Toxicological Assessment of E-liquid Formulations Using In Vitro Genotoxicity and Cytotoxicity Assays

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Abstract

In the Electronic Nicotine Delivery Systems (ENDS) Premarket Tobacco Application (PMTA; 2016) Draft Guidance, the FDA recommends a full assessment of the toxicological profile associated with new tobacco products, using in vitro toxicology (e.g., genotoxicity and cytotoxicity) studies. As part of a toxicological hazard assessment, we tested flavor varieties of e-liquids used in MarkTen® e-vapor products (a total of 14 formulations) and two carrier formulations (propylene glycol, glycerin, with 0% or 5% nicotine) to a standard battery of in vitro cytotoxicity (Neutral Red Uptake [NRU]) and genotoxicity (Ames and micronucleus [MN]) assays according to OECD guidelines and using the maximum doses suggested for mixtures. The e-liquid formulations were characterized for key ingredients (propylene glycol, glycerin, and nicotine). All the formulations were non-cytotoxic per NRU assay (viability >80%). None of the e-liquids were mutagenic in Ames assay, however some reduction in background lawn was observed with carrier formulation at the high (5%) nicotine content. In the MN assay, 3/14 MarkTen® flavor formulations induced a weak but statistically significant increase in micronuclei formation, resulting in positive or equivocal findings according to OECD 487: All three flavor formulations were further evaluated in an in vivo combined genotoxicity (MN and Comet; OECD 474/489) assay and found to be negative for genotoxic endpoints. Therefore, consistent with International Conference on Harmonization (ICH) S2(R1) genotoxicity testing guideline (2012), the tested e-liquids were regarded as negative for genotoxicity under the conditions of the assays.

Experimental Method

Test Articles

Total of 14 e-liquids used in MarkTen® products containing various levels [up to 4% nicotine; flavors; propylene glycol (PG); glycerol (VG)] and carrier (PG/VG with 0% and 5% nicotine).

Neutral Red Uptake (NRU) Assay

Balb/c 3T3 cells were incubated either in the presence of vehicle control (DMSO) or increasing concentrations of positive control (sodium lauryl sulfate) or e-liquid for ~48 h according to OECD 129[2]. The maximum concentration of e-liquid was up to 0.5% (v/v).

Salmonella Mutagenicity (Ames) Assay

Five *Salmonella typhimurium* strains (TA1537, TA98, TA100, TA1535 and TA102) were used according to OECD 471[3]. Cytotoxicity was assessed to determine the doses with the maximum concentration tested up to 100 µL/plate. Testing was performed in triplicate in presence and absence of metabolic activation (S9). DMSO was used as vehicle control.

In Vitro Micronucleus (MN) Assay

E-liquids were evaluated for micronucleus induction according to OECD 487[4] in TK6 cells during short (4 h) incubations with and without S9, and long (27 h) incubations without S9, followed by an extended recovery of 40 h. Cytotoxicity was assessed to determine the doses, with the maximum concentrations tested up to 1% (v/v).

In Vivo Micronucleus (MN) & Comet Assay (Follow up)

For 3/14 formulations, in vivo genotoxicity study was performed per OECD 474 (2016)[5] and OECD 489 (2016).[6] Male and female rats were used (data shown for male rats only).

In Vivo Genotoxicity		
Groups	**Test Materials**	**Animal Number (M/F)**
Negative Control	Filtered Air	6/6
Test Article (TA)	TA-Low (~¼ MTD)	6/6
	TA-Mid (~½ MTD)	6/6
	TA-High (MTD)	8/8
Reference	Base Formulation (PG/G/Nicotine, flavor free) (MTD)	8/8
Positive Control	CP 20 mg/kg/day (2 d); EMS: 200 mg/kg (1 d)	6/6

Exposure regimen
- Nose-only inhalation, up to 6 hrs/day, 4 days
- Aerosol generated by a CAG: ~ 275 ◦C
- Particle size: MMAD 0.7-1.1 µm (GSD 1.6 - 2.2)

Sample collection
- Positive control: 2-4 hrs after EMS (18-24 hrs after the 2nd CP)
- Post-exposure plasma: nicotine and cotinine (within 5 min)
- MN: bone marrow
- Comet: nasal, liver, and lung tissue

Observations & Conclusions

- All e-liquid formulations tested were negative for cytotoxicity under the conditions of the NRU assay.
- All e-liquid formulations tested were negative for mutagenic activity under the conditions of the Ames assay.
- In the in vitro MN assay, 11 / 14 e-liquid formulations were negative for inducing micronuclei in TK6 cells: The remaining 3 formulations (MarkTen® Bold Classic, Summer Fusion and Menthol) were positive according to the OECD 487. These 3 formulations were further evaluated in the in vivo genotoxicity (MN and Comet) OECD assays. There was no increase in two in vivo genotoxicity endpoints (bone marrow MN ;and DNA breakage in the site of entry (nasal tissue), lung and liver (Comet assay)) in these 3 formulations and their base formulations, compared to the negative control (filtered air). In summary, under the tested conditions, negative results in the combined in vivo assays, demonstrated absence of genotoxic risk.

References

1. FDA (2016) Guidance for Industry: Premarket Tobacco Product Applications for Electronic Nicotine Delivery Systems, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Tobacco Products, May.
2. OECD (2010) OECD Guidance Document on Using Cytotoxic Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests.
3. OECD (1997) OECD Guidance Document for Testing Chemicals Test Guideline 471, Bacterial Reverse Mutation Assay.
4. OECD (2016) OECD Guidance Document for Testing Chemicals Test Guideline 487, In Vitro Mammalian Cell Micronucleus Test.
5. OECD (2016) OECD Guidance Document for Testing Chemicals Test Guideline 474, In Vivo Mammalian Erythrocyte Micronucleus Test.
6. OECD (2016) OECD Guidance Document for Testing Chemicals Test Guideline 489, In Vivo Mammalian Alkaline Comet Assay.
7. ICH (2012) S2(R1) - Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use.

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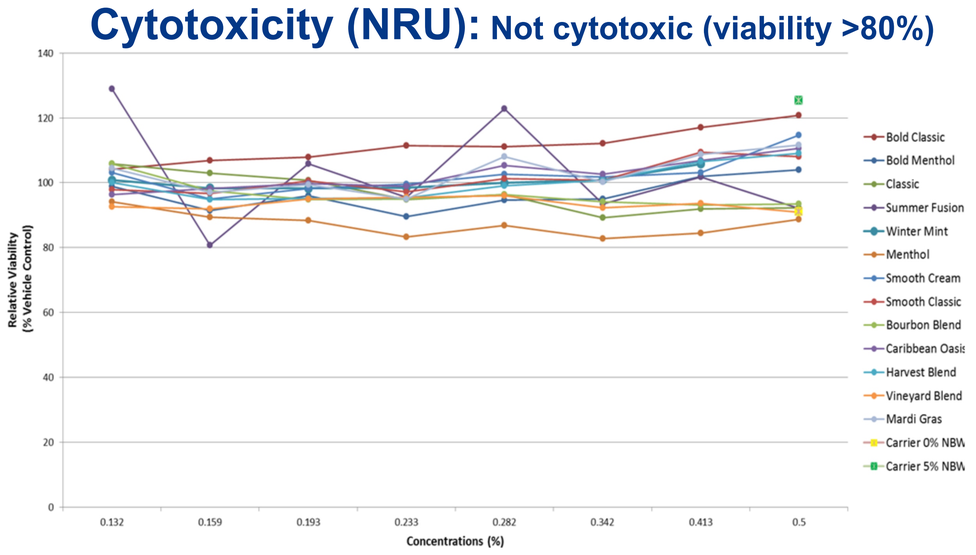
Results

Cytotoxicity (NRU): Not cytotoxic (viability >80%)

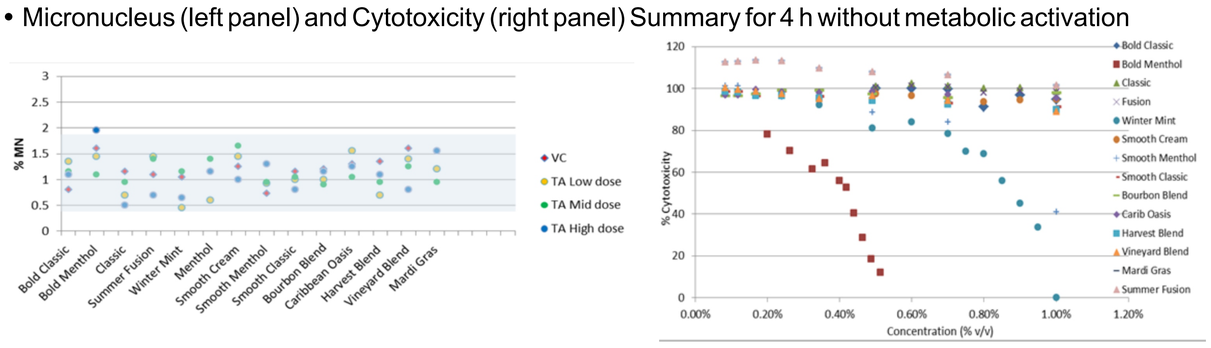


Mutagenicity (Ames): Negative in all strains (±S9)

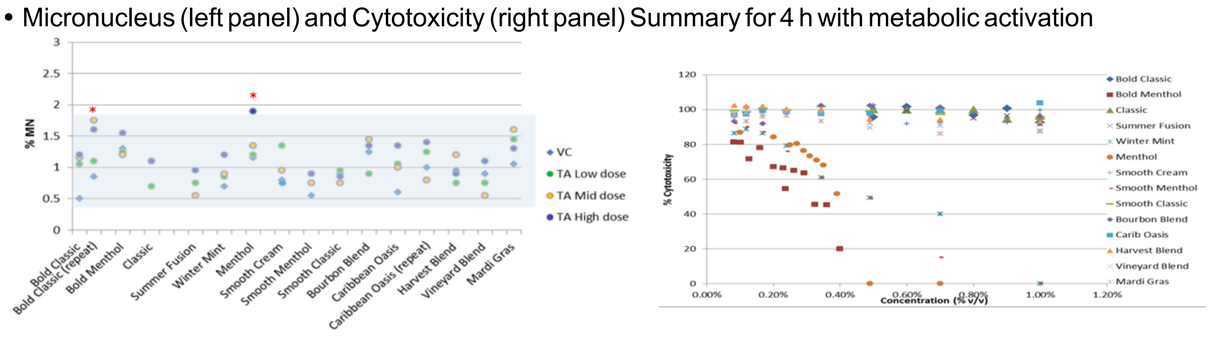
Current Brand Name	Cytotoxicity Results
Carrier (PG/VG/0% NBW)	No cytotoxicity observed
Carrier (PG/VG/5% NBW)	Cytotoxicity in strains TA1537, TA100 and TA1535 –S9 at 100 µL/plate
Classic	No cytotoxicity observed
Summer Fusion	Cytotoxicity in strains TA1537, TA100 and TA1535 –S9 at ≥90 µL/plate
Winter Mint	Cytotoxicity in strains TA1537 and TA100 ± S9 at ≥12.5 µL/plate; Cytotoxicity in strains TA98, TA102 and TA1535 ± S9 at ≥25 µL/plate
Menthol	Cytotoxicity in strains TA1537, TA98 and TA1535 –S9 and TA 100 ±S9 at ≥6.25 µL/plate; Cytotoxicity in strains TA1537, TA98 and TA1535 +S9 and TA 102 ±-S9 at ≥12.5 µL/plate
Bold Classic	Cytotoxicity in strain TA1537 –S9 at 100 µL/plate
Bold Menthol	Cytotoxicity in strains TA1537, TA98, TA100, TA1535 and, TA102 ±S9 at ≥20 µL/plate
Smooth Cream	No cytotoxicity observed
Caribbean Oasis	No cytotoxicity observed
Mardi Gras	No cytotoxicity observed
Smooth Menthol	Cytotoxicity in all strains –S9 at ≥12.5 µL/plate; Cytotoxicity in strains TA1537, TA98 and TA100 +S9 at ≥12.5 µL/plate; Cytotoxicity in strains TA1535 and TA102 +S9 ≥25 µL/plate
Harvest Blend	Cytotoxicity in strains TA1537, TA98, TA100, TA1535 and TA102 –S9 at ≥50 µL/plate and TA1535 +S9 at 100 µL/plate
Vineyard Blend	No cytotoxicity observed
Bourbon Blend	Cytotoxicity in strain TA1535 –S9 at 100 µL/plate
Smooth Classic	No cytotoxicity observed

Genotoxicity (in vitro MN): 3/14 e-liquids, positive/equivocal

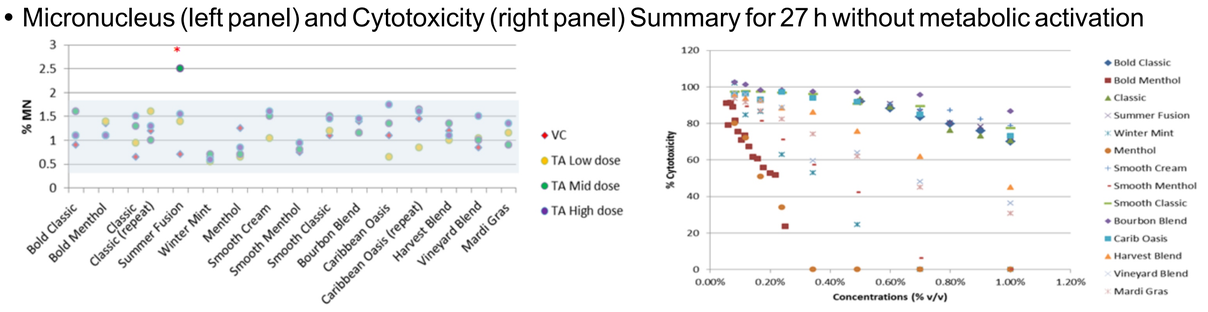
- Micronucleus (left panel) and Cytotoxicity (right panel) Summary for 4 h without metabolic activation



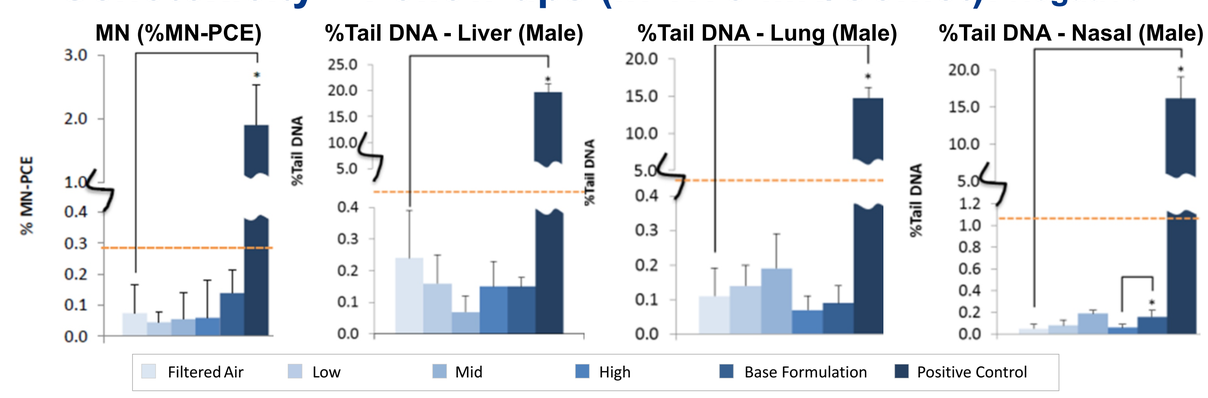
- Micronucleus (left panel) and Cytotoxicity (right panel) Summary for 4 h with metabolic activation



- Micronucleus (left panel) and Cytotoxicity (right panel) Summary for 27 h without metabolic activation



Genotoxicity – Follow ups (in vivo MN/Comet): Negative



Data shown for Summer Fusion.
Bold Classic and Menthol were also negative in vivo genotoxicity assays (data not shown)