In vivo Genotoxicity Testing of Aerosolized ENDS E-liquids

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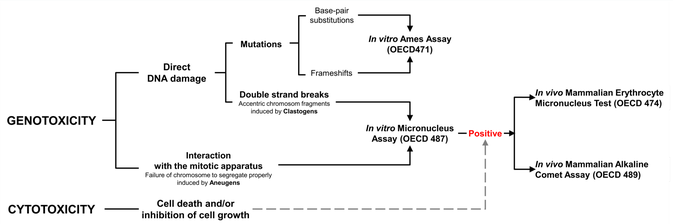
Abstract

The FDA draft guidance (2016) on premarket tobacco product application for electronic nicotine delivery systems (ENDS) recommends toxicity assessment including *in vitro* genotoxicity. As part of due diligence hazard assessment, we subjected e-liquids used in MarkTen® e-vapor products to standard *in vitro* genotoxicity (Ames and micronucleus [MN]) testing. None of e-liquids were mutagenic in Ames assay, however some e-liquids induced a weak but statistically significant increase in MN, resulting in positive or equivocal findings according to OECD487. Herein, we performed follow-up *in vivo* genotoxicity testing (a combined MN and Comet test) according to ICH guidance S2 (R1) to evaluate the biological relevance of *in vitro* MN results. Three different e-liquids were tested under the combined *in vivo* study design based on OECD489 (Comet) and 474 (MN). Male and female Crl:CD(SD) rats were exposed to filtered air (negative control) or e-liquid aerosols via nose-only inhalation for up to 6 hrs/day, 4 consecutive days. The capillary aerosol generator (CAG) was used to generate the aerosols with the particle size (MMAD) of 0.7-1.1µm (GSD 1.6-2.2). The highest exposure concentrations (up to 2 mg/L total particulate matter [TPM]) were selected for each e-liquid based on the respective maximum tolerated dose. The study included concurrent positive controls (cyclophosphamide [CP] and ethyl methanesulfonate [EMS], administered by oral gavage). Blood samples were collected immediately after the last exposure and analyzed for biomarkers of exposure (nicotine and cotinine). At necropsy, bone marrow samples were collected for MN evaluation and the liver, lung, and nasal tissue samples were collected for the Comet assay (DNA breakage). In all three studies, the plasma nicotine and cotinine levels increased with increasing aerosol exposure concentration (TPM). The male groups tolerated higher TPM exposures than the female groups. For the three e-liquids tested, there were no significant increases in the %MN in the bone marrow and the % Tail DNA (DNA breakage) in liver, lung, and nasal tissues compared to the negative control group. Therefore, under the tested *in vivo* condition, these e-liquids were negative for genotoxicity, implying no biological relevance of weak *in vitro* genotoxicity signals.

Genotoxicity Testing Battery for ENDS E-liquids



In vitro Testing of Three ENDS E-liquids (PG/G/Flavors/Nicotine)

ENDS E-liquids	Nicotine by weight (NBW)	NRU (Balb/c 3T3 Fibroblasts)	Ames (5 Stains of S. Typhimurium)	MN (TK6, a human lymphoblast cell line)
1	2.5%	> 80% viability	Negative	Positive 27 hr w/o S9 [a,b]
2	4%	> 90% viability	Negative	Positive 4 hr w/ S9 [a]
3	3.5%	> 80% viability	Negative	Positive 4 hr w/ S9 [a, b, c]

a. Significant increase compared to the concurrent vehicle control; b. Positive for dose-response trend with Cochran Amitage test; c. Significant increase compared to published historical control for the vehicle (0.4-1.8%, Sobol et al. 2012)

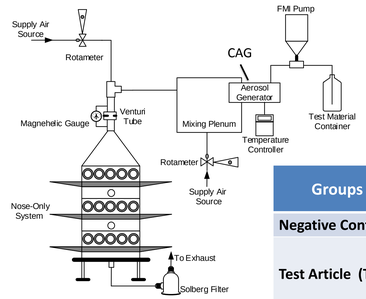
Reference

1. OECD (2014), *Test No. 474: Mammalian Erythrocyte Micronucleus Test*, OECD Publishing, Paris.
2. OECD (2016), *Test No. 489: In Vivo Mammalian Alkaline Comet Assay*, OECD Publishing, Paris.
3. Sobol Z, Homiski, M. L., Dickinson, D.A., et al. (2012). Development and validation of an in vitro micronucleus assay platform in TK6 cells. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 746 (1): 29-34
4. U.S. Department of Health and Human Services/Food and Drug Administration/Center for Tobacco Products. (2016). Guidance for industry (DRAFT): Premarket Tobacco Product Applications for Electronic Nicotine Delivery Systems .
5. International Conference on Harmonisation. (2011). S2(R1): Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use.
6. Lee, K. M. (2017) A combined In Vivo Micronucleus and Comet Study of an Aerosolized ENDS Formulation. ICEM-ACEM 2017, Seoul, Korea.

Study Design for Combined In Vivo Genotoxicity Testing

Topic	Suggested by ICH Guidance	Study Design Used	Note
Study duration	Single or repeated	Repeated (3-4 days)	Can be part of safety tox study
Animals, sex	Young rodents M (unless sex-specific)	Rats, M/F (~7 week at start)	The sex with reduced exposure may not be scored
Top dose	Max. tolerated dose (MTD)	MTD (range-finding)	Max. feasible/ possible dose
Route of exposure	Clinically relevant	Nose-only inhalation	Aerosol exposures
Endpoints	DNA break; cytogenetics	Comet & MN	Preferable in a single study
Target tissues	Clinical relevant; site of contact	Nasal, lung, liver; bone marrow	Exposure-relevant
Exposure confirmation	Cytotoxicity or exposure	Plasma nicotine & cotinine	Systemic exposures similar or higher than clinical
Positive controls	Not always; other route acceptable	PC for each endpoints; oral	If established, not always

Exposure System and Definitive Study



Exposure regimen
- Nose-only inhalation, up to 6 h/day, 4 days
- Aerosol generated by a Capillary Aerosol Generator (CAG)
- Particle size: MMAD 0.7-1.1 µm (GSD 1.6 - 2.2)

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

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)	8/8
Positive Control	CP 20 mg/kg/day (2 d); EMS: 200 mg/kg (1 d)	6/6

Conclusion

- Three ENDS e-liquids were tested in combined *in vivo* genotoxicity study via inhalation according to ICH S2(R1) guidance, as a follow-up of positive *in vitro* MN results.
- Exposure concentrations were set to the MTD, based on mortality and abnormal clinical signs. Males groups were found to be able to tolerate higher TPM (total particulate matter, aerosol mass) exposure levels.
- The plasma nicotine and cotinine levels increased with increasing TPM exposure concentration in the three studies.
- There was no increase in two genotoxicity endpoints (MN and Comet) in all three e-liquids 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, with the examined target tissues and the markers of exposure, demonstrated absence of significant genotoxic risk.**

Acknowledgement

Thanks

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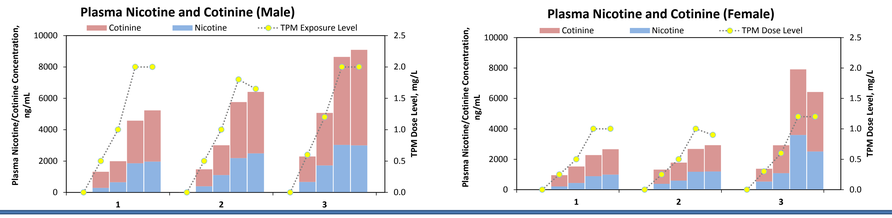
The poster can be found at www.altria.com/ALCS-Science
www.criver.com

Results

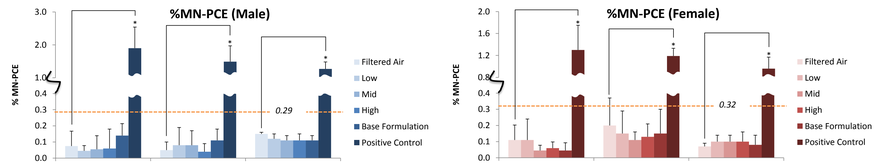
Biomarkers of Exposure: Plasma Nicotine and Cotinine

(Filtered air, Low, Mid, High, and Base Formulation from left to right for each test article.)



Micronucleus (Bone Marrow)

(The dotted line shows the upper limit of the 95% confidence interval for the negative historical control.)



Comet (Liver, Lung, and Nasal)

(The dotted line shows the upper limit of the 95% confidence interval for the negative historical control.)