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

Utkarsh Doshi

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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)

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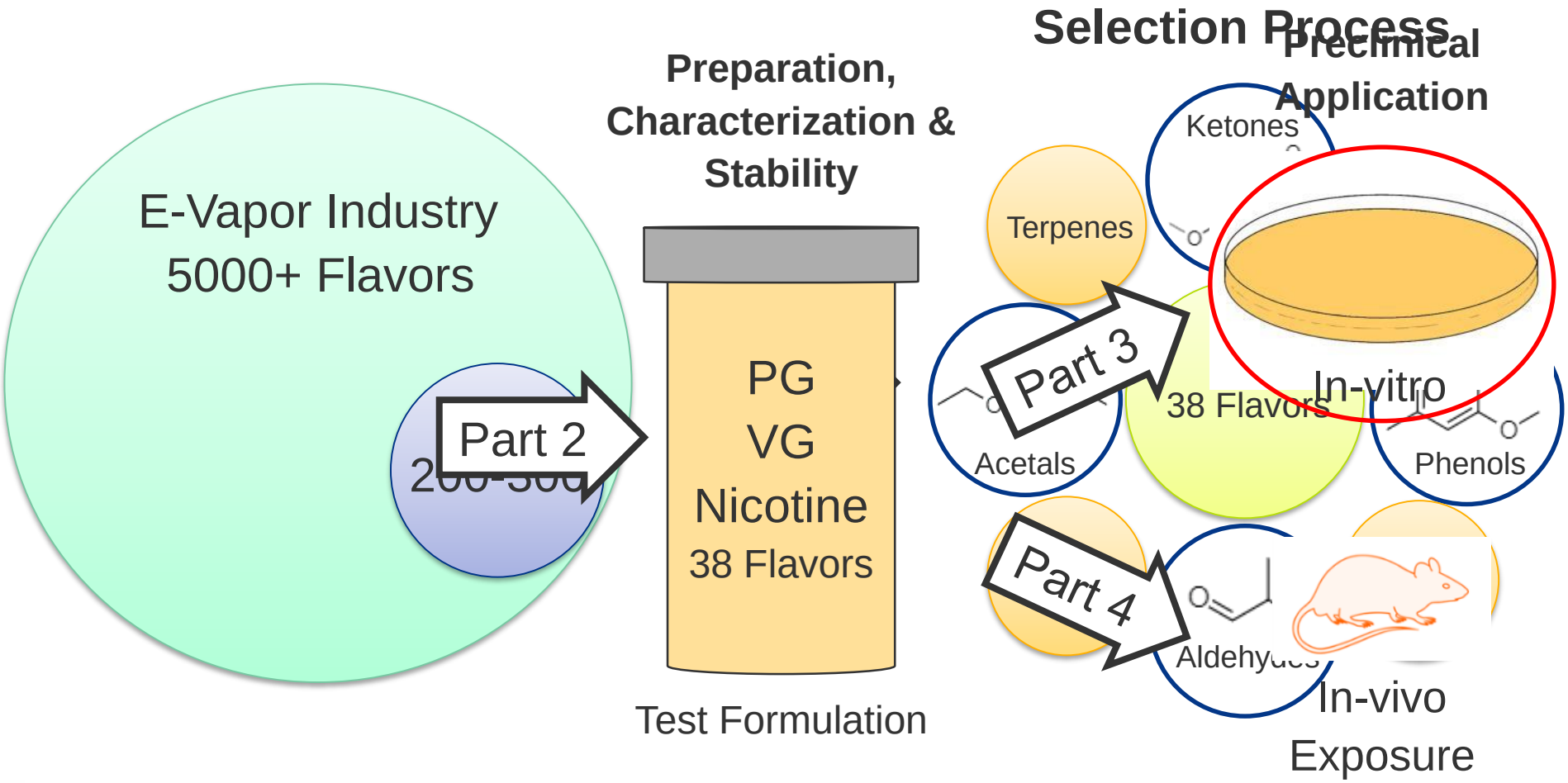
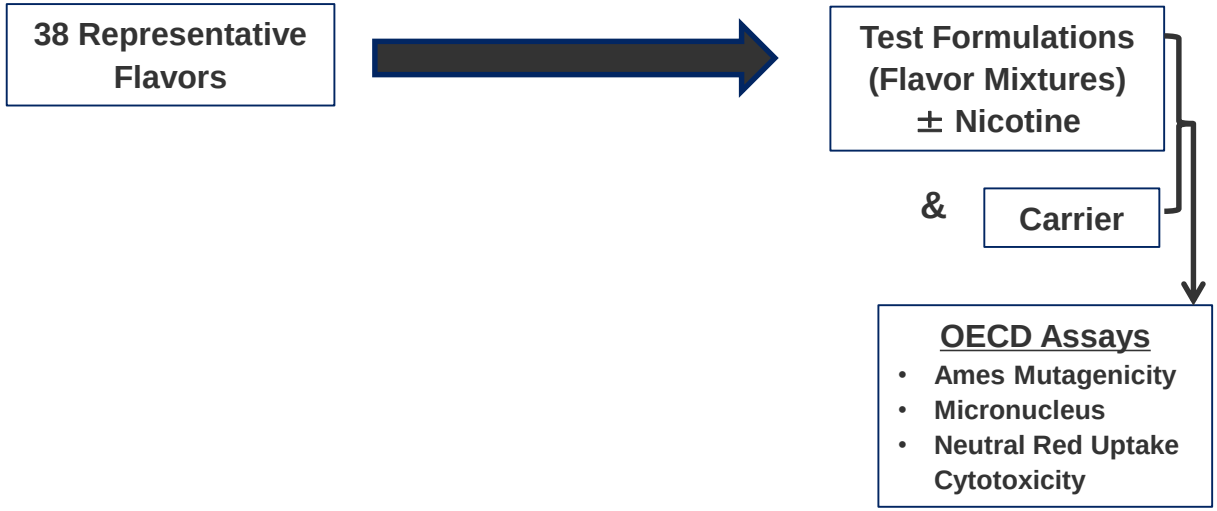
Preclinical Testing of Flavors in E-vapor Products: Overview



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

Background (cont)



- Test Articles:
  - Carrier (PG:VG (80:20) + 2% Nicotine)
  - Test Formulation (18.6% flavor)
  - Test Formulation (18.6% flavor) + 2% Nicotine

OECD: Organization for Economic Cooperation & Development

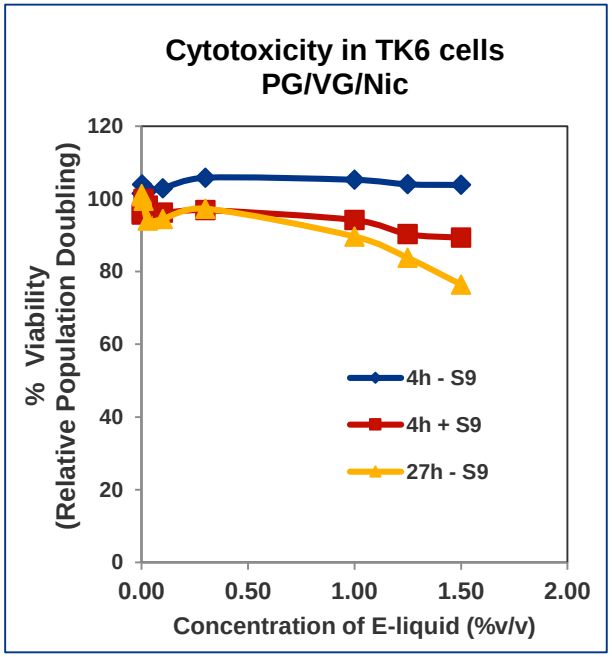
Mutagenicity Assessment

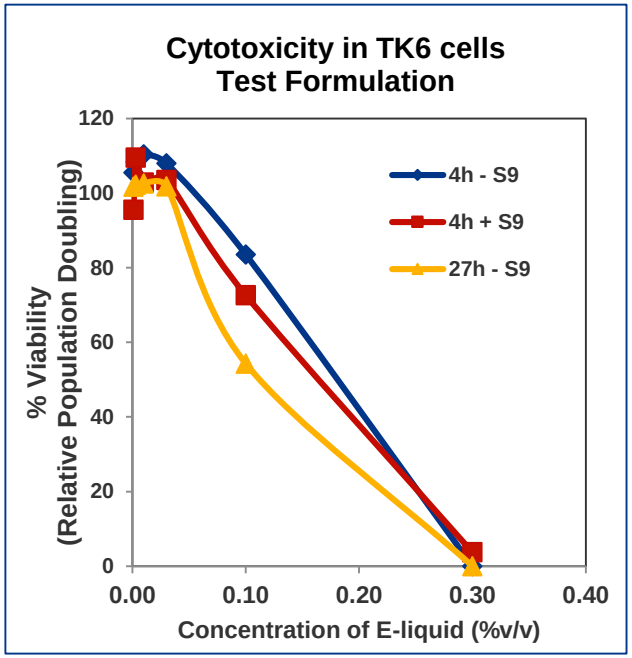
- **Ames Assay: OECD 471 Test Guidance (1997).**
- **Detects compounds ability to cause mutations (point or frame-shift).**
- **Carrier & test formulations ±nicotine were tested in 5 strains of *Salmonella typhimurium* TA98, TA100, TA102, TA1535 & TA1537 in absence and presence of metabolic activation (Aroclor induced rat liver S9).**

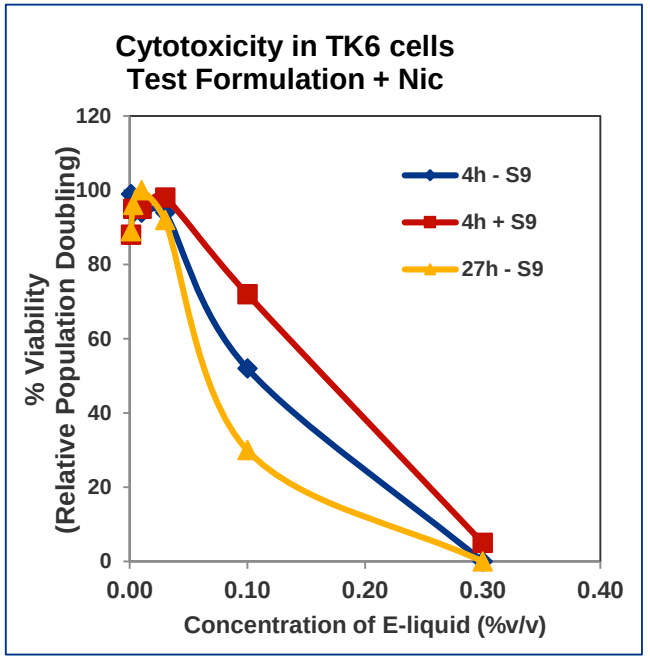
Test Articles	Mutagenicity
Carrier (PG/VG/Nicotine)	Negative
Test Formulation	Negative
Test Formulation + Nicotine	Negative

Genotoxicity Assessment

- **Mammalian in vitro micronucleus assay: OECD 487 Test Guidance (2016).**
- **TK6, human lymphoblast cell line.**
- **Three treatment conditions: Short term (±S9), long term (-S9).**







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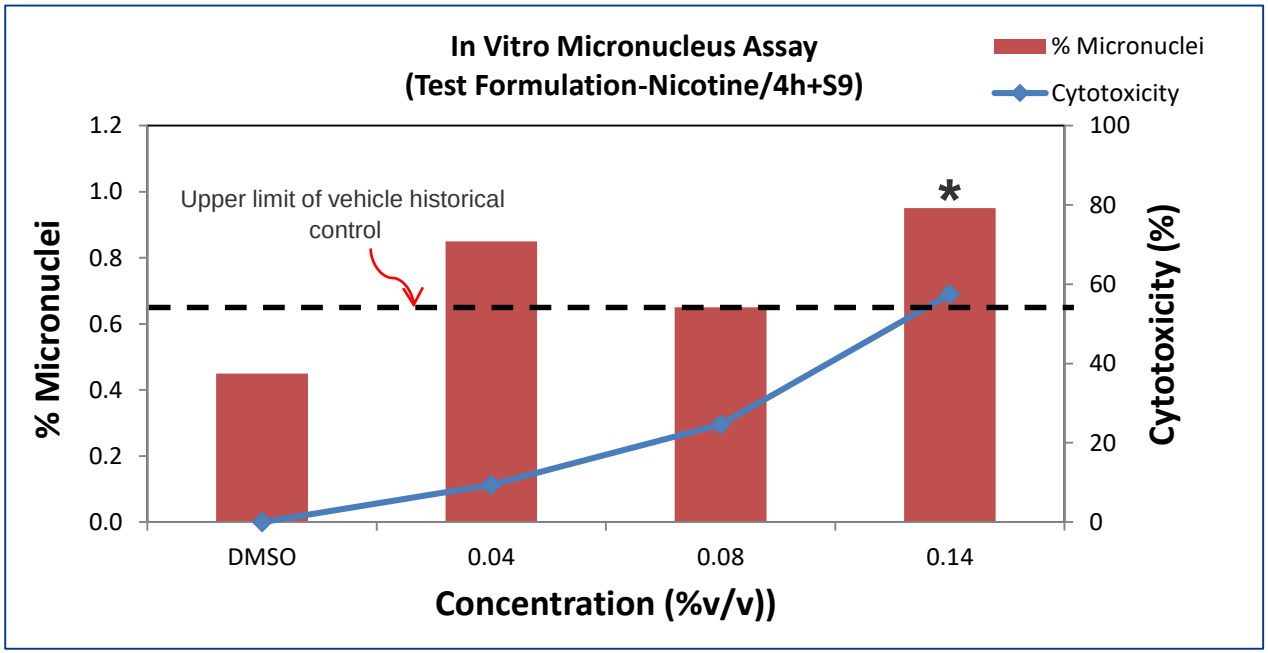
Genotoxicity Assessment (cont)

Test Articles	Genotoxicity
Carrier (PG/VG/Nicotine)	Negative
Test Formulation	Equivocal
Test Formulation + Nicotine	Negative



* p≤0.05, Fisher exact test

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

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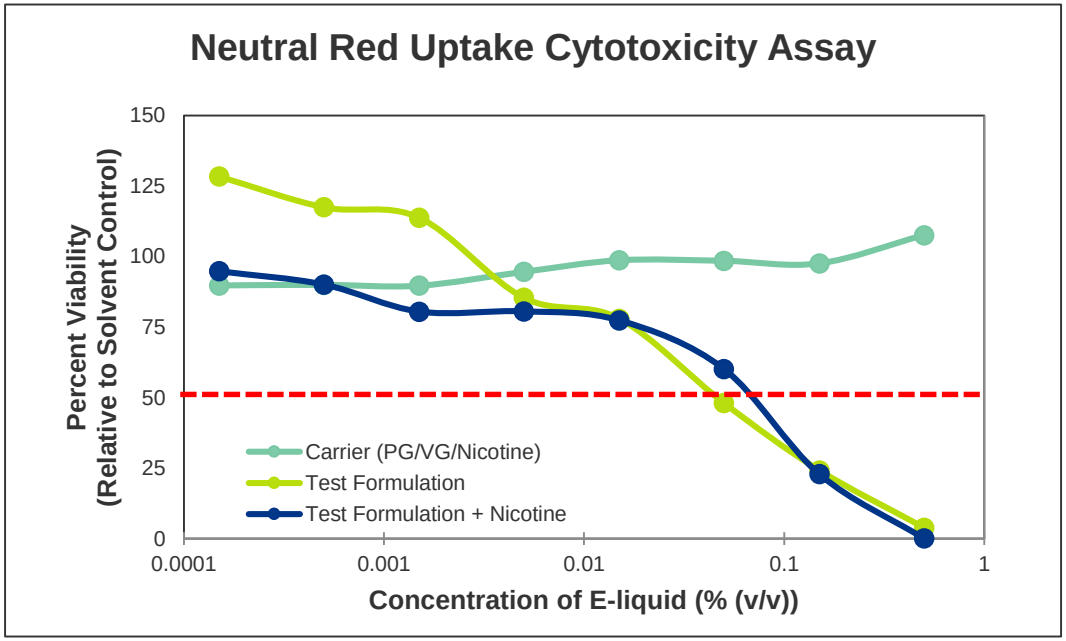
Cytotoxicity Assessment

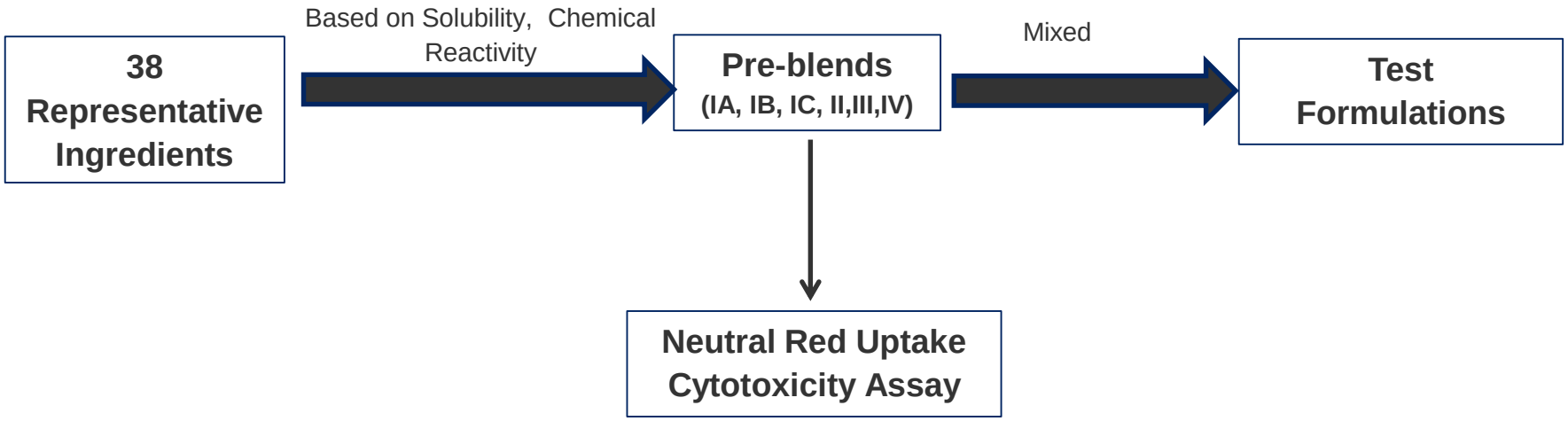
- **Neutral Red Uptake Assay: OECD 129 Test Guidance (2010)**
- **Murine fibroblast cell line (BALB/c 3T3 cells, clone 31)**
- **48 hr treatment**



50% Viability

Identifying Drivers of Cytotoxicity

- Cytotoxicity was 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.



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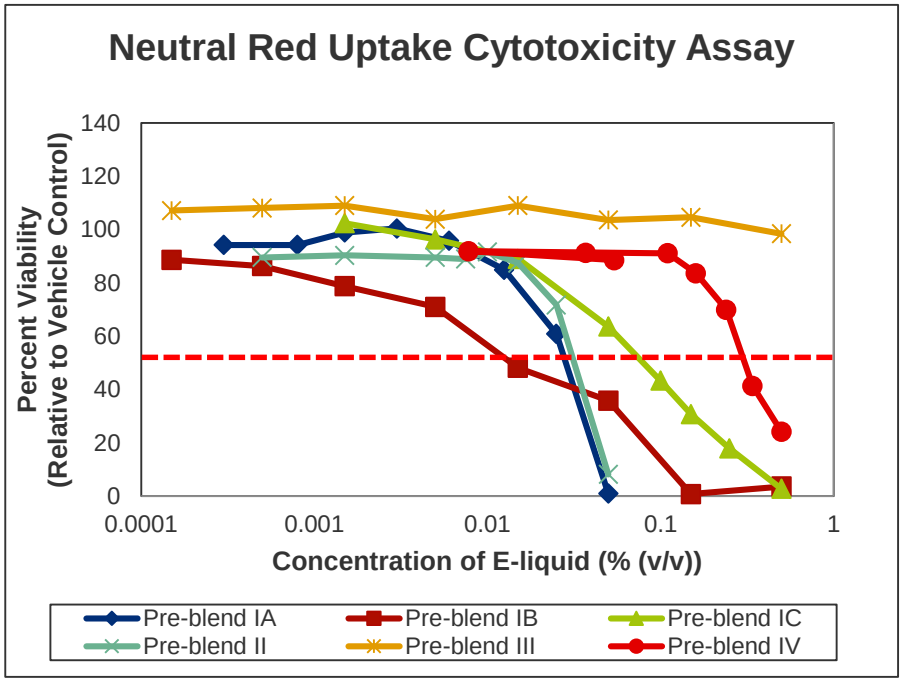
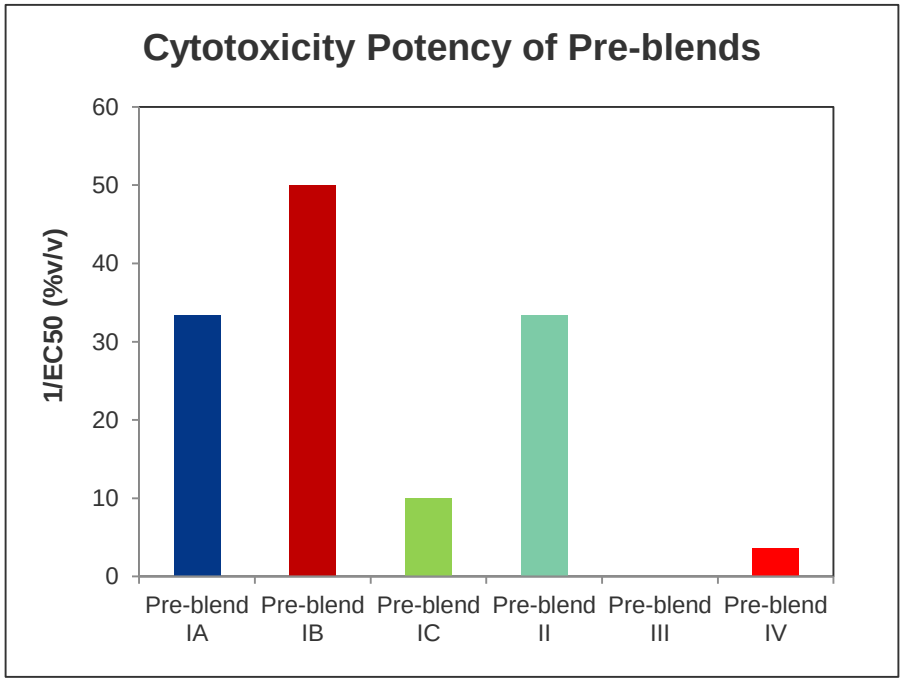
Cytotoxicity Assessment of Pre-blends





- 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)

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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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