Preclinical Testing of Flavors in E-vapor Products, Part 3:
In Vitro Cytotoxicity and Genotoxicity of Representative Flavor Mixtures

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Overview of Session

- Part 1: Selection of Representative Flavor Mixtures Using a Structural Grouping Approach (Kim Ehman)

- Part 2: Preparation and Stability Characterization of Representative Flavor Mixtures (Cameron Smith)

- Part 3: *In Vitro* Cytotoxicity and Genotoxicity of Representative Flavor Mixtures (Utkarsh Doshi)

- Part 4: Flavor Transfer from the Liquid to the Aerosol for Inhalation Exposure (Jingjie Zhang)
Preclinical Testing of Flavors in E-vapor Products: Overview

Part 1
In-vitro Exposure

Part 2
E-Vapor Industry 5000+ Flavors

Part 3
PG VG Nicotine 38 Flavors

Part 4
Selection Process
Preclinical Application

Preparation, Characterization & Stability

E-Vapor Industry 5000+ Flavors
Background

- Flavor compounds for oral consumption fall within “generally recognized as safe (GRAS)” category

- Limited safety data exists for inhalation route of exposure

- Many flavor compounds in e-vapor products are commonly used as mixtures which makes their hazard characterization resource and time-demanding

- Alternative approach (part 1):
  - Evaluate structural similarities to develop representative flavor mixtures for preclinical toxicity testing

- Representative flavor mixtures were tested for in vitro cytotoxicity and genotoxicity
Test Articles:
- Carrier (PG:VG (80:20) + 2% Nicotine)
- Test Formulation (18.6% flavor)
- Test Formulation (18.6% flavor) + 2% Nicotine

OECD Tests:
- Ames Mutagenicity
- Micronucleus
- Neutral Red Uptake Cytotoxicity
Mutagenicity Assessment

- Detects compounds ability to cause mutations (point or frame-shift).
- Carrier & test formulations ±nicotine were tested in 5 strains of *Salmonella typhimurium* TA98, TA100, TA102, TA1535 & TA1537 in absence and presence of metabolic activation (Aroclor induced rat liver S9).

<table>
<thead>
<tr>
<th>Test Articles</th>
<th>Mutagenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrier (PG/VG/Nicotine)</td>
<td>Negative</td>
</tr>
<tr>
<td>Test Formulation</td>
<td>Negative</td>
</tr>
<tr>
<td>Test Formulation + Nicotine</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Genotoxicity Assessment

- TK6, human lymphoblast cell line.
- Three treatment conditions: Short term (±S9), long term (-S9).

![Cytotoxicity in TK6 cells](image)

<table>
<thead>
<tr>
<th>Concentration of E-liquid (%v/v)</th>
<th>% Viability (Relative Population Doubling)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>100</td>
</tr>
<tr>
<td>0.50</td>
<td>80</td>
</tr>
<tr>
<td>1.00</td>
<td>60</td>
</tr>
<tr>
<td>1.50</td>
<td>40</td>
</tr>
<tr>
<td>2.00</td>
<td>20</td>
</tr>
</tbody>
</table>

- Cytotoxicity in TK6 cells PG/VG/Nic
- Cytotoxicity in TK6 cells Test Formulation
- Cytotoxicity in TK6 cells Test Formulation + Nic

![Cytotoxicity plots](image)
Genotoxicity Assessment (cont)

<table>
<thead>
<tr>
<th>Test Articles</th>
<th>Genotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrier (PG/VG/Nicotine)</td>
<td>Negative</td>
</tr>
<tr>
<td>Test Formulation</td>
<td>Equivocal</td>
</tr>
<tr>
<td>Test Formulation + Nicotine</td>
<td>Negative</td>
</tr>
</tbody>
</table>

In Vitro Micronucleus Assay (Test Formulation-Nicotine/4h+S9)

- **% Micronuclei**
  - Upper limit of vehicle historical control

- **Cytotoxicity (%)**
  - DMSO
  - 0.04
  - 0.08
  - 0.14

* Criteria For Positive Genotoxicity Call

All 3 criteria have to be met:
- Statistical Significance (p≤0.05, Fisher exact)
- Outside of vehicle historical control
- Significant for trend

* p≤0.05, Fisher exact test
Cytotoxicity Assessment

- Murine fibroblast cell line (BALB/c 3T3 cells, clone 31)
- 48 hr treatment
Identifying Drivers of Cytotoxicity

- Cytotoxicity was a common trend observed in all 3 assays.

- To understand the drivers of cytotoxicity, 38 flavor ingredients were divided into sub-group mixtures (called pre-blends) based on their solubility and chemical reactivity (part 2) and tested using NRU assay.
Cytotoxicity Assessment of Pre-blends

- Pre-blends IA, IB and II were the major contributors to toxicity.
- Examples of flavors reported to be in vitro cytotoxic/irritant:
  - IA (isopulegol)
  - II (furaneol, ethyl maltol)
Conclusions

- Representative flavor mixtures did not show mutagenicity and genotoxicity in the in vitro assays.

- Representative flavor mixtures showed cytotoxicity in the in vitro assay, however the cytotoxicity was driven by few selected flavors or flavor groups.

- Use of read across approach in combination with systematic toxicity evaluation (deconstructing mixtures into subsets of flavors) can reduce the list of compounds for thorough toxicological evaluation.
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