Risk Assessment Strategy of Flavor Ingredients in e-Vapor Products

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Beyond Science & Decisions...*Flavor Ingredients in e-Vapor Products*

- A real-time compendium of practical, problem-driven approaches for “fit for purpose” risk assessments
- Links novel and pragmatic scientific methods and approaches with specific problems faced by risk assessors and risk managers
- Enhanced communication and collaboration across various stakeholders (e.g., regulatory, and industry, academic community)
I. INTRODUCTION

The Importance of Flavor Ingredients in Harm Reduction

Donna Smith
Current Situation

- Cigarette Smoking is still the leading cause of major preventable diseases, morbidity and mortality worldwide.
- The current prevalence of smoking in the US is ~14%\(^\text{(1-2)}\)
- Quit attempts often fail, and long-term cessation is low\(^\text{(3-5)}\)


Why Harm Reduction?

• "A centerpiece of [FDA’s] comprehensive regulatory plan is acknowledging that nicotine, while highly addictive, is delivered through products on a continuum of risk. And it’s the delivery mechanism – not the nicotine itself – that is truly the issue at-hand.”

  Scott Gottlieb, M.D.
  Former Commissioner of Food and Drugs

• Of those smokers in the US who are unable or unwilling to quit, the majority are interested in “less harmful” tobacco products

According to data from the FDA’s PATH study, over half of adult smokers would consider using a tobacco product if it had a reduced harm claim. This equates to about 22 million adult smokers who are interested in less harmful tobacco products, particularly if they receive truthful and accurate risk information.
The Continuum of Risk

• A strong public health consensus has formed that not all tobacco products present the same risk
• These authorities agree that there is a broad continuum of risk among tobacco products, with cigarettes at the highest end of that spectrum
• This continuum recognized that most of the harm caused by tobacco results from the burning of tobacco

Continuum of Risk

Harm Reduction Equation

- The availability of acceptable combustion-free alternatives to smoking is important.
- It is paramount that these alternatives be both:
  - Satisfying
  - Sensorially acceptable
Is the **Availability** of Reduced Risk Products Enough?

- **Smokeless** tobacco products are widely available in the US, but consumer acceptance on a national level is very low.
- Analyses of available epidemiological data show that smokeless tobacco products are significantly less harmful than cigarettes.

Multidimensional Framework for Nicotine Containing Products
Pharmacokinetics

- E-vapor products more closely mimic the PK of cigarettes than smokeless tobacco or NRTs


Flavor Ingredients Selection is Important to Realize the Greatest Harm Reduction on a Population Level

Data analyzed from the Population Assessment of Tobacco and Health (PATH) at Wave 2 from current adult dual consumers of cigarettes and e-vapor, where this is defined as having used more than 100 cigarettes in their lifetime and now using cigarettes every day or some days, and having ever used e-vapor fairly regularly and now using e-vapor every day or some days.

Non-menthol smokers
Flavor Ingredients in E-vapor Products

- Most e-vapor products contain flavor ingredients
- While these flavor ingredients are GRAS for use in food, their inhalation toxicity is generally unknown
- E-vapor products deliver a mixture of flavor ingredients along with carriers such as propylene glycol, glycerine, acids and nicotine
- There are thousands of flavor ingredients that could be used in e-vapor products

Toxicological Considerations for Flavor Ingredients

- Route of exposure is inhalation
- Complex mixtures
- Stability
- Flavor ingredient transfer from the e-liquid to the aerosol
- Aerosol particle size and resulting deposition
- Extrapolation of data from animal studies to human exposure
- Long-term health effect
II. CASE STUDY – Flavor Ingredients in e-Vapor Products

Flavor Group Representatives (FGRs): Selection Based on Structural Grouping Approach

Davide Sciuscio
Some Considerations….

- Typical flavor mixtures contain 20 flavors
- Food approved flavor ingredients are often used in e-cigarettes
- 2500 flavor ingredients have been approved by EFSA (for food)
- Today >5000+ Flavors are available on the market (growing)

Imperative to acquire safety data on flavor ingredients used by inhalation in a fast and agile way.

- No Inhalation data available for the vast majority of flavor ingredients
- GRAS status for the use of flavor ingredients in food does not mean that GRAS flavor ingredients are safe for use in ENDS
- Lack of standards for flavor testing

Classical approaches for evaluating safety require a series of in vitro and in vivo studies on individual flavors and definition of safe-use levels (not suitable)

- Costly and time consuming (years of animal testing)
- Single Flavor ingredients or Mixtures (numerous flavor combinations possible)
- Additive, synergistic or antagonistic effects?
Some Examples to Acquire Safety Data on Chemicals

In recent years, the use of alternative low-testing and/or non-testing methods for the hazard assessment of substances has been promoted by several regulatory frameworks across different sectors and countries, in order to minimize monetary, timing and ethical costs associated with *in vivo* testing.

Read-across is one of the most commonly used alternative approaches for filling data gaps in registrations submitted under REACH. This approach uses relevant information from analogous (‘source’) substances to predict the properties of ‘target’ substances.

EFSA have used a Flavoring Group Evaluation (FGE) approach to assess flavor ingredients in food. The Procedure is a stepwise approach that integrates information on intake from current uses, structure-activity relationships, metabolism and, when needed, toxicity.

Structurally related compounds are expected to show some metabolic and biological behavior in common.
Combinatorial Flavor-Group-Based Approach

Flavor compounds available

2500+ Substances

Selection

FEMA GRAS

CMR
classified respiratory sensitizers
oils/extracts

NO

245 Substances

Example Flavor Toolbox

Clustering

Groups of Relevant Flavor Ingredients

Rank based on potential for inducing toxicity

1) Acetal
2) Linalool
3) Ethyl lactate
38) Furaneol

(1) Literature (ECHA, ToxPlanet)
(2) In vitro data (RTCA, HCS)
(3) Toxicological prediction (e.g. with TOPKAT)
(4) Predicted HCS (Reg. Mod. ToxPi)
(5) Cramer classes

Experimental data

Predicted data

1 Representative for Each Group (38)

2500+ Substances

245 Substances

38 Groups

1) Linalool
2) Ethyl lactate
3) Acetal
38) Furaneol

1 Representative for Each Group (38)
Example for Selection of an FGR: Group 8

EU definition for group 8:
• Secondary alicyclic saturated and unsaturated alcohols/ketones/ketals/esters with ketals containing alicyclic alcohols or ketones and esters containing secondary alicyclic alcohols. Esters may contain aliphatic acyclic or alicyclic acid component
Example for Selection of an FGR: Group 8

EU definition for group 8:
- Secondary alicyclic saturated and unsaturated alcohols/ketones/ketals/esters with ketals containing alicyclic alcohols or ketones and esters containing secondary alicyclic alcohols. Esters may contain aliphatic acyclic or alicyclic acid component.
Example for Selection of an FGR: Group 8a Data Acquisition

- Oral LD$_{50}$, mutagenicity and genotoxicity data (ECHA or ToxPlanet database)
- *In vitro* cytotoxicity (internal data)
- DNA Damage, Oxidative Stress, Inflammation, etc. (internal data)

<table>
<thead>
<tr>
<th>Flavoring substance</th>
<th>CAS</th>
<th>EU Chemical group</th>
<th>PMI/ALCS Chemical group</th>
<th>ECHA LD$_{50}$ mg/kg</th>
<th>Toxplana n LD$_{50}$ mg/kg</th>
<th>NOAEL</th>
<th>Repeated dose toxicity oral</th>
<th>Interpretation Mutagenicity*</th>
<th>Interpretation Genotoxicity*</th>
<th>EC$_{50}$ ratio</th>
<th>ToxPiScore (HCS)</th>
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<td>.</td>
<td>1670</td>
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<td>2.35 mg/kg bw/day</td>
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<td>Equivocal</td>
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<td>0.29</td>
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<td>DAMASCENONE, BETA-</td>
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<td>.</td>
<td>&gt; 2000</td>
<td>2.35</td>
<td>mg/kg/day</td>
<td></td>
<td></td>
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<td>4590</td>
<td>.</td>
<td>.</td>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td>0.86</td>
<td></td>
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<td>4590</td>
<td>3290</td>
<td>.</td>
<td></td>
<td>Negative</td>
<td>Negative</td>
<td>0.48</td>
<td>0.23</td>
</tr>
<tr>
<td>IRONE, ALPHA-</td>
<td>79-69-6</td>
<td>8</td>
<td>8A</td>
<td>&gt;5000</td>
<td>.</td>
<td>.</td>
<td></td>
<td>Negative</td>
<td></td>
<td>0.82</td>
<td></td>
</tr>
</tbody>
</table>

* Literature data from different studies (Ames, MLA, MN, SCE etc.) have been reviewed and interpreted providing a final recommendation
Example for Selection of an FGR: Group 8a
FGRs Data Integration (2)

- Mechanistic data completion using Toxicological Priority Index (ToxPi) developed by EPA and predictive modelling
- A predictive model was developed in order to complement HCS data for all flavor ingredients: pCramer, pIrritancy, pChronicLOAEL, pExpCarcinogenicity and pXCelligence were retained in the final model

**Mechanistic Based Screening**

**Toxicological Priority Index (ToxPi)**

HCS data were available for 35 Flavorings

<table>
<thead>
<tr>
<th>Flavored substance</th>
<th>CAS</th>
<th>Predicted ToxPi</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALPHA-DAMASCONE</td>
<td>43052-87-5</td>
<td>0.23</td>
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<tr>
<td>DAMASCENONE, BETA-</td>
<td>23696-85-7</td>
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<td>DAMASCONE, BETA-ISOMER 1</td>
<td>23726-92-3</td>
<td>0.16</td>
</tr>
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<td>0.17</td>
</tr>
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<td>IONONE, ALPHA-</td>
<td>127-41-3</td>
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</tr>
<tr>
<td>IONONE, BETA-</td>
<td>14901-07-6</td>
<td>0.19</td>
</tr>
<tr>
<td>IRONE, ALPHA-</td>
<td>79-69-6</td>
<td>0.14</td>
</tr>
</tbody>
</table>

The model based on the attributes above was the best predictive model (based on CV-RMSE, final model $R^2=0.87$) and selected for predicting the ToxPi for all the Flavor ingredients.
### Example for Selection of an FGR: Group 8a

**FGRs Data Integration (1)**

- Predictive *in vivo* toxicity modeling (*TOPKAT*\(^{(1)}\))
- Cramer Classes (OECD QSAR Toolbox\(^{(2)}\))

<table>
<thead>
<tr>
<th>Flavoring substance</th>
<th>CAS</th>
<th>EU Chemical group</th>
<th>PMI/ALCS Chemical group</th>
<th>Cramer Class</th>
<th>TOPKAT Ocular Irritancy</th>
<th>TOPKAT Rodent Carcinogenicity</th>
<th>TOPKAT Chronic LOAEL (mg/kg b.w.)</th>
<th>TOPKAT Develop. Toxicity</th>
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<tbody>
<tr>
<td>ALPHA-DAMASCONE</td>
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<td>Class I</td>
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<tr>
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<td>8A</td>
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<td>true</td>
<td>26.93</td>
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<td>DAMASCONE, BETA- ISOMER 2</td>
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<td>8A</td>
<td>Class I</td>
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<td>false</td>
<td>true</td>
<td>7.24</td>
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</table>

\(^{(1)}\) TOPKAT (TOxicity Prediction by Komputer Assisted Technology) employs robust and cross-validated Quantitative Structure Toxicity Relationship (QSTR) models for assessing various measures of toxicity and utilizing the patented Optimal Predictive Space validation method to assist in interpreting the results.

\(^{(2)}\) https://qsartoolbox.org/
Example for Selection of an FGR: Group 8a
Ranking and FGR Selection

Flavor within each group was ranked based on:

1. pLD50, pDevToxicity, PredictedToxPi, pChronicLOAEL and pIrritancy scores
2. For each flavor, the average rank is computed which is used to generate the final ranking (FinalGroupRank)

<table>
<thead>
<tr>
<th>Flavoring substance</th>
<th>LD50_GroupRank</th>
<th>pDevToxicity_GroupRank</th>
<th>PredictedToxPi_GroupRank</th>
<th>pChronicLOAEL_GroupRank</th>
<th>pIrritancy_GroupRank</th>
<th>AverageGroupRank</th>
<th>FinalGroupRank</th>
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<tr>
<td>ALPHA-DAMASCONE</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1,5</td>
<td>1,5</td>
<td>1</td>
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<tr>
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<td>2</td>
<td>7</td>
<td>3</td>
<td>1,5</td>
<td>3,2</td>
<td>2</td>
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<tr>
<td>DAMASCONE, BETA- ISOMER 1</td>
<td>4</td>
<td>5,5</td>
<td>5</td>
<td>5,5</td>
<td>5</td>
<td>5</td>
<td>6</td>
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<tr>
<td>DAMASCONE, BETA- ISOMER 2</td>
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<td>5,5</td>
<td>4</td>
<td>5,5</td>
<td>5</td>
<td>4,5</td>
<td>5</td>
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<td>IONONE, ALPHA-</td>
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<td>2</td>
<td>4</td>
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<td>4</td>
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<td>5,5</td>
<td>3</td>
<td>7</td>
<td>5</td>
<td>5,2</td>
<td>7</td>
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<tr>
<td>IRONE, ALPHA-</td>
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<td>2</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td>4,2</td>
<td>3</td>
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</table>

Worst case of the group 8A
## Flavor Group Representatives – Final Selection

<table>
<thead>
<tr>
<th>GROUP NUMBER</th>
<th>PMI/ALCS GROUP NAME</th>
<th>FLAVOR GROUP REPRESENTATIVES</th>
<th>GROUP NUMBER</th>
<th>PMI/ALCS GROUP NAME</th>
<th>FLAVOR GROUP REPRESENTATIVES</th>
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<tbody>
<tr>
<td>1</td>
<td>GROUP 1</td>
<td>ACETAL</td>
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<td>GROUP 13</td>
<td>FURANEOL</td>
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<td>2</td>
<td>GROUP 1-2 a</td>
<td>ISOBUTYRALDEHYDE</td>
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<td>GROUP 15</td>
<td>2-METHYL-4-PHENYL-2-BUTANOL</td>
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<tr>
<td>3</td>
<td>GROUP 1-2 b</td>
<td>ISOAMYL ALCOHOL</td>
<td>22</td>
<td>GROUP 16</td>
<td>AMBROX</td>
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<td>4</td>
<td>GROUP 1-2 c</td>
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<td>GROUP 18</td>
<td>EUGENYL ACETATE</td>
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<tr>
<td>5</td>
<td>GROUP 1-2 d</td>
<td>ETHYL 2-METHYL BUTYRATE</td>
<td>24</td>
<td>GROUP 20</td>
<td>P-MENTHA-8-THIOL-3-ONE</td>
</tr>
<tr>
<td>6</td>
<td>GROUP 3</td>
<td>(E,Z)-2,6-NONADIENAL</td>
<td>25</td>
<td>GROUP 21</td>
<td>ACETANISOLE</td>
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<tr>
<td>7</td>
<td>GROUP 3-4</td>
<td>CITRONELLOL, D-L-</td>
<td>26</td>
<td>GROUP 22</td>
<td>METHYL CINNAMATE</td>
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<td>8</td>
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<td>10</td>
<td>GROUP 5 b</td>
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<td>29</td>
<td>GROUP 24</td>
<td>2,5-DIMETHYL PYRIMIZINE</td>
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<td>11</td>
<td>GROUP 6</td>
<td>LINALOOL</td>
<td>30</td>
<td>GROUP 25</td>
<td>2-METHOXY-4-METHYLPHENOL</td>
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<td>12</td>
<td>GROUP 8 a</td>
<td>ALPHA-DAMASCONE</td>
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<td>GROUP 26</td>
<td>PARA-DIMETHOXYBENZENE</td>
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<td>13</td>
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<td>38</td>
<td>GROUP 31 b</td>
<td>PARA-CYMENE</td>
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</tbody>
</table>
Flavor Group Representative Assessment

38 Flavor group representatives (test mixtures)

Preparation, characterization & stability

In vitro cytotoxicity and genotoxicity

Aerosol generation & characterization

In vivo inhalation
II. CASE STUDY – Flavor Ingredients in e-Vapor Products

Flavor Group Representatives (FGRs): Preparation and Stability Characterization

Cameron Smith
Definition: Pre-Blends

- Basic concept: concentrated ingredients (flavors) are diluted and combined to make a final mixture or product

- **Pre-blends** used in this study are concentrated (5–20 × more than the test formulation) mixtures containing PG, ethanol, and selected flavor compounds

- Pre-blends can increase shelf life and aid in the repetitive and time-consuming batch characterization necessary in preclinical studies
Study Design

Longer Stability (Weeks)
- Pre-blend IA – 9
- Pre-blend IB – 7
- Pre-blend IC – 6
- Pre-blend II – 7
- Pre-blend III – 2
- Pre-blend IV – 6

Shorter Stability (Days)
- Dilute with PG, VG, Water, Nicotine
  +1 Flavor
- Test Formulation
  PG
  VG
  Nicotine
  38 Flavors
Grouping into Stable Pre-Blends

- Evaluated reactivity of compounds based on functional group characteristics
- Define the minimum number of categories as possible
- Ensured compounds within each grouping had limited reactivity
Stability Study Design

- Evaluate using gas chromatography-mass spectrometry (GC-MS)

- 1 Month Stability for Pre-blends
  - Refrigerated and Room Temperature Conditions

- 10 Days Stability for Test Formulations (All 38 FGRs)
  - Refrigerated and Room Temperature Conditions
Example: Pre-blend 1A Stability

Aldehydes, Alcohols, Acetals, Ketones, Hydrocarbons

Graph showing the percent of initial concentration over time for various compounds. The graph includes data for p-cymene, 1-penten-3-one, isopulegol, isobutyraldehyde, D-L-citronellol, ethyl lactate, cis-3-hexenol, acetal, and 2-methyl-4-phenyl-2-butanol.
# Test Formulation Without Nicotine

<table>
<thead>
<tr>
<th>Group #</th>
<th>Flavor Group Representatives</th>
<th>T0</th>
<th>T1 - 1 day</th>
<th>T2 - 7 days (± 1 day)</th>
<th>T3 - 11 days (± 1 day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>acetal</td>
<td>100%</td>
<td>102%</td>
<td>107%</td>
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# Test Formulation With Nicotine

## Addition of nicotine shortens stability period

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</table>
Analytical Learnings and Optimization

Abundance

Time (mins.)
Analytical Learnings and Optimization

• Develop one all encompassing method – All 38 Flavor ingredients
• Develop method using common GC/MS
• Ensure solvent is unreactive
• Full Scan is well suited for identifying impurities
• Selective Ion Monitoring (SIM) useful for co-eluting peaks
• Method is well suited for verifying vendor supplied pre-blend formulations are prepared according to COA and reproducible from batch to batch
Stability Summary

- Depending on the test formulation ingredients, pre-blends are stable for a matter of months in refrigerated conditions.
- All test formulation flavor ingredients used in the study were stable for at least 3 days in the presence of nicotine and 10 days without nicotine at refrigerated conditions.
- Test formulation was stable for at least 1 day at room temperature.
- Based on the stability data, test formulations containing nicotine was prepared fresh every 3 days during pre-clinical testing.
Flavor Group Representative Assessment

38 Flavor group representatives (test mixtures) → Preparation, characterization & stability → Aerosol generation & characterization → In vitro cytotoxicity and genotoxicity → In vivo inhalation
II. CASE STUDY – Flavor Ingredients in e-Vapor Products
Flavor Group Representatives (FGRs): *In Vitro* Toxicity Screening

Davide Sciuscio
GOALS

• Define a panel of *in vitro* tests to assess flavor mixtures and enable initial decision making process in product development
• Characterize the biological activity of the test mixture (FGRs)
• Identify the major contributors of the test mixture to biological effects
### In Vitro Toxicity Screening

#### METHODS

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<th>Cytotoxicity</th>
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<th>Genotoxicity</th>
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<td>• NRU (OECD TG129)</td>
<td>• AMES (OECD TG 471)</td>
<td>• MN (OECD TG 487)</td>
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<tr>
<td>• RTCS</td>
<td></td>
<td>• ToxTracker™</td>
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<td>• phosphoH2AX</td>
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#### TEST ITEMS

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<th>FINAL MIXTURE (38 FGRs)</th>
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<table>
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<th>SINGLE FGRs</th>
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Pre-Blend and FGR Mixtures: *In Vitro* Regulatory Assays

- Neutral Red Uptake (NRU) Cytotoxicity Assay (OECD TG129)

- Ames Mutagenicity Assay (OECD TG 471)

- Micronucleus (MN) Assay (OECD TG 487)
Pre-Blend and FGR Mixtures: Additional In Vitro Assays

- Real Time Cell Analyzer (RTCA) Cytotoxicity Assay
- ToxTracker™ Carcinogenicity Assay
- High Content Screening γH2Ax
### Positive FGRs *In vivo* Findings

<table>
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<td>2-methoxy-4-propylpheno</td>
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<tr>
<td>3-methyl-2,4-nonadieno</td>
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<td>NA</td>
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</tbody>
</table>
General Considerations And Conclusions

- ToxTracker™ and pH2Ax gave a better characterization of the genotoxic effects of test mixture and FGRs
- The in vitro panel of tests provided useful information about the hazards associated with the single FGRs, pre-blends and with the test mixture, and might be used to quickly characterize new flavor systems and drive product development
- It is important to highlight that the concentrations tested in vitro are often one or more orders of magnitude higher than those achievable in vivo, thus the in vitro results alone should not be interpreted in isolation to make statements about the safety of flavor ingredients
Flavor Group Representative Assessment

- 38 Flavor group representatives (test mixtures)
- Preparation, characterization & stability
- Aerosol generation & characterization
- In vitro cytotoxicity and genotoxicity
- In vivo inhalation
II. CASE STUDY – Flavor Ingredients in e-Vapor Products
Flavor Group Representatives: Aerosol Generation and Characterization

Patrick Vanscheeuwijck
Various Types of E-vapor Generation Systems

- Adjustable voltage (3–6 V)
- Varying resistance (1.0–6.5 Ω)

→ Potential for user-driven changes in delivered power

- 8000 flavors available, and numbers are increasing

What shall be used?

Multichannel e-cigarette vaping machines

Capillary aerosol generator (CAG)

E-liquid nebulization collision nebulizer
The CAG produces a stream of well controlled aerosol by heating and vaporization of a liquid, followed by nucleation and condensation of the vapor. Liquid is pumped into an electrically heated capillary and hot, saturated vapor exiting from the tip of the capillary is cooled down, leading to homogeneous nucleation of vapors and condensational growth of generated nuclei to form an aerosol.
Capillary Aerosol Generator (CAG)

Benefits of using the CAG for e-vapor inhalation studies:

• Ability to assess e-liquid formulations independently of e-cigarette device specificities
• Ability to simulate the operating conditions (temperature) of e-cigarette devices
• Continuous production, over several hours, of a controlled aerosol similar to e-vapor
• Simplified logistics and less labor intensive

Invented by Philip Morris, Inc. (Howell and Sweeney, 1998)

Further developed as a novel aerosol generator for pharmaceutical drug delivery
Prototype e-Cigarette and the Capillary Aerosol Generator (CAG) Comparison and Qualification for Use in Sub-Chronic Inhalation Exposure Testing

Chemical composition  •  Analytical fingerprint chemical analysis: nearly identical number of known and unknown compounds
  •  Good correlation of the aerosol levels of formulation constituents. Statistically significant difference in levels of PG will not be seen at the nose-only exposure ports

Particle size measurements  •  Similarity in MMAD and GSD

Port-to-port variability  •  Differences in exposure port homogeneity below ± 10% and generally not statistically significant

Chemical by-products  •  Acetaldehyde below the LOQ for both generators
  •  Acrolein levels not statistically significantly different
  •  About eight times higher level of formaldehyde from the prototype e-cigarette compared with the CAG

CAG is suitable for use in 28-day, 90-day or longer inhalation studies
## Aerosol Generation & Characterization

### Analyte Liquid Aerosol Transfer

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<th>Test Formulation w/o Nicotine (N = 3)</th>
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<td>Glycerol (mg/g)</td>
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<td>146.1±0.5 147.1±3.1</td>
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<tr>
<td>Nicotine (mg/g)</td>
<td>20.21±0.17 20.61±0.25</td>
<td>ND ND NA</td>
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<tr>
<td>PG (mg/g)</td>
<td>580.6±2.14 611.2±14.2</td>
<td>625.3±0.99 656.3±26.5</td>
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<tr>
<td>Water (mg/g)</td>
<td>63.11±0.89 79.90±2.37</td>
<td>55.81±0.71 73.81±0.71</td>
</tr>
</tbody>
</table>

### Analyte Liquid Aerosol Transfer

- **Transfer**

\[
\text{Transfer} = \frac{\text{Concentration in Aerosol (mg/g)}}{\text{Concentration in E-liquid (mg/g)}} \times 100\%.
\]

- **Water exceeded 100%** by a wide margin due to the hygroscopicity of PG and Glycerin.

**Notes:**
- **NA = not applied; ND = not detected; BLOQ = below the limit of quantification.**
- **a.** The values were normalized by the collected aerosol mass.
- **b.** The transfer was calculated as \[\text{Transfer} = \frac{\text{Concentration in Aerosol (mg/g)}}{\text{Concentration in E-liquid (mg/g)}} \times 100\%\].
- **c.** Water exceeded 100% by a wide margin due to the hygroscopicity of PG and Glycerin.

---

### Test Formulation w/ Nicotine (n = 4) vs. Test Formulation w/o Nicotine (n = 4)

<table>
<thead>
<tr>
<th>Test Formulation w/ Nicotine (n = 4)</th>
<th>Test Formulation w/o Nicotine (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMAD (µm)</td>
<td>0.97 ± 0.07</td>
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<tr>
<td>GSD</td>
<td>1.77 ± 0.18</td>
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<tr>
<td></td>
<td>1.23 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>1.82 ± 0.13</td>
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</table>
Flavor Transfer

![Graph showing Flavor Transfer with time (mins.) on the x-axis and response on the y-axis. The graph includes peaks labeled 'Liquid/Aerosol'.]
## Selected Carbonyls in the Aerosol

<table>
<thead>
<tr>
<th></th>
<th>Blank (n = 3)</th>
<th>Carrier (PG/VG/Nicotine/Water) (n = 3)</th>
<th>High w/ Nicotine (n = 3)</th>
<th>High w/o Nicotine (n = 3)</th>
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</thead>
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<tr>
<td>Aerosol Mass (mg)</td>
<td>100</td>
<td>107.2 ± 5.4</td>
<td>106.7 ± 1.3</td>
<td>116.1 ± 1.5</td>
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<tr>
<td>Formaldehyde (µg/g) c</td>
<td>&lt; LOQ</td>
<td>8.71 ± 0.57</td>
<td>4.98 ± 0.15</td>
<td>5.88 ± 0.24</td>
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<tr>
<td>Acetaldehyde (µg/g) c</td>
<td>3.09 ± 0.11</td>
<td>8.34 ± 0.89</td>
<td>Above 1000 b</td>
<td>Above 1000 b</td>
</tr>
<tr>
<td>Acrolein (µg/g) c</td>
<td>&lt; LOD</td>
<td>1.63 ± 0.20</td>
<td>5.36 ± 0.65</td>
<td>2.37 ± 0.13</td>
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<tr>
<td>Crotonaldehyde (µg/g) c</td>
<td>&lt; LOD</td>
<td>&lt; LOD</td>
<td>10.57 ± 0.75</td>
<td>8.18 ± 0.17</td>
</tr>
</tbody>
</table>

- a. Assumes 100 mg for calculation purposes;
- b. Approximations - Above Calibration Curve;
- c. Reported values were normalized to the collected aerosol mass.

**Where did acetaldehyde come from?**
1,1-Diethoxyethane is Detected as Acetaldehyde (Artifact of Method)

1,1-diethoxyethane detected as acetaldehyde in the carbonyl analysis due to the sampling limitation

1,1-diethoxyethane as a flavor was transferred to the aerosol around 100% by GC-MS method.
Summary

• Flavor transfer from liquid formulation into the aerosol was confirmed
• Particle size for both formulations (high with and without nicotine) tested were in the desired range
• Nicotine, PG and glycerol matched in liquid and CAG aerosol for the test formulations
• Selected carbonyls measured in CAG generated aerosols were consistent with previous studies
Flavor Group Representative Assessment

38 Flavor group representatives (test mixtures) → Preparation, characterization & stability → In vitro cytotoxicity and genotoxicity → Aerosol generation & characterization → In vivo inhalation
II. CASE STUDY – Flavor Ingredients in e-Vapor Products

Flavor Group Representatives (FGRs):
5-Week Range-Finding Inhalation Study in A/J Mice

Patrick Vanscheeuwijk
Mouse Model of Disease

- Smoke-induced lung cancers in human:
  - Human adenocarcinoma frequently carries \textit{Kras} mutations
- A/J mouse model develops cigarette smoke-induced lung adenocarcinoma, with increased transcription rate of mutated \textit{Kras}
- Suitable to study co-morbidities: inflammation and oxidative stress associated with pathogenesis of lung cancer and COPD

Steinn (2013) – Could not find reference
Dose Selection and Human Relevance

- To derive the test atmosphere concentrations to be used in the A/J study, the following human-relevant approach was used, for the high concentration mixture:
  - Use the ‘maximum use level’ of the flavoring ingredients, and apply to FGR
  - Assume 4 ml of e-liquid use per day for adults
  - Calculate human dose
  - Calculate corresponding mouse dose [Alexander formula, CDER conversion factor based on body surface area\(^{1,2,3}\)]
  - Calculate required test atmosphere concentration to achieve the dose
    - Taking into account: 60% transfer rate, required quantity of aerosol to expose animals in whole body chamber (800L)
  - Medium and low concentration mixtures for the A/J mouse study were created by applying a 4-fold serial dilution from the “high mixture”

1 Alexander et al., 2008, Inhal. Toxicol. 20, 1179-89
2 Bide et al., 2000, J. Appl. Toxicol. 20, 273-90
3 CDER, 2005
Objective

- Perform a Dose Range Finding Study on CAG-aerosolized e-liquids with flavor ingredients from the Flavor ‘Toolbox’ mixture in preparation of a combined chronic toxicity/carcinogenicity study
Study Design and Endpoints

A/J mice (female/male*)

• Exposure: 6 hours/day, 5 days/week for 5 weeks
• Sham (fresh air)
• Control groups: CAG-generated aerosol PG/VG/N, 3R4F cigarette smoke (CS) (Health Canada Intense conditions)
• Test item groups: CAG-generated PG/VG/N/F – Flavor ‘toolbox’ mixture, Low, Medium, High
• All Nicotine-containing groups: 15.0 µg/L

Endpoints:

• Lung inflammation: free lung cells, cytokines/chemokines in BALF (n=10)
• Histopathology evaluation of respiratory tract (n=11)
• Systems toxicology respiratory tract (n=8)

*for male mice: limited study design: Sham, PG/VG/N, and PG/VG/N/F-H groups only
## Composition Inhalation Formulations

<table>
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<th>Inhalation formulation</th>
<th>Component (g/100g)</th>
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<tr>
<td></td>
<td>PG</td>
<td>VG</td>
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<tr>
<td>PG/VG/N</td>
<td>71.7</td>
<td>17.9</td>
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<td>PG/VG/N/F Low</td>
<td>68.0</td>
<td>17.0</td>
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<tr>
<td>PG/VG/N/F Med</td>
<td>64.3</td>
<td>16.1</td>
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<tr>
<td>PG/VG/N/F High</td>
<td>56.9</td>
<td>14.2</td>
</tr>
</tbody>
</table>

- Typical commercial products (liquid) contain 1g to 3g flavor/100g
Aerosol Generation and Sampling of Aerosol

Whole Body Exposure Chambers

Exposure Room

Sampling of diluted aerosol in WBEC

Control Room
Aerosol composition reflects that of formulation

TPM: total particulate matter; LOD, limit of detection
Aerosol Uptake: Urine Nicotine Metabolites

Similar uptake of nicotine by mice exposed to nicotine-containing aerosols, incl. smoke

Higher nicotine metabolites in male PG/ VG/N/F-H group because of two outliers.

Total Nicotine Metabolites = 6 major nicotine metabolites

NM, not planned for measurement
Analysis of 24-h urine samples (n=3) shows good uptake of flavor ingredients

- **Methyl Antranilate** (ng, mean±SEM):
  - Sham: <LOQ
  - PGVGNF-H: 6000 ± 200

- **Ethyl Maltol** (µg, mean±SEM):
  - Sham: <LOQ
  - PGVGNF-H: 30 ± 5

- **Ethyl vanillic acid** (µg, mean±SEM):
  - Sham: 20 ± 3
  - PGVGNF-H: <LOQ

- **Eugenol** (ng, mean±SEM):
  - Sham: <LOD
  - PGVGNF-H: 4000 ± 200
In-Life Body Weight Progression

Transient weight loss was observed during weeks 1-2 and most prominent in 3R4F CS-exposed group.

Body weight measurement were performed twice per week. N=29/group.
Lung Inflammation Determined in Lavage Fluid

Lung inflammation was prominent in the 3R4F CS-exposed mice but not in the e-vapor exposed groups.

Cytokines/chemokines

Free Lung cells (lavage fluid)
Histopathology Evaluation of the Nose and Larynx

Typical adaptive changes observed in the nasal respiratory epithelium in the 3R4F group – severity higher than in Sham and e-vapor groups.

Changes at most sensitive sites of the larynx: Concentration-response in flavor ingredient-exposed groups; much less pronounced than after 3R4F exposure.

No other noteworthy epithelial changes in e-vapor exposed groups.
Conclusions

- 3R4F cigarette smoke causes known adaptive changes in the nasal and laryngeal epithelia, and lung inflammation
- The flavored e-liquid aerosols were well tolerated by the mice, without signs of severe toxicity
- The flavored e-liquid aerosols, even at the highest flavor concentration, did not cause lung inflammation
- Few respiratory tract epithelial changes were observed in mice exposed to aerosols from flavored e-liquids, and when observed, their severity was much lower than in mice exposed to 3R4F cigarette smoke
- The flavor ingredients concentrations used in this dose range finding study are deemed suitable to be used in a chronic toxicity study
Key Takeaways

• Implemented a structural flavor grouping approach to assess flavor ingredients used in e-vapor products
• Flavors and flavor mixtures are well characterized chemically and biologically (in vitro)
• The aerosol dynamics are well characterized
• The results from a 5-week study of the complex flavor mixtures show no effects at human relevant doses
III. BEYOND SCIENCE

Julia Hoeng
Data Transparency Inspires Confidence in Research

Majority of Americans say they are more apt to trust research when the data is openly available

% of U.S. adults who say when they hear each of the following, they trust scientific research findings ...

- Data is openly available to the public
  - Less: 8%
  - More: 57%
  - Makes no difference: 34%

- Reviewed by an independent committee
  - 10%
  - 52%
  - 37%

Note: Respondents who did not give an answer are not shown.
Source: Survey conducted Jan. 7-21, 2019.
“Trust and Mistrust in Americans’ Views of Scientific Experts”

PEW RESEARCH CENTER
Bias Against Industry-Funded Research in Public Opinion

% of U.S. adults who say when they hear each of the following, they trust a science practitioner’s recommendation ...

- Open to getting a second opinion
  - Less: 7%
  - More: 68%
  - Makes no difference: 23%

- Based on review from an independent committee
  - Less: 17%
  - More: 43%
  - Makes no difference: 38%

- Received financial incentives from the government
  - Less: 37%
  - More: 14%
  - Makes no difference: 48%

- Received financial incentives from an industry group
  - Less: 62%
  - More: 10%
  - Makes no difference: 27%

Note: Respondents who did not give an answer are not shown.
Source: Survey conducted Jan. 7-21, 2019.
“Trust and Mistrust in Americans’ Views of Scientific Experts”

Pew Research Center
Science & Society
Independent Peer Review of the Toxicological Assessment of Tobacco Heating System 2.2

Independent Peer Review of the Toxicological Assessment of Tobacco Heating System 2.2 (Continued)

INTERVALS - a Data & Results Sharing Platform, Aimed at Improving Transparency in Industry-Funded Research

https://www.intervals.science/

https://sciences.altria.com/

• Reproducible assessment of alternative products
• Enable evidence-based decisions
• Foster the development of a Smoke Free Future
Considerations for the Development of INTERVALS

“It is not enough to do your best; you must know what to do and then do your best”

W. Edwards Deming

There are many products & flavors to be tested, rapid innovation with many new emerging assay protocols, technologies, and no real data standards

Need a platform that demonstrates the scientific rigor, thoroughness, precision required in Inhalation Toxicology of candidate reduced risk products to:

- Ensure quality of the data and that the adequate testing strategies are used
- Enable reuse of data sets (3Rs, generation of new hypotheses)
- Inform the scientific community
INTERVALS: Scientific Data Transparency Applied to Industry

Aim: establish a community and a public repository for 21st-century preclinical and clinical (systems) inhalation toxicology assessment data and results that supports open data principles

The INTERVALS Community/Ecosystem

Overview of the Platform

- Faceted search enables quick retrieval of resource of interest
- Detailed protocols
- Clear contact detail
- Community features (news/commenting/events)
Detailed Study Results and Direct Link to Data

Method: Plaque size measurements - planimetry and microCT

Planimetry
After removal of the aortic arch, the aortic wall was opened longitudinally, stained with Oil Red O, and the intimal area covered by plaques normalized to the whole area was determined from digital images. The intimal area covered by plaques was determined by planimetry and normalized to the whole area of the aortic wall.

Figures 2 - Micro-computed tomography (micro-CT) based aortic arch (in situ) plaque measurements:
A. Plaque volume, B. Plaque surface area, C. Aortic occlusion (mean ± SEM), D. Representative micro-CT images.

Gray, the plaque in dark yellow. A centerline embedded in the aorta is pseudo-colored to indicate the cross-sectional area of plaque at each point along the aorta. At the bottom of this frame, the silica distance and plaque cross-sectional area are reported, as well as total measurements (average occlusion, total plaque volume, total plaque surface area) for each of the regions (sinus, aortic arch, thoracic aorta, brachiocephalic trunk).

- Linear Distance Measurements (top-right) for each slice along the curved centerline, the average aorta radius, maximum plaque thickness, and average plaque thickness are plotted. As the animation proceeds, a black time-bar indicates the current slice distance along the graph.
- Percent Measurements (middle-right) for each slice along the curved centerline, the percent coverage (percent of the vessel wall that has plaque attached) and percent occlusion (percent of the vessel cross-section that is occluded with plaque) are plotted.
- Two planar silicas (bottom-right) - the grayscale silicas cut through the aorta in an orientation centered around and perpendicular to the centerline. The right side is displayed with segmented aortic plaque overlaid in red, and segmented brachiocephalic trunk plaque overlaid in blue.

All metrics and 3D movies were created for the aortas using SCIRun (Scientific Computing and Imaging Institute, University of Utah). All samples were scanned and analyzed blind to treatment assignment.
A Mine of Data
### Studies Published on INTERVALS

The numbers indicate the number of published studies for each test item/type of study.

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<th>In situ</th>
<th>In vitro</th>
<th>In vivo</th>
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Published Study Titles

- Comparative assessment of HPHC yields in THS 2.2 and commercial cigarettes
- 3D vasculature-on-a-chip model to assess the effect of THS 2.2 exposure on monocyte-to-endothelium adhesion \textit{in vitro}
- 6-month Systems Toxicology Inhalation/Cessation Study with CHTP 1.2 and THS 2.2 in Apoe\textsuperscript{−/−} Mice
- 8-month Systems Toxicology Inhalation/Cessation Study with THS 2.2 in Apoe\textsuperscript{−/−} Mice
- 90-day OECD Rat Inhalation Study with THS 2.2 (TG413 Guideline)
- A 2-year clinical study evaluating the safety profile of an electronic vapor product
- A Cross-sectional Study of the Socio-demographic and Other Determinants of Chronic Obstructive Pulmonary Disease (COPD) Among Those Who Smoke, Quit Smoking and Never-smoking Cigarettes
- A lung/liver-on-a-chip platform for acute and chronic toxicity studies
- A system toxicology approach to investigate the impact of an acute exposure to cigarette smoke and electronic cigarette on human lung and oral \textit{in vitro}
- Acute exposure of human organotypic buccal epithelium cultures to e-liquid aerosols – Comparison with cigarette smoke by using a systems toxicology approach
- Assessment of acute CHTP 1.2 aerosol exposure in \textit{in vitro} human buccal epithelial cultures
- Assessment of Acute THS 2.2 Aerosol Exposure in \textit{in vitro} Human Bronchial Epithelial Cultures
- Assessment of Acute THS 2.2 Aerosol Exposure in \textit{in vitro} Human Buccal Epithelial Cultures
- Assessment of Acute THS 2.2 Aerosol Exposure in \textit{in vitro} Human Nasal Epithelial Cultures
- Assessment of Repeated CHTP 1.2 Aerosol Exposure in \textit{in vitro} Human Gingival Epithelial Cultures
- Assessment of repeated THS 2.2 aerosol exposure in \textit{in vitro} human gingival epithelial cultures
- Atherogenesis Study \textit{in vitro} – Transendothelial Migration Assay with THS 2.2
Published Study Titles (Continued)

• Cigarette smoke reduces colitis severity in mice
• Cigarette smoke vs. e-cigarette aerosol: toxicological comparison with a 3D in vitro human respiratory model
• Clinical reduced exposure study with 5 days in a confinement setting (REX-C) – EU
• Clinical reduced exposure study with 5 days in a confinement setting (REX-C) – Japan
• Determination of eight carbonyls in aerosols trapped in PBS for in vitro assessment
• Effect of 3R4F smoke and THS 2.2 aerosol on the color stability of teeth.
• Effects of 3R4F smoke and THS 2.2 aerosol on the properties of dental resin composites
• Effects of cigarette smoke and electronic cigarette aerosol on the coloration of dental hard tissues and composite resin restorations
• Evaluation of a Novel Tobacco Vapor (NTV) product impact on the indoor air quality (IAQ)
• Heat-not-burn tobacco products: a systematic literature review (up to Nov 2017)
• IIS.PML.2017.16 Research on the Effects of Exhaled Pollutant from Tobacco Heating System (THS) on Indoor Air Quality
• Impact of E-vapor aerosols on the cardiovascular and respiratory systems in ApoE−/− mice
• Impact of THS 2.2-generated environmental aerosol on indoor air quality in comparison with smoke from a commercial cigarette.
• In vitro biological effects of selected individual smoke constituents and mixtures of smoke constituents
• In vitro systems toxicology assessment of selected flavoring substances in e-liquid formulations (flavor toolbox)
• In vitro toxicological and biological responses of aerosols from a novel hybrid tobacco product as compared with two tobacco heating products and a reference cigarette
• Investigation of Solid Particles in the Mainstream Aerosol of THS 2.2 and 3R4F
• Long-term exposure to THS 2.2 of human bronchial epithelial cells
• Nicotine pharmacokinetic profile and safety of the THS 2.2 Menthol - ZRHM-PK-05-JP
• Nicotine pharmacokinetic profile and safety of the Tobacco Heating System (THS) 2.2 - ZRHR-PK-02-JP
• Novel Tobacco Vapor product aerosol: chemistry analysis and in vitro toxicological evaluation in comparison with 3R4F cigarette smoke
Physico-chemical studies of direct interactions between components of electronic cigarette liquid mixtures and lung surfactants
Systems toxicology assessment of the biological effects of an e-liquid and its corresponding aerosol using 2D and 3D airway epithelial cultures
Systems Toxicology Meta-Analysis: Biological Impact of a Candidate MRTP Aerosol on Human Organotypic Cultures of the Aerodigestive Tract
THS 2.2 Menthol: Aerosol in vitro toxicology (Neutral Red Uptake, Ames assay and Mouse Lymphoma Assay), in comparison with 3R4F.
THS 2.2 Menthol: Chemical composition of aerosol in comparison with the mainstream smoke constituents of 3R4F.
THS 2.2 regular: Aerosol in vitro toxicology (Neutral Red Uptake, Ames assay and Mouse Lymphoma Assay), in comparison with 3R4F.
THS 2.2 regular: Chemical composition and physical properties of the aerosol in comparison with the mainstream smoke of 3R4F.
THS 2.2 regular: influence of tobacco blends on aerosol composition
Tier I peer review of toxicological assessment of the Tobacco Heating System 2.2.
Tier II peer-review of toxicological assessment of the Tobacco Heating System 2.2.
Acknowledgements
BACK UP SLIDES
Questions
Questions

• Describe the dose-response relationship in the dose range relevant to human exposure?

• Address human variability and sensitive populations?

• Incorporate existing biological understanding of the likely mode of action?
Flavor Group Representatives (FGRs) Selection Based on Structural Grouping Approach

- Question 1:
  Is the clustering approach appropriate? What would you add to strengthen the approach?
- Question 2:
  Is the FGR selection appropriate?
- Question 3:
  What would you do differently?
- Question 4:
  Are you familiar with similar approaches for the assessment of complex mixtures?
- Question 5:
  We consider the approach is applicable to other flavoring ingredients with further supporting *in vitro* work to establish specificity and sensitivity beyond the 246 flavoring ingredients evaluated in this study.
Representative Flavor Mixtures (RFMs): *In Vitro* Toxicity Screening

- **Question 1:**
  Do you consider the *in vitro* methods used appropriate for the flavor ingredient hazard characterization?

- **Question 2:**
  Do you consider a battery of *in vitro* tests (informed with *in vivo* data) appropriate to drive flavor system development?

- **Question 3:**
  What would you do differently?
Representative Flavor Mixtures (RFMs): Aerosol Generation and Characterization

• Question 1:
  Do you consider the aerosol generation by CAG appropriate for animal testing?

• Question 2:
  Do you consider the aerosol characterization in this project sufficient?

• Question 3:
  What would you do differently?
Representative Flavor Mixtures (RFMs):
5-Week Range-Finding Inhalation Study in A/J Mice

• Question 1:
• Question 2:
• Question 3:
• Question 4: