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E-Cigarette Liquids and Aldehyde Flavoring Agents Inhibit CYP2A6 Activity in Lung Epithelial Cells

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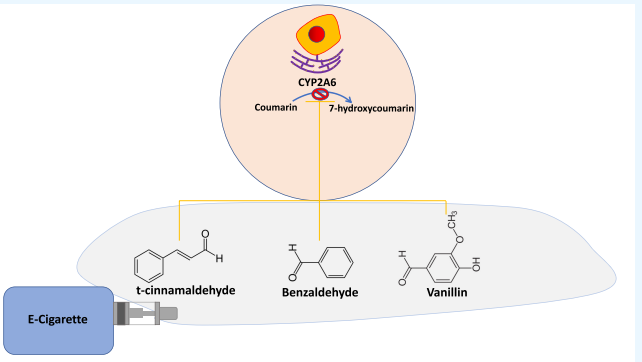
ABSTRACT: Certain e-liquids and aromatic aldehyde flavoring agents were previously identified as inhibitors of microsomal recombinant CYP2A6, the primary nicotine-metabolizing enzyme. However, due to their reactive nature, aldehydes may react with cellular components before reaching CYP2A6 in the endoplasmic reticulum. To determine whether e-liquid flavoring agents inhibited CYP2A6 in a cellular system, we investigated their effects on CYP2A6 using BEAS-2B cells transduced to overexpress CYP2A6. We demonstrated that two e-liquids and three aldehyde flavoring agents (cinnamaldehyde, benzaldehyde, and ethyl vanillin) exhibited dose-dependent inhibition of cellular CYP2A6.



1. INTRODUCTION

A subset of e-cigarette liquids (e-liquids) and common e-liquid flavoring agents were previously shown to inhibit microsomal recombinant cytochrome P450 2A6 (CYP2A6), the enzyme primarily responsible for the metabolism of nicotine to cotinine.[1] The overarching concern of CYP2A6 inhibition by e-liquids and flavoring agents is the potential for reduced metabolism of nicotine present in most e-liquids. Previous research has identified multiple cases where pharmaceuticals, environmental agents, or cigarette additives have inhibited CYP2A6, resulting in a drug−drug interaction that ultimately can increase serum nicotine levels.[2−5]

Microsomal recombinant enzyme screening systems are an ideal solution for rapidly screening numerous compounds or mixtures at multiple concentrations.[6−8] These systems generally result in less variability than cell-based or in vivo studies. However, screening using a microsomal recombinant cytochrome P450 enzyme system is not without drawbacks. In a cell-free microsomal cytochrome P450 system, the enzyme is freely accessible to the test chemical. In contrast, cellular cytochrome P450s, including CYP2A6, are predominantly located at the endoplasmic reticulum (ER).[9,10] For a test chemical to directly affect CYP2A6 in a cell-based system, the chemical must first be capable of reaching the cytochrome P450 at the ER. Due to their reactive nature, aldehydes may react with components within the cell before reaching cytochrome P450.[11−15] However, we were unable to identify any available literature on the screening of e-liquids or common aldehyde flavoring agents for CYP2A6 inhibition using a cell-based system. We hypothesized various e-liquids and aldehyde flavoring agents would inhibit CYP2A6 in intact cells, albeit at higher concentrations than those previously identified using a microsomal recombinant CYP2A6 system.

A cell-based assay to screen for perturbation of CYP2A6 by e-liquids and reactive flavoring agents is valuable as it addresses data gaps on the ability of flavoring agents, specifically reactive aromatic aldehydes, to enter cells and target CYP2A6 at the ER. Thus, a cell-based assay may provide more biologically relevant pharmacokinetic parameters such as the half-maximal inhibition concentration (IC_{50}) than those previously derived using recombinant microsomal CYP2A6.[16]

2. EXPERIMENTAL SECTION

2.1. E-Cigarette Reagents and Supplies. E-liquids were purchased from three individual e-cigarette shops. To ensure possible variations in nicotine concentrations did not impact experiments, only e-liquids labeled as nicotine-free were purchased. The absence of nicotine was subsequently confirmed using mass spectrometry (data not shown). E-cigarette liquids were selected to present a diversity of flavor profiles. Additionally, popular flavors were selected when possible. High Caliber Flamethrower was purchased from an

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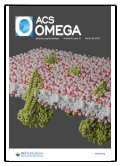

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online vendor (myvaporstore.com), while Strawberry Poptart and Reds apple Watermelon were purchased from local vape shops (The Vapor Girl, Chapel Hill, NC & Local Liquids, Chapel Hill, NC, respectively). E-liquids were stored in glass bottles and away from light until experimental use. The following flavoring agents were purchased from Sigma-Aldrich (St. Louis, MO): food-grade trans-cinnamaldehyde (≥95% pure), GC-grade benzaldehyde (≥99% pure), GC-grade isoamyl acetate (≥99% pure), and GC-grade vanillin (≥99% pure). A mixture of 60% propylene glycol (PG) and 40% vegetable glycerin (VG) obtained from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, MA) was utilized as a vehicle control for e-liquids. *trans*-2-Phenylcyclopropylamine hydrochloride (tranylcypromine) was acquired from Sigma (Sigma-Aldrich, St. Louis, MO).

2.2. Viral Transduction and Confirmation of Metabolic Activity. To overexpress CYP2A6, BEAS-2B cells were transduced using a lentiviral vector encoding human *CYP2A6* (GenBank Accession: BC096253) at a multiplicity of infection of 5. BEAS-2B cells obtained from Dr. Curtis Harris (NCI, Rockville, MD) were incubated in a T25 cell culture flask with 1 mL of serum-free Lonza KBM medium containing CYP2A6 lentiviral vectors for 3 h, with rocking every 30 min. After 3 h, an additional 4 mL of Lonza Gold keratinocyte growth medium (KGM) medium was added to the T25, and the cells were cultured in the lentiviral vector-containing medium for 48 h.

Successful CYP2A6 transgene cassette integration was confirmed by visually identifying the expression of a secondary green fluorescent protein reporter gene (GFP). No GFP expression was observed in nontransduced, parental BEAS-2B cells. Transduced BEAS-2B cells fixed using 4% paraformaldehyde were stained with nuclear-staining dye Hoechst, and actin-staining dye phalloidin. GFP expression was monitored using fluorescence microscopy at excitation λ = 485, emission λ = 525. Transgenic cells were positively selected using 50 μg/mL hygromycin B for 5 days and maintained at this concentration.

To ensure CYP2A6-transduced BEAS-2B cells were metabolically competent, and expressed sufficient CYP2A6 and cytochrome P450 cofactors, the catabolism of coumarin, a pro-fluorogenic CYP2A6 substrate, by both nontransduced and CYP2A6-transduced cells was examined using a method outlined by Donato et al.[17,18] Cells were cultured in separate 12-well plates in 37 °C in 5% CO_2 incubator to confluence. At confluence, the cells were treated with either a strong CYP2A6 inhibitor, 50 μM tranylcypromine (positive control), or no CYP2A6 inhibitor (negative control) for 1 h. After 1 h, BEAS-2B and CYP2A6-transduced BEAS-2B cells were incubated with 100 μM coumarin. After 1 h, 200 μL of cell culture supernatant was transferred to Costar 96-well fluorescence-compatible black flat-bottom plate and measured for the presence of the fluorescent metabolite, 7-hydroxycoumarin using excitation of λ = 365 and emission of λ = 450 using a BMG LabTech CLARIOstar microplate reader (Cary, NC).

To confirm that increased fluorescence in the medium of CYP2A6-transduced BEAS-2B cells was due to the metabolism of pro-fluorogenic coumarin to 7-hydroxycoumarin (Figure 1), high-performance liquid chromatography (HPLC) was performed to detect 7-hydroxycoumarin (standard from Sigma Chemical Co.) using a modified method of von Weyman and Murphy.[19] An isocratic separation of coumarin and the metabolite was achieved using a C18 Ultrasphere ODS column

Figure 1. Metabolism of coumarin to 7-hydroxycoumarin by CYP2A6. Coumarin is a pro-fluorogenic substrate that is highly selective for CYP2A6-mediated metabolism. The substrate undergoes metabolic hydroxylation of the benzene ring by CYP2A6 to form 7-hydroxycoumarin. 7-Hydroxycoumarin is fluorescent at λ = 450 when excited at λ = 365, whereas the parent substrate exhibits negligible emission at λ = 450.

(250 × 4.6 mm^2) with a 70:30:0.2 water/acetonitrile/acetic acid solvent pumped at 1.0 mL/min by a 2695 HPLC (Waters Associates, Milford, MA) with Waters 2487 UV module detection at 324 nm.

2.3. Preparation of E-Cigarette Liquids and Flavoring Agents. E-liquid dilutions were formed immediately prior to the experiment each day. 0.5% stock solutions of e-liquids were prepared in Lonza keratinocyte basal medium (KBM) prior to experimental use. E-liquids were screened for CYP2A6 inhibition at six separate concentrations ranging from 0.00049 to 0.5% (final; v/v) using a fourfold serial dilution scheme. 2 mM stock solutions of individual flavoring agents were prepared in Lonza KBM medium. Flavoring agents were screened for CYP2A6 inhibition at six concentrations ranging from 0.49 to 500 μM using a fourfold serial dilution scheme. The E-liquid and flavoring agent concentration ranges were based on E-liquid flavoring agent concentrations identified in previous literature and levels of flavoring agents shown to inhibit microsomal recombinant CYP2A6. Aromatic aldehydes have been found in e-liquids at concentrations up to 1.1 M.[20]

2.4. Cell Culture Viability. Cell viability was monitored by measuring lactate dehydrogenase (LDH) activity after exposure to e-liquids and flavoring agents at the highest tested concentration by utilizing Promega CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI). CYP2A6-transduced BEAS-2B cells were cultured in a sterile Costar high-binding clear-bottom 96-well plate using Lonza KGM medium. Upon confluence, the cells were cultured in Lonza KBM medium and subsequently exposed to e-liquid at 0.5% or flavoring agent at 500 μM, the highest concentration tested for CYP2A6 inhibition, for 1 h in triplicate wells. At 1 h, coumarin was added to each test well to 50 μM, for a total exposure time of 2 h. Promega cell lysis buffer was utilized to determine maximal LDH activity.

At 2 h, 50 μL of cell culture medium from each well was transferred to a 96-well flat clear-bottom plate, which was then incubated with tetrazolium salt substrate for 30 min. Red formazan product is formed from the tetrazolium salt in proportion to the amount of LDH present in the cell culture medium. After 30 min incubation, absorbance was measured at 490 nm using a BMG LabTech CLARIOstar microplate reader (Cary, NC). The percent of maximal LDH release was calculated using the equation listed in eq S1.

2.5. Cellular CYP2A6 Inhibition Screening. To screen e-liquids and flavoring agents for inhibition of cellular CYP2A6, transduced BEAS-2B cells overexpressing CYP2A6 were first cultured to confluence in a 24-well polystyrene plate using Lonza Gold keratinocyte growth medium (KGM). At confluence, cells were incubated with the test chemical or e-liquid for 60 min in Lonza KBM medium. Following the 60 min incubation, nonfluorescent CYP2A6 substrate coumarin was added to each well at a final concentration of 50 μM.

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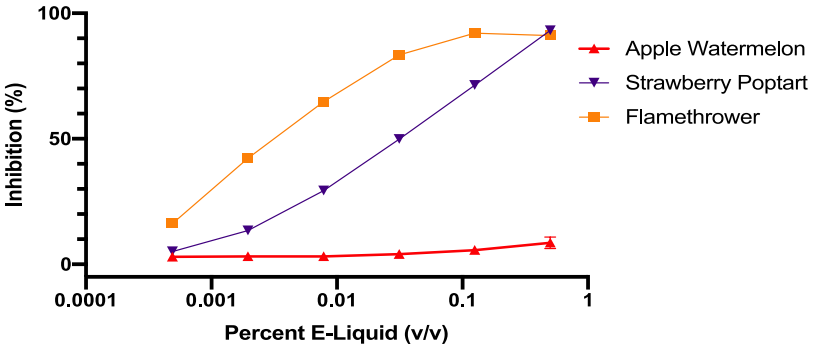

Figure 2. E-liquid inhibition of cellular CYP2A6 at 60 min. E-Cigarette liquids were tested for CYP2A6 inhibition at concentrations ranging from 0.00049 to 0.5% (v/v) in triplicate wells. Percent inhibition calculated from the PG/VG vehicle baseline value. Mean ± SEM, n = 3 independent experiments per treatment group.

CYP2A6 activity was kinetically monitored by measuring the presence of the fluorescent 7-hydroxycoumarin metabolite every minute for 1 h (excitation at λ = 365, emission λ = 450) using a BMG LabTech CLARIOstar microplate reader (Cary, NC). Each concentration was run in triplicate wells and in three independent experiments (n = 3).

Flavoring agents were screened using the same methods as described above for e-liquids. Because flavoring agents were dissolved directly into Lonza KBM medium prior to experimental use, a PG/VG vehicle control was not necessary. Therefore, the fluorescence intensity in the absence of a known CYP2A6 inhibitor or test chemical was treated as the maximal cellular CYP2A6-mediated 7-hydroxycoumarin formation.

As a positive control, the known CYP2A6 inhibitor *trans*-2-phenylcyclopropylamine (tranylcypromine) was administered at 500 μM (final) to induce maximal CYP2A6 inhibition for use in a standard percent inhibition equation (eq S1).

2.6. Statistical Analysis. Unless otherwise stated, data were generated from experiments conducted on three separate days and were performed in triplicate each day. When suitable, data were compared to PG/VG vehicle control and positive inhibition control (500 μM tranylcypromine). Fluorescence in the presence of 500 μM tranylcypromine was considered maximal inhibition for cell-based CYP2A6. Fluorescence for each well was calculated using eq S2 after a 1 h incubation time.

GraphPad Software, Inc. Prism 8.0.1 (San Diego, CA) was used for statistical analyses. Results are expressed as mean ± standard error of the mean (SEM). Results with a p-value of < 0.05 were considered statistically significant. p-Values were calculated in GraphPad Prism 8.0.1 using a two-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test to compare case samples to appropriate controls.

CYP2A6 IC_{50} values were calculated for each e-liquid and flavoring agent using a four-parameter variable slope function in GraphPad Prism 8.0.1. IC_{50} 95% confidence intervals are listed after each IC_{50}.

3. RESULTS AND DISCUSSION

3.1. CYP2A6-Transduced BEAS-2B Cells Metabolize Coumarin to Fluorescent 7-Hydroxycoumarin. After a 1 h incubation with 100 μM coumarin, there was a considerable increase in fluorescence in the CYP2A6-transduced cell culture supernatant compared to the nontransduced cells, indicating an elevation of metabolism of coumarin to 7-hydroxycoumarin by CYP2A6 in the CYP2A6-transduced BEAS-2B cells (Figure S1). When CYP2A6-transduced cells were pretreated with 50 μM tranylcypromine for 1 h, the fluorescence was strongly reduced in the CYP2A6-transduced wells and remained low in both the tranylcypromine-treated and untreated nontransduced cells.

HPLC analysis confirmed that increased fluorescence in the medium of CYP2A6-transduced BEAS-2B cells was due to the metabolism of pro-fluorogenic coumarin to 7-hydroxycoumarin. A KBM medium component elicited a peak at approximately 11.4 min (Figure S2A). 7-Hydroxycoumarin elution time was identified as approximately 13.8 min using 50 μM 7-hydroxycoumarin standard (Figure S2B). Coumarin elution time was identified as approximately 21.8 min using 50 μM coumarin standard (Figure S2C). The area of 7-hydroxycoumarin peak in CYP2A6-transduced cell culture medium was elevated (Figure S2D) in comparison to the medium of CYP2A6-transduced BEAS-2B cells cultured in 500 μM tranylcypromine (Figure S2E). The HPLC results suggest that the fluorescence identified in the supernatant of coumarin-treated CYP2A6-transduced BEAS-2B cells is due to the metabolism of coumarin into fluorescent 7-hydroxycoumarin by CYP2A6. This shows that inhibition of cellular CYP2A6 in the transduced BEAS-2B cells is measurable using a fluorescence-based approach.

Collectively, these results demonstrate that the CYP2A6-transduced BEAS-2B cells have considerable CYP2A6 enzymatic activity compared to the nontransduced BEAS-2B cells. Additionally, the findings show that coumarin in conjunction with CYP2A6-transduced BEAS-2B cells can serve as a system for monitoring CYP2A6 activity.

3.2. Certain E-Liquids and Flavoring Agents Inhibit Cellular CYP2A6 Activity. In this study, we investigated the impact of three e-liquids and four e-cigarette flavoring agents on cellular CYP2A6 activity for comparison to previous findings using recombinant microsomal CYP2A6.[16] At the highest concentration tested (0.5% [v/v]), the e-liquid "apple watermelon" (AW) exhibited an average CYP2A6 inhibition of 8.6% relative to maximal inhibition. As illustrated in Figure 2, lower concentrations exhibited negligible reduction in cellular CYP2A6-mediated 7-hydroxycoumarin formation. The e-liquid strawberry poptart (SP) exhibited near-maximal inhibition of cellular CYP2A6 at 0.5% (v/v) and 0.031% (v/v). The e-liquid "flamethrower" (FT) exhibited the most potent inhibition of cellular CYP2A6. Near-complete inhibition of cellular CYP2A6 was identified down to 0.031% (v/v). GraphPad Prism 8.0.1 was used to calculate the half-maximal inhibitory concentration (IC_{50}) for each e-liquid. The IC_{50} values (95% CI) of SP and

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FT were calculated as 0.071% (v/v) (0.044, 0.22%) and 0.0021% (v/v) (0.00099, 0.0032%), respectively. Due to limited inhibition of CYP2A6 by AW, no IC_{50} was calculated. IC_{50} values are summarized in Table 1.

Table 1. Summary of IC_{50} Values of Cellular CYP2A6 by Selected E-Liquids at 60 min[a]

concentration range tested (%)	e-liquid name	IC_{50} (%) (95% CI)
0.00049−0.5	apple watermelon	>0.125(-)
0.00049−0.5	strawberry poptart	0.071(0.044−0.22)
0.00049−0.5	flamethrower	0.0021(0.00099−0.0032)

[a]E-liquid IC_{50} values (% [v/v]) obtained from cellular CYP2A6 assay. E-liquids tested at six concentrations ranging from 0.00049 to 0.5% in triplicate wells; n = 3 independent experiments per treatment group.

Of the flavoring agents, benzaldehyde and cinnamaldehyde were the most potent inhibitors of cellular CYP2A6 (Figure 3). Both benzaldehyde and cinnamaldehyde exhibited a strong dose-dependent increase in cellular CYP2A6 inhibition. Cinnamaldehyde was a strong inhibitor of cellular CYP2A6 with an IC_{50} of 7.4 μM (1.3, 13.8 μM). Benzaldehyde also appeared to be a strong inhibitor of cellular CYP2A6, with a similar IC_{50} of 7.6 μM (3.7, 11.4 μM). While also a CYP2A6 inhibitor, vanillin exhibited considerably lower potency compared to benzaldehyde and cinnamaldehyde, with near-maximal inhibition occurring only at 500 μM and an IC_{50} value of 200 μM (136, 1,576 μM). Isoamyl acetate, the sole nonaromatic aldehyde tested, exhibited negligible inhibition of cellular CYP2A6 even at 500 μM, suggesting that aldehyde flavoring agents with an aromatic group are more likely to inhibit cellular CYP2A6. IC_{50} values of flavoring agents are summarized in Table 2. Due to limited inhibition of cellular CYP2A6, an IC_{50} was not calculated for isoamyl acetate.

We assessed cytotoxicity, pH changes, and chemical autofluorescence to eliminate the possibility that the results presented in this study were confounded by the factors. As illustrated in Figure S3, none of the three e-liquids exhibited statistically significant LDH release above vehicle control at 0.5%. The two most potent cellular CYP2A6 inhibitors, benzaldehyde and cinnamaldehyde, exhibited a mean percent of maximal LDH of 8.9 and 6.6%, respectively (Figure S4).

Table 2. Summary of IC_{50} Values of Cellular CYP2A6 by Flavoring Agents at 60 min[a]

concentration range tested (μM)	flavoring agent	IC_{50} (%) (95% CI)
0.49−500	isoamyl acetate	>500(-)
0.49−500	vanillin	200(136−1576)
0.49−500	benzaldehyde	7.6(3.7−11.4)
0.49−500	cinnamaldehyde	7.4(1.3−13.8)

[a]IC_{50} values (μM) of flavoring agents obtained from cellular CYP2A6 assay. Flavoring agents tested at six concentrations ranging from 0.49 to 500 μM; n = 3 independent experiments per treatment group.

Vanillin exhibited the highest mean percent of maximal LDH of e-cigarette flavorings. Isoamyl acetate exhibited negligible increase in LDH release above background. Further, alteration of Lonza KBM medium pH was not adversely affected by the presence of any e-liquid at 0.5% (data not shown). Additionally, background fluorescence was measured for each e-liquid and was determined to have a negligible effect for each e-liquid (data not shown).

Winters et al. found a number of e-liquids and numerous flavoring agents are capable of inhibiting microsomal recombinant CYP2A6 using the pro-fluorogenic CYP2A6 substrate 3-cyanocoumarin at 10 μM.[16] Recapitulating our previous findings in a cell-based assay using a different probe substrate provides additional evidence that some e-liquids and flavoring agents inhibit CYP2A6. The results of the present study indicate two of the three tested e-cigarette liquids—FT and SP—and aromatic aldehyde flavoring agents are cell-permeable, sufficiently stable to reach the ER, and capable of inhibiting CYP2A6. These findings are especially relevant for reactive aromatic aldehydes, which have the potential to bind other cellular components before reaching CYP2A6 at the ER.

It is worth noting that the calculated IC_{50} of each e-liquid and flavoring agent was ∼2.5- to 12-fold higher in the CYP2A6-transduced BEAS-2B cell assay than in the microsomal recombinant CYP2A6 assay, which was conducted at a 5-fold higher substrate concentration (K_m of 3-cyanocoumarin is not known).[16] However, it cannot yet be concluded that the aromatic aldehydes studied react with cellular components before reaching CYP2A6 in the ER. Additionally, the role of protective factors (e.g., cell membrane barrier) against these compounds cannot be excluded.

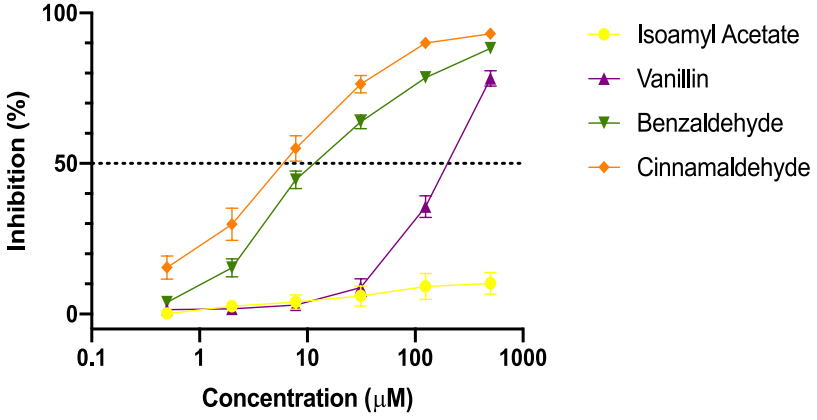

Figure 3. Flavoring agent inhibition of cellular CYP2A6 at 60 min. Flavoring agents were tested for CYP2A6 inhibition at concentrations ranging from 0.49 to 500 μM in triplicate wells. Percent inhibition calculated from the media baseline value. Mean ± SEM, n = 3 independent experiments per treatment group.

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4. CONCLUSIONS

While the data presented here support the position that chemicals in vaping products have the potential to modify cellular nicotine metabolism, the ability for e-cigarette flavoring agents to be absorbed at levels sufficient to inhibit CYP2A6 in vivo remains unanswered. To fully understand any potential impact of e-cigarette liquids on the metabolism of nicotine in vivo, additional research on absorption and distribution of inhaled e-cigarette flavoring agents is necessary. Further, this study only examined a small subset of common e-cigarette flavoring agents; however, the inhibitory effects of these flavoring agents on CYP2A6, the enzyme primarily responsible for the metabolism of nicotine, pose the potential for a drug−drug interaction as they are delivered concomitantly with nicotine.[21]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c08258.

Confirmation of CYP2A6 activity in CYP2A6-transduced BEAS-2B cells (Figure S1); HPLC-UV identification of 7-hydroxycoumarin (Figure S2); percent of maximum LDH activity equation (Equation S1); cytotoxicity of e-liquids (Figure S3); cytotoxicity of flavoring agents (Figure S4); and percent inhibition equation (Equation S2) (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

95% CI, 95th percentile confidence interval; CYP2A6, cytochrome P450 2A6; CYP450, cytochrome P450; IC_{50}, ER, endoplasmic reticulum; half-maximal inhibitory concentration; mM, millimolar; nm, nanometer; PG, propylene glycol; SEM, standard error of the mean; μM, micromolar; VG, vegetable glycerin

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