fig. 4 C).

IL-6

BEAS-2B cells showed no significant changes in IL-6 production at 4 and 24 hours (Table 2, Fig. 5 A). Naïve THP-1 cells showed elevation in IL-6 with alpha-pinene at 4 hours and after 24 hours, ethyl butyrate (Table 3, Fig. 5 B). Activated THP-1 cells demonstrated much more IL-6 activity with significant suppression at 4 h with many ELFCs, including 2,3-heptanedione, 2,3-hexanedione, 2,3-pentanedione, alpha-pinene, cinnamaldehyde, decanal, eugenol, hexanal, limonene, linalool, nonanal, and trans-2-hexen-1-al. After 24 h,

the same ELFCs significantly suppressed IL-6 production with the inclusion of 2,3-butanedione, acetoin, isoamyl acetate, l-carvone, and vanillin (Table 4, Fig. 5 C).

IL-8

BEAS-2B showed little activity with IL-8 with only nonanal eliciting significant production at 4 h; at 24 h no significant changes were found (Table 2, Fig. 6 A). Naïve THP-1 cells exposed to fufural induced production of IL-8 at 4 hours while at 24 hours it was ethyl butyrate, ethyl maltol and hexanal significantly effecting IL-8 (Table 3, Fig. 6 B). The activated THP-1 cells did not exhibit significantly increased IL-8 production compared to the vehicle control, however, many ELFCs significantly decreased IL-8 production. The activated THP-1 cells were significantly affected by alpha-pinene, cinnamaldehyde, decanal, eugenol, hexanal, limonene, nonanal, and trans-2-hexen-1-al at 4 h. The same ELFCs also significantly decreased IL-8 production at 24 h (Table 4, Fig. 6 C).

TNF- α

BEAS-2B cells showed no significant changes at 4 or 24 h for any of the ELFCs (Table 2, Fig. 7 A). Naïve THP-1 cells showed varied significant effects with ethyl maltol eliciting higher TNF- α , whereas alpha-pinene, cinnamaldehyde, decanal, eugenol, nonanal, and trans-2-hexen-1-al had suppressive effects at 4 h. At 24 h, only ethyl maltol and hexanal treated cells produced significant increases in levels (Table 3, Fig. 7 B). Activated THP-1 cells showed far more significant effects from the ELFCs than the naïve form with ethyl maltol being the only one to significantly increase TNF- α , whereas 2,3-butanedione, 2,3-heptanedione, 2,3-hexanedione, 2,3-pentanedione, alpha-pinene, butyraldehyde, cinnamaldehyde, decanal, ethyl butyrate, eugenol, hexanal, l-carvone,

limonene, linalool, nonanal, and trans-2-hexen-1-al all suppressed TNF- α at 4 h. The same pattern was seen at 24 h (Table 4, Fig. 7 C).

Modeling

Across all cell types and time points canonical SMILES and functional groups were the most important physicochemical properties in accurately predicting a compound's toxicity grouping, which were determined via clustering based on average assay results (Fig. 8). Canonical SMILES and functional groups were also the top two variables of importance when Mean Decrease in Gini Index was evaluated. Other physicochemical properties, such as solubility, density, molecular weight, and SDS hazard were consistently less important and did not display a uniform trend for prediction of toxicity grouping across cell types and time points.

DISCUSSION

Our study demonstrates that, regarding viability, alpha-pinene, decanal, eugenol, and nonanal had the greatest detrimental effects across both cell lines, causing cell death in a short amount of time (4 hours). Interestingly, the naïve THP-1 cells were more sensitive than the activated version, as they were also susceptible to limonene where the activated were not, and the BEAS-2B cells were less sensitive overall. Cinnamaldehyde and hexanal were only harmful to cells over a longer exposure (24 hours). Alpha-pinene and limonene had large variations in their effects, likely due to their high volatility. Causes of cell death could be attributed to induction of apoptosis or necrosis, or loss of organelle function. Further studies are needed to determine which mechanisms are responsible for ELFC induced cell death. Many of the cytotoxic ELFCs have very low solubility in water and are more lipophilic in nature, allowing them to interact with and alter cell membranes. That combined with their relatively low molecular weights also allows them to readily cross the phospholipid bilayers. These interactions can destabilize both the outer membrane of the cell and organelle membranes, leading to cytoplasm leakage, loss of membrane potential, and mitochondrial dysfunction (Wei and Shibamoto, 2010; Kumar et al, 2015). In our study, this effect was seen by measuring lactate dehydrogenase leakage from the interior of the cell into the culture medium. The ELFCs with low solubility in water were unsurprisingly the most potent cell membrane disruptors (alpha-pinene, decanal, eugenol, hexanal, limonene, and nonanal).

ELFC compounds have the potential to act as both pro- and antioxidants. When testing for reactive oxygen species generation, the most powerful prooxidants were vanillin, ethyl maltol, 2,3-pentanedione, 2,3-hexanedione, and 2,3-heptanedione. The diketones share a similar structure but differ in carbon chain length. 2,3-Pentanedione was initially used as a supposedly safer substitute to the known toxicant diacetyl (2,3-butanedione),

however it was recently found to be just as damaging to lung tissue (Morgan et al., 2016). Other studies have shown the potential of other e-liquids for inducing reactive oxygen species in multiple cell types (Farsalinos et al., 2013; Lerner et al., 2015). The consequences of this oxidative stress can result in apoptosis and sustained inflammation, which are implicated in a number of pulmonary diseases including asthma, fibrosis, chronic obstructive pulmonary disease, and lung cancer (Sundar et al, 2013). The mechanism of ROS induction of the diketones is likely similar due to their shared chemical properties. Vanillin acts as a prooxidant at higher concentrations and antioxidant at low concentrations, likely due to the radical-scavenging activity via the homolytic fragmentation of the O-H bond (Bezerra et al., 2016). Our study only demonstrated its prooxidant effects due to the relatively high dose used. Ethyl maltol and vanillin were found to produce ROS in cell-free systems (Bitzer et al, 2018), possibly suggesting similar mechanisms of ROS production between the two. ROS overproduction can also be a consequence of peroxisome dysfunction, which normally regulates ROS generation and quenching within the cell (Bonekamp et al., 2009), which may be affected by ELFC exposure.

When examining inflammatory cytokines, Ween et al., (2017) uncovered that certain e-liquid flavoring chemicals in conjunction with nicotine increased levels of IL-8 while decreasing levels of TNF- α , IL-6, and IL-1 β in differentiated THP-1 cells. Other papers have shown similar findings, IL-8 was increased in neutrophils exposed to E-cigarettes (Higham et al., 2016) while IL-1 β and TNF- α was decreased in PBMCs exposed to cigarette extracts (Ouyang et al., 2000). IL-6 was also found to be decreased in the BALF of E-cigarette exposed mice (Lerner et al., 2015). Our experiment evaluated ELFC exposure to BEAS-2B cells and differentiated THP-1 cells, with the THP-1 cells being transformed into naïve and activated subtypes. The BEAS-2B cell line did not produce a large amount of cytokines compared to the THP-1 cells, which is to be expected as THP-1 are immune

cells. Alpha-pinene and ethyl maltol were the most potent inducers of IL-1 β at 4 and 24 hours respectively. This indicates ethyl maltol having a slow-acting, but potent and lasting effect, with similar results seen with TNF- α at 24 hours. With the activated THP-1 cells, many of the ELFCs associated with essential oils had a suppressive effect on inflammatory cytokine secretion, which coincides with findings of essential oils having anti-inflammatory properties (Wei and Shibamoto, 2010). While some decreases in cytokine production can be attributed to cell death, robust anti-inflammatory effects seen with alpha-pinene and cinnamaldehyde may be due to the inhibition of the NF- κ B pathway (Kim et al., 2015). Interestingly, the suppression of IL-6 and TNF- α appear to be linked for the same ELFCs, vanillin and the diketones, which coincidentally induced large amounts of ROS. This pathway could potentially involve peroxisome proliferator-activated receptors (PPARs), although further testing is required to elucidate this (Aleshin & Reiser, 2013). In some cases, anti-inflammatory effects could be beneficial and even therapeutic, but dysregulation of cytokine production in the lung due to ELFC exposure could potentially make the user susceptible to infection as well as other immunological issues.

Due to the vast amount of e-cigarette flavoring chemicals available on the market, we decided to employ a data model to be able to extrapolate toxicological data from the chemicals we tested and potentially apply it to untested chemicals in order to predict their relative toxicity based on shared characteristics. After grouping and analyzing the ELFCs by solubility, M.W., density, functional groups, and canonical SMILES, we determined that the most effective way to accurately predict the toxicity of an ELFC is by assessing the canonical SMILE signature for effects on BEAS-2B cells and functional group for THP-1 cells.

The ELFCs which appear to affect cells the most fall into the categories of aldehydes with large carbon chains attached, compounds containing benzene rings, and chemicals classified as monoterpenes, which contain two isoprene groups. Terpenes and modified terpenes (terpenoids) are key ingredients of essential oils, volatile organic compounds derived from plants and used widely as fragrances and for their potential medicinal properties. Essential oils have historically been used to treat diseases of the respiratory tract, digestive system, gynecological, andrological, endocrine, cardiovascular, nervous system, and skin infections (Firenzuoli et al, 2014). Of the flavors tested, alpha-pinene, cinnamaldehyde, decanal, DL-menthol, eugenol, L-carvone, limonene, linalool, methyl salicylate, and nonanal are primary constituents of their respective essential oils. Of these select chemicals, alpha-pinene, cinnamaldehyde, decanal, eugenol, and limonene exhibited significant toxicity to the cell lines tested. Although these chemicals exhibit therapeutic benefits in certain scenarios, their use in e-liquids potentially poses a significant health risk to the e-cigarette user. In a recent case study, e-cigarette users exhibited symptoms of lipoid pneumonia, an inflammatory response to the presence of lipids within the alveolar space, which is typically caused by aspirating hydrocarbons or oil-based products (Henry et al., 2019). More comprehensive studies are required to elucidate the acute and chronic effects of ELFCs on respiratory pathology. In Table 1, 10 ELFCs (bolded) have been selected for further study due to their significant effects on both cell lines across multiple assays.

CONCLUSIONS

To conclude, cell viability was most affected by alpha-pinene, decanal, eugenol, hexanal, nonanal, and trans-2-hexen-1-al. The THP-1 cells demonstrated more sensitivity to cinnamaldehyde, ethyl maltol, and limonene compared to BEAS-2B cells.

Cell membrane damage was elevated in both cell lines after treatment with alpha-pinene, decanal, and nonanal. The THP-1 cells were also more sensitive to ethyl maltol, eugenol, and hexanal.

Vanillin, ethyl maltol, and the diketones (2,3-pentanedione, 2,3-heptanedione, and 2,3-hexanedione) elicited high amounts of ROS from both cell lines.

The BEAS-2B cells did not produce large amounts of inflammatory cytokines. Naïve THP-1 cells produced high levels of IL-1 β , IL-8, and TNF- α when exposed to ethyl maltol and hexanal. Activated THP-1 cells also had increased IL-1 β and TNF- α when exposed to ethyl maltol, but interestingly many ELFCs had a suppressive effect on inflammatory cytokines produced by activated macrophages. In many cases this was independent of reduced cell viability.

These findings provide insight into the potential lung pathology that e-cigarette and vape users are at risk for and provide a basis for future experiments with ELFC exposures. However, more research is needed to elucidate the various mechanisms of toxicity. Possible mechanisms of ELFC induced toxicity based on the endpoints assessed and literature research are outlined in figure 9. Potential future *in vitro* assays would assess DNA damage, mitochondrial integrity, glutathione (GSH) and superoxide dismutase (SOD) activity, NF- κ B and caspase-1 activation, as well as apoptosis and necrosis.

FIGURE LEGENDS

Table 1) Flavoring chemical names showing associated flavors, solubility, functional group and molecular structures.

Table 2) BEAS-2B cells 1000 μ M exposure profile at all time points and end points.

* $p < 0.1$, ** $p < 0.01$, *** $p < .001$, () significance is lower than negative control.

Table 3) Naïve THP-1 cells 1000 μ M exposure profile at all time points and end points.

* $p < 0.1$, ** $p < 0.01$, *** $p < .001$, () significance is lower than negative control.

Table 4) Activated THP-1 cells 1000 μ M exposure profile at all time points and end points.

* $p < 0.1$, ** $p < 0.01$, *** $p < .001$, () significance is lower than negative control.

Table 5) 1000 μ M exposure for all cell types at all time points and end points. Arrows represent magnitude of significant change compared to vehicle control. * $p < 0.05$

Figure 1 A) Viability of BEAS-2B epithelial cells after 4 and 24 hours measured by AlamarBlue fluorescence. B) Viability of naïve THP-1 cells after 4 and 24 hours. C) Viability of activated THP-1 cells after 4 and 24 hours. Significant p-values are displayed in graph.

Figure 2 A) Membrane damage was assessed by lactate dehydrogenase (LDH) release at 4 and 24 hours in BEAS-2B cells, B) naïve THP-1 cells, and C) activated THP-1 cells.

Figure 3 A) ROS were measured with cell-permeable dye 2',7'-Dichlorofluorescein diacetate and read at 6 h for BEAS-2B cells, B) naïve THP-1 cells, and C) the activated THP-1 cells.

Figure 4 A) IL-1 β in cell supernatants after 4 and 24 hours for BEAS-2B cells, B) naïve THP-1 cells, and C) activated THP-1 cells. *Indicates no significant decrease in viability accompanying decrease in cytokine production.

Figure 5 A) IL-6 in cell supernatants after 4 and 24 hours for BEAS-2B cells, B) naïve THP-1 cells, and C) activated THP-1 cells. *Indicates no significant decrease in viability accompanying decrease in cytokine production.

Figure 6 A) IL-8 in cell supernatants after 4 and 24 hours for BEAS-2B cells, B) naïve THP-1 cells, and C) activated THP-1 cells. *Indicates no significant decrease in viability accompanying decrease in cytokine production.

Figure 7 A) TNF- α in cell supernatants after 4 and 24 hours for BEAS-2B cells, B) naïve THP-1 cells, and C) activated THP-1 cells. *Indicates no significant decrease in viability accompanying decrease in cytokine production.

Figure 8) Important physicochemical properties in accurately predicting a compound's toxicity.

Figure 9) Speculation on possible mechanisms of ELFC induced toxicity based on endpoints assessed and literature research.

Table 1) List of flavoring chemicals and their characteristics

ELFC	Flavor Profile	Solubility in H ₂ O (g/L)	Functional Groups	Molecular structure
2,3,5-Timethylpyrazine	Cocoa, Nutty	15.2	pyrazine
2,3-Butanedione	Buttery, Sweet	200.0	acetyl, carbonyl
2,3-Heptanedione	Buttery, Dairy	71.4	acetyl, carbonyl
2,3-Hexanedione	Creamy, Fruity	211.3	acetyl, carbonyl
2,3-Pentanedione	Buttery, Caramel	66.7	acetyl, carbonyl
2-Aceylpyrazine	Bready, Nutty	66.5	acetyl, pyrazine
Acetaldehyde	Tart, Green Apple	1000.0	acetyl, aldehyde
Acetoin	Creamy, Dairy	1000.0	acetyl, hydroxyl
Alpha-pinene ([-]-α-pinene)	Cedarwood, Pine	0.00249	isoprene
Benzyl Alcohol	Cherry, Almond	42.9	benzene, hydroxyl, isoprene
Butyraldehyde	Cocoa, Green	71.0	aldehyde
Cinnamaldehyde	Cinnamon, Spice	1.42	aldehyde, benzene, isoprene
Decanal	Citrus, Orange peel	0.0435	aldehyde
DL-Menthol	Menthol, Minty	0.456	hydroxyl, isoprene
Ethyl Acetate	Fruity, Grape	80.0	carboalkoxy
ELFC	Flavor Profile	Solubility in H ₂ O (g/L)	Functional Groups	Molecular structure
Ethyl Butyrate	Fruity, Apple	4.9	carboalkoxy
Ethyl Maltol	Sweet, Sugary	24.2	hydroxyl, pyrone
Eugenol	Clove, Spice	2.46	benzene, hydroxyl, isoprene, methoxy
Furfural	Bready, Nutty	77.0	aldehyde, furan
Hexanal	Fruity, Green	5.64	aldehyde
Isoamyl Acetate	Fruity, Banana	2.0	carboalkoxy
Isopropyl Myristate	Cheesy, Dairy	0.0000135	carboalkoxy
L-Carvone	Spearmint, Caraway	1.31	carbonyl, isoprene
Limonene ([R]-[+]-Limonene)	Citrus, Orange	0.0138	isoprene
Linalool	Lemon, Lavender	1.59	hydroxyl, isoprene
Methyl Salicylate	Wintergreen, Peppermint	0.70	benzene, carboalkoxy, hydroxyl, isoprene
Nonanal	Green, Lemon	0.096	aldehyde
Propionaldehyde	Floral, Grape	306.0	aldehyde
Trans-2-Hexen-1-al	Fruity, Green	5.26	aldehyde
Vanillin	Sweet, Vanilla	6.88	aldehyde, benzene, hydroxyl, isoprene, methoxy

Table 2) BEAS-2B results summary

BEAS-2B (1000 μ M)			4 Hour				6 hour	24 Hour					
Chemical	Viability	LDH	IL-1 β	IL-6	IL-8	TNF- α	Intra ROS [#]	Viability	LDH	IL-1 β	IL-6	IL-8	TNF- α
2,3,5-Trimethylpyrazine													
2,3-Butanedione													
2,3-Heptanedione							***						
2,3-Hexanedione							***						
2,3-Pentanedione							***						
2-Acetylpyrazine													
Acetaldehyde													
Acetoin													
Alpha-Pinene	(**)	*	*					(***)					
Benzyl alcohol													
Butyraldehyde													
Cinnamaldehyde													
Decanal		*						(***)					
DL-Menthol													
Ethyl Acetate													
Ethyl Butyrate													
Ethyl Maltol							*						
Eugenol	(***)							(***)					
Furfural													
Hexanal								(**)					
Isoamyl Acetate													
Isopropyl Myristate													
L-Carvone													
Limonene													
Linalool													
Methyl Salicylate													
Nonanal	(***)	*	*		*			(***)					
Propionaldehyde													
Trans-2-Hexen-1-al	(***)							(***)					
Vanillin							***						
*** p < 0.001													
** p < 0.01													
* p < 0.1													
() sig dif lower													

Table 3) Naïve THP-1 results summary

Naïve THP-1 (1000 μ M)			4 Hour				6 hour	24 Hour					
Chemical	Viability	LDH	IL-1 β	IL-6	IL-8	TNF- α	Intra ROS [#]	Viability	LDH	IL-1 β	IL-6	IL-8	TNF- α
2,3,5-Trimethylpyrazine													
2,3-Butanedione													
2,3-Heptanedione							***						
2,3-Hexanedione							***						
2,3-Pentanedione							***						
2-Acetylpyrazine													
Acetaldehyde													
Acetoin													
Alpha-Pinene	(***)		***	*		(***)		(***)	***				
Benzyl alcohol													
Butyraldehyde													
Cinnamaldehyde						(***)		(***)					
Decanal	(***)					(***)		(***)	**				
DL-Menthol													
Ethyl Acetate													
Ethyl Butyrate											***	***	
Ethyl Maltol						***	***	(*)	**	***		***	***
Eugenol	(***)					(***)		(***)	***				
Furfural					*								
Hexanal			***					(***)	***	***		***	***
Isoamyl Acetate													
Isopropyl Myristate													
L-Carvone													
Limonene	(**)		***					(**)	***				
Linalool													
Methyl Salicylate													
Nonanal	(***)					(***)		(***)	***				
Propionaldehyde													
Trans-2-Hexen-1-al						(***)		(**)					
Vanillin							***						
*** p < 0.001													
** p < 0.01													
* p < 0.1													
() sig dif lower													

Table 4) Activated THP-1 results summary

Activated THP-1 (1000 μ M)		4 Hour					6 hour	24 Hour					
Chemical	Viability	LDH	IL-1 β	IL-6	IL-8	TNF- α	Intra ROS [†]	Viability	LDH	IL-1 β	IL-6	IL-8	TNF- α
2,3,5-Trimethylpyrazine													
2,3-Butanedione						(***)					(**)		(**)
2,3-Heptanedione				(*)		(***)	***				(**)		(***)
2,3-Hexanedione				(*)		(***)	***				(**)		(***)
2,3-Pentanedione				(*)		(***)	***				(***)		(**)
2-Acetylpyrazine													
Acetaldehyde													
Acetoin											(**)		
Alpha-Pinene	(*)		**	(**)	(**)	(***)		(*)			(***)	(***)	(***)
Benzyl alcohol													
Butyraldehyde						(*)							(*)
Cinnamaldehyde			(***)	(***)	(**)	(***)		(***)		(***)	(***)	(***)	(***)
Decanal	(***)		(***)	(**)	(**)	(***)		(***)		(***)	(***)	(***)	(***)
DL-Menthol													
Ethyl Acetate													
Ethyl Butyrate						(***)							(***)
Ethyl Maltol			***			*	***		***	***			*
Eugenol	(***)		(***)	(**)	(**)	(***)		(***)	*	(*)	(***)	(***)	(***)
Furfural													
Hexanal				(**)	(**)	(***)		(**)			(***)		(***)
Isoamyl Acetate											(*)		
Isopropyl Myristate													
L-Carvone			(***)			(***)				(*)	(***)		(***)
Limonene				(**)	(**)	(***)					(***)	(***)	(***)
Linalool				(*)		(***)					(***)		(***)
Methyl Salicylate													
Nonanal	(***)		(***)	(**)	(**)	(***)		(***)	***	(*)	(***)	(***)	(***)
Propionaldehyde													
Trans-2-Hexen-1-al			(***)	(***)	(**)	(***)				(*)	(***)	(***)	(***)
Vanillin						(*)	***				(*)		
*** p < 0.001													
** p < 0.01													
* p < 0.1													
() sig dif lower													

Table 5) Results summary expressing magnitude of change compared to vehicle control

BEAS-2B													
THP-1 Naïve													
THP-1 Activated													
↓	lower than vehicle control												
↕	≥0.5 fold lower than vehicle control												
↑	higher than vehicle control												
↗	≥2 fold higher than vehicle control												

ELFC	Viability	LDH	4 Hours						6 Hours		24 Hours					
			IL-1β	IL-6	IL-8	TNF-α	Intra ROS	Viability	LDH	IL-1β	IL-6	IL-8	TNF-α			
2,3,5-Trimethylpyrazine	B	N	A	B	N	A	B	N	A	B	N	A	B	N	A	A
2,3-Butanedione																↓
2,3-Heptanedione																↓
2,3-Hexanedione																↓
2,3-Pentanedione																↓
2-Acetylpyrazine																↓
Acetaldehyde																
Acetoin																
Alpha-Pinene	↓	↓	↓													↓
Benzyl alcohol																
Butyraldehyde																↓
Cinnamaldehyde																↓
Decanal																↓
DL-Menthol																
Ethyl Acetate																
Ethyl Butyrate																↓
Ethyl Maltol																↓
Eugenol	↓	↓	↓													↓
Furfural																
Hexanal																↓
Isoamyl Acetate																↓
Isopropyl Myristate																
L-Carvone																↓
Limonene																↓
Linalool																↓
Methyl Salicylate																
Nonanal	↓	↓	↓													↓
Propionaldehyde																↓
Trans-2-Hexen-1-al	↓															↓
Vanillin																

Figure 1) Cell Viability

A.) BEAS-2B

Figure 1) Cell Viability

B.) Naïve THP-1

Figure 1) Cell Viability

C.) Activated THP-1

Figure 2) LDH

A.) BEAS-2B

Figure 2) LDH

B.) Naïve THP-1

Figure 2) LDH

C.) Activated THP-1

Figure 3) Intracellular ROS

A.) BEAS-2B

Figure 3) Intracellular ROS

B.) Naïve THP-1

Figure 3) Intracellular ROS

C.) Activated THP-1

Figure 4) IL-1 β

A.) BEAS-2B

Figure 4) IL-1 β

B.) Naïve THP-1

Figure 4) IL-1 β

C.) Activated THP-1

Figure 5) IL-6

A.) BEAS-2B

Figure 5) IL-6

B.) Naïve THP-1

Figure 5) IL-6

C.) Activated THP-1

Figure 6) IL-8

A.) BEAS-2B

Figure 6) IL-8

B.) Naïve THP-1

Figure 6) IL-8

C.) Activated THP-1

Figure 7) TNF- α

A.) BEAS-2B

Figure 7) TNF- α

B.) Naïve THP-1

Figure 7) TNF- α

C.) Activated THP-1

Figure 8) Important physicochemical properties in accurately predicting a compound's toxicity.

Figure 9) Speculation on possible mechanisms of ELFC toxicity

Possible Mechanisms of ELFC Toxicity

Supplementary Table 1) Calculations for estimated e-liquid flavoring exposure

ELFC exposure calculations	
Input parameters	
Chemical	Linalool
MW	182.3 $\mu\text{g}/\mu\text{mol}$
Concentration in e-liquid	30 mg/mL
% absorbed by lung	85 %
# puffs/hour	35
Volume of e-liquid consumed per puff	0.2 mL/puff
Volume of e-liquid consumed per day	1.5 mL/day
Fixed parameters	
Inhalation rate	16.3 m^3/day
Inhalation rate	679.2 L/hr
Respiratory tidal volume	0.5 L/breath
Total lung surface area	80 m^2
Deposition area fraction (% of lung surface)	0.1
Surface liquid (mucous) thickness	0.03 cm
Lung surface liquid volume estimated	2400 cm^3
Calculated Results	
Dose per puff	6 mg/puff
Dose per day	3360 mg/day
Retained dose per puff	5.1 mg
Retained hourly dose	178.5 mg/hour
Maximal estimated lung surface concentration	0.00223 mg/cm^2
	0.07438 mg/mL
	74.375 $\mu\text{g}/\text{mL}$
	0.40798 $\mu\text{mol}/\text{mL}$
	407.981 μmol

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention. The mention of brand name does not constitute product endorsement.

Conflicts of interest

The authors of this manuscript do not have any conflicts of interest, financial or otherwise, to declare.

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