Systems Toxicology Assessment of Flavor Compounds Present in E-Vapor Products Using Human Primary Bronchial Epithelial Cells

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Introduction and Objective

Assessing e-cigarette toxicity is challenging, considering the lack of standardized testing methods and the large variety of commercially available flavored e-liquid mixtures and devices. While some flavors used in e-liquids are on the U.S. Food and Drug Administration's "generally considered as safe" list when ingested or applied topically, limited data exist about their toxicity when they are inhaled. We recently developed a high-throughput approach to assess the biological impact of e-liquid ingredients on primary human lung epithelial cells (using an advanced cellular assay platform). The objective of this ongoing study is to assess the potential toxicity of flavor mixtures and individual flavor ingredients in this *in vitro* model using a step-wise approach (Figure 1) combining cellular toxicity assays and gene expression analysis.

A primary list of flavor compounds of interest was first established. Flavors were grouped in 34 clusters, as defined by the European Food Safety Authority, based on common physicochemical properties (vapor pressure, boiling point, enthalpy of vaporization, log P, polar surface area, etc.) and available toxicological data. Excluded from this list were compounds known to be potential carcinogens, mutagens, reproductive toxicants, or respiratory sensitizers as well as natural extracts, essential oils, and enantiomers. All flavor compounds (initially dissolved in propylene glycol (PG)) were freshly prepared in a matrix containing PG (41%), vegetable glycerin (VG) (38%), and nicotine (0.6%) in phosphate buffered saline (with a final pH value ranging from 6.5 to 7.8) before adding them to the normal human bronchial epithelial (NHBE) cell culture medium for exposure experiments.

In the first step, a series of different concentrations of each tested compound (defined based on maximum level used in Philip Morris International's products) were tested in NHBE cells over a 24-hour exposure period, using a real-time, impedance-based assay, and results were compared with the corresponding doses of non-flavored matrix. In the second step, selected flavors, based on their additional contribution to matrix toxicity, identified in STEP 1 (Figure 1), were evaluated further using a battery of high-content screening (HCS) endpoints (Figure 1, STEP 2). In this poster, the results of the assessment of four flavors widely used in e-vapor products (guaiacol, whiskey lactone, diacetyl, and cinnamaldehyde) are presented (Figures 4 through 6). In a third step, the study will be complemented by gene expression analysis of the exposed NHBE cells (after 4- and 24-hour exposure) followed by a computational approach leveraging mechanistic network models to identify and quantify perturbed molecular pathways.

Methods

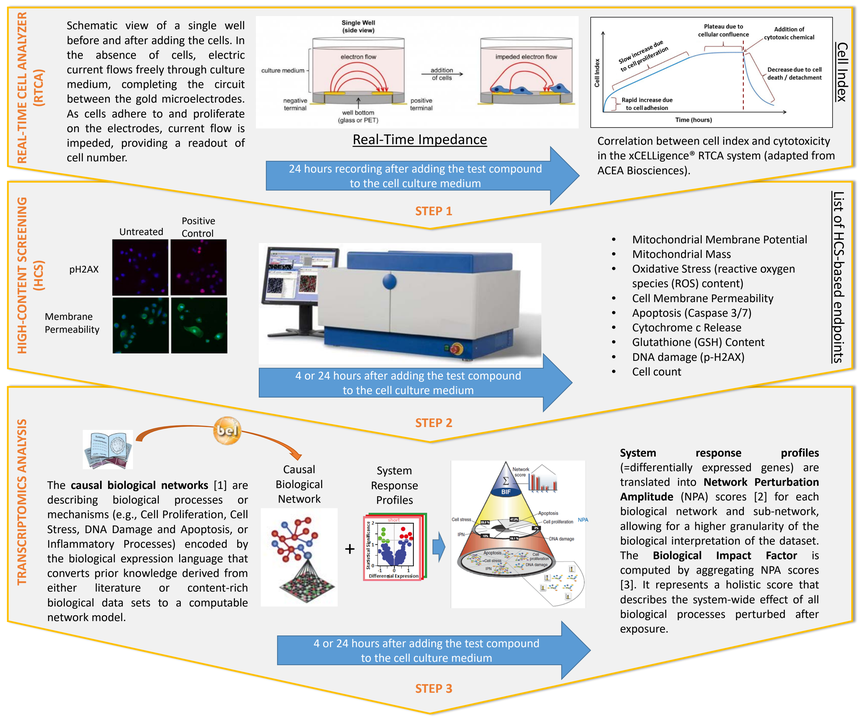

Figure 1. *The Flavor Assessment Workflow is a three-step approach designed to assess the toxicity of flavor compounds in NHBE cells. STEP 1 corresponds to a dose-range finding experiment using a real-time, impedance-based measurement system that will determine the toxic index (see Figure 4) of each flavor compound. STEP 2 provides further information on the mechanism of toxicity triggered by the flavor compound exposure and is based on HCS image analysis (see Figures 5 and 6). Only compounds with a toxic index lower than 1 were tested in STEP 2. Finally, STEP 3 complements the mechanistic understanding of the flavor exposure effect using a systems toxicology approach based on transcriptomic data and computable biological networks.*

1. S. Boué et al. ***Causal biological network database: a comprehensive platform of causal biological network models focused on the pulmonary and vascular systems.*** Database, 2015, 1-14.
2. F. Martin et al. ***Assessment of network perturbation amplitude by applying high-throughput data to causal biological networks.*** BMC Systems Biology 2012, 6:54.
3. F. Martin et al. ***Quantification of biological network perturbations for mechanistic insight and diagnostics using two-layer causal models assessment of biological impact using transcriptomics data and mechanistic network models.*** BMC Bioinformatics 2014, Jul 11; 15(1): 238.
4. I. Gonzales Suarez et al. ***In vitro systems toxicology assessment of non flavored e-cigarette liquids in primary lung epithelial cells.*** Applied In Vitro Toxicology 2017, Mar 1;3(1):41-55.
5. L.S. Birnbaum, T. A. Burke & J.J. Jones**. Informing 21st-Century Risk Assessments with 21st-Century Science**. Environmental Health Perspectives 2016, 124(4), A60–A63.

Results

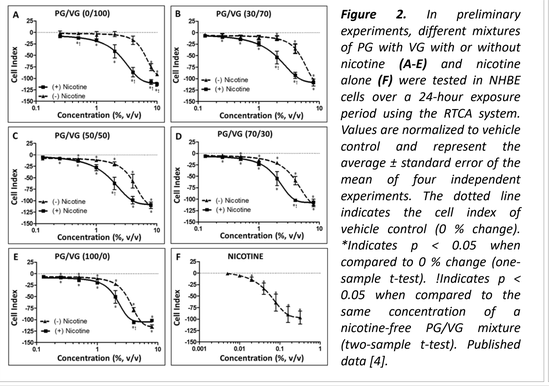

Figure 2. *In preliminary experiments, different mixtures of PG with VG with or without nicotine **(A-E)** and nicotine alone **(F)** were tested in NHBE cells over a 24-hour exposure period using the RTCA system. Values are normalized to vehicle control and represent the average ± standard error of the mean of four independent experiments. The dotted line indicates the cell index of vehicle control (0 % change). *Indicates p < 0.05 when compared to 0 % change (one-sample t-test). !Indicates p < 0.05 when compared to the same concentration of a nicotine-free PG/VG mixture (two-sample t-test). Published data [4].*

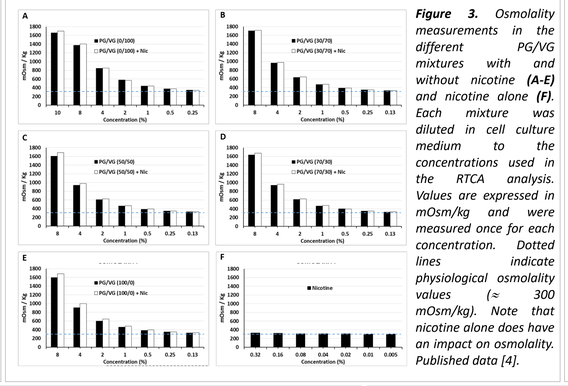

Figure 3. *Osmolality measurements in the different PG/VG mixtures with and without nicotine **(A-E)** and nicotine alone **(F)**. Each mixture was diluted in cell culture medium to the concentrations used in the RTCA analysis. Values are expressed in mOsm/kg and were measured once for each concentration. Dotted lines indicate physiological osmolality values (≈ 300 mOsm/kg). Note that nicotine alone does have an impact on osmolality. Published data [4].*

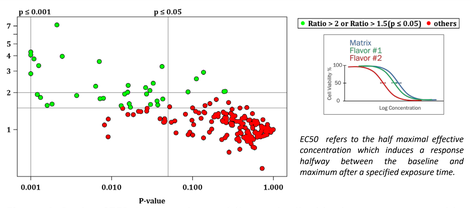

Figure 4. *During STEP 1, seven doses of flavored e-liquid mixtures were tested in NHBE cells over a 24-hour exposure period using the RTCA system. Dose-response curves for the cell indices were plotted, and EC50 was calculated.*
Toxic indices of flavor compounds (i.e., the ratios of EC50 matrix/EC50 flavor mixture (y-axis)) were plotted over p-values (x-axis in log scale). Dots represent the various tested flavor compounds. The two horizontal lines indicate EC50 ratios of 1.5 and 2.0, respectively. The vertical line indicates the p-value cut-off at ≤ 0.05. Flavor compounds showing a toxic index above 2.0 or a toxic index above 1.5 with a p-value ≤ 0.05 are highlighted in green. These compounds were selected for follow-up investigations in STEP 2.

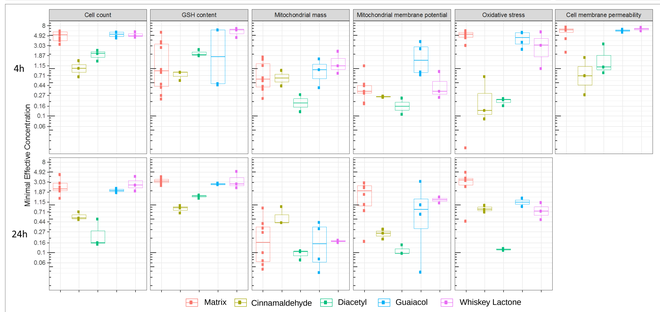

Figure 5. *Minimal effective concentration values (MEC) for selected HCS endpoints (indicated in top gray boxes from left to right: cell count, cell membrane permeability, glutathione content, mitochondrial mass, mitochondrial membrane potential, and oxidative stress (ROS levels)) after exposure to the matrix or to the matrix + flavor (i.e., cinnamaldehyde, diacetyl, guaiacol, or whiskey lactone) for four (top panel) or 24 hours (bottom panel). Data of three independent experiments are presented (except for the matrix alone); each dot corresponds to the MEC50 value measured in one experiment.*

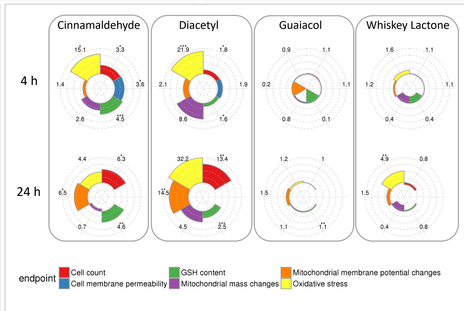

Figure 6. *Pie chart of HCS endpoint ratios. Each slice of the chart represents the MEC ratios for any given endpoint, with the size of the slice proportional to the ratio. The ratios are computed as means (of three experimental replicates) of MEC values of the matrix versus the matrix + flavor (measured within the same experiment) and are shown in parentheses. For comparison, a t-test was computed with the null hypothesis: mean matrix MEC is higher than mean flavor MEC. The t-test p-values are reported as follows: **** p< 0.0001 *** p< 0.001; ** p< 0.01; * p< 0.05.*

Conclusions

A list of flavoring compounds frequently used in e-liquid formulations is currently being assessed to investigate their potential toxicity in human bronchial epithelial cells using a step-wise approach described in Figure 1. A dose-range assessment of each flavor is first performed using a real-time, impedance-based measurement system that allow the determination of the toxic index (Figure 4) of each flavor compound. A second step using a panel of HCS-based endpoints (STEP 2 in Figure 1) is then performed to further understand the effect of selected flavor on NHBE cells. An example of four different flavor compounds assessed in STEP 2 are reported in Figure 5 and 6. Guaiacol and whiskey lactone showed a low cellular and mitochondrial toxicity comparable to the matrix alone at the tested concentrations, independently of the time point tested. Only after 24 hours of whiskey lactone exposure, a sign of oxidative stress was observed compared to the matrix alone. Two other flavors, diacetyl and cinnamaldehyde, showed a similar toxic profile at the tested concentrations, and appeared to exert their effect mainly at the mitochondrial level, as demonstrated by a consistent decrease of mitochondrial membrane potential after 24 hours exposure. In addition, evidence of oxidative stress was also observed for both flavors after 4 hours and 24 hours exposure together with a consistent cellular antioxidant glutathione depletion. The results of this study will be complemented by microarray-based transcriptomic analysis, followed by a computational approach leveraging mechanistic network models to identify and quantify biological perturbations. In conclusion, this approach could contribute to the classification of inhalation toxicants among flavor ingredients typically found in e-liquid mixtures and may prove useful in establishing a list of flavor ingredients that can be used safely in e-cigarettes. This assessment method is aligned with mechanism-based toxicity testing and Risk Assessment in the 21st Century *[5]*.

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Competing Financial Interest

Philip Morris International is the sole source of funding and sponsor of this project.