# Toxicological Assessment of E-liquid Formulations Using In Vitro Genotoxicity and Cytotoxicity Assays Bonnie G. Coffa, Utkarsh Doshi, Jingjie Zhang, Willie J. McKinney, K. Monica Lee



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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In the Electronic Nicotine Delivery Systems (ENDS) Premarket Tobacco Application (PMTA; 2016) Draft Guidance, the FDA recommends a full assessment of the toxicological profile associated with new tobacco products, using in vitro toxicology (e.g., genotoxicity and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<sup>®</sup> 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<sup>®</sup> 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<sup>®</sup> 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<sup>2</sup>. 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<sup>3</sup>. Cytotoxicity was assessed to determine the doses with the maximum concentration tested up to 100 µL/plate. Testing 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<sup>4</sup> 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)<sup>5</sup> and OECD 489 (2016).<sup>6</sup> Male and female rats were used (data shown for male rats only).

In Vivo Genotoxicity					
Groups	Test Ma	Animal Number (M/F)			
<b>Negative Control</b>	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		
<b>Positive Control</b>	CP 20 mg/kg/day (2 d); EMS: 200 mg/kg (1 d)		6/6		
Exposure regimen		Sample collection			

Exposure regimen			Sample collection	
• •	Nose-only inhalation, up to 6 hrs/day, 4	•	Positive control: 2-4 hrs after EMS	
c	lays		hrs after the 2 <sup>nd</sup> CP)	
• A	Aerosol generated by a CAG: ~ 275 °C	•	Post-exposure plasma: nicotine and	
• F	Particle size: MMAD 0.7-1.1 µm (GSD		cotinine (within 5 min)	
1	1.6 - 2.2)	•	MN: bone marrow	
		•	Comet: nasal, liver, and lung tissue	

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Mutagenicity (Ames): Neg				
Current Brand Name	Cytotoxicity Results			
Carrier (PG/VG/0% NBW)	No cytotoxicity observed			
Carrier (PG/VG/5% NBW)	Cytotoxicity in strains TA1537, T			
Classic	No cytotoxicity observed			
Summer Fusion	Cytotoxicity in strains TA1537, T			
Winter Mint	Cytotoxicity in strains TA1537 ar Cytotoxicity in strains TA98, TA1			
Menthol	Cytotoxicity in strains TA1537, T $\geq$ 6.25 µL/plate; Cytotoxicity in and TA 102 ± S9 at $\geq$ 12.5 µL/pla <sup>2</sup>			
Bold Classic	Cytotoxicity in strain TA1537 –S			
Bold Menthol	Cytotoxicity in strains TA1537, T ≥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 TA1537, TA98 and TA100 +S9 at TA1535 and TA102 +S9 at <u>&gt;</u> 25 μ			
Harvest Blend	Cytotoxicity in strains TA1537, The second strains TA1537 at $\geq$ 50 $\mu$ L/plate and TA1535 +S9 at			
Vineyard Blend	No cytotoxicity observed			
Bourbon Blend	Cytotoxicity in strain TA1535 –S			
Smooth Classic	No cytotoxicity observed			

• 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.

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.

## **Observations and Conclusions**

• In the in vitro MN assay, 11 / 14 e-liquid formulations were negative for inducing micronuclei in TK6 cells: The rest of 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

Base Formulation

Positive Control