Comparative studies on the genotoxic and cytotoxic potential of mainstream smoke condensate from menthol and non-menthol cigarettes which burn or primarily heat tobacco.

B R Bombick, J T Avalos, K P Putnam, D L Bowman, J B Mabe, K W Fowler, D W Bombick, W T Morgan, and D J Doolittle. Product Evaluation, R J Reynolds Tobacco Company, Winston-Salem, NC, USA.

53030 1900

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187

ABSTRACT

The genotoxic potential of mainstream cigarette smoke condensate (CSC) from Eclipse menthol cigarettes, which primarily heat tobacco, was compared to that of CSC from Eclipse non-menthol cigarettes. Matched tobacco-burning menthol and non-menthol ultralight cigarettes were also compared. CSCs from all four cigarettes were evaluated in an *in vitro* toxicology test battery which included sister chromatid exchange (SCE) and Neutral Red cytotoxicity assays in Chinese Hamster ovary cells, and the Ames bacterial mutagenicity assay. CSCs from the tobacco-burning menthol and non-menthol cigarettes were positive in the Ames assay (TA98, TA100, TA1538, and TA1537), SCE, and Neutral Red cytotoxicity assays. CSC from the two Eclipse cigarettes was positive in the Ames assay in the presence of S9 activation and in the SCE assay without activation, but yielded negative results in the cytotoxicity assay. CSCs from the Eclipse cigarettes were significantly lower ($p<0.05$) in biological activity than CSC from tobacco-burning cigarettes in all assays. However, there were no significant differences between Eclipse menthol and non-menthol cigarettes (at $p<0.05$), and no significant differences (at $p<0.05$) between the menthol and non-menthol tobacco-burning cigarettes. These data indicate that the use of menthol does not significantly increase the genotoxic potential of CSC as measured in the Ames and SCE tests, or cytotoxicity as measured in the Neutral Red assay.

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187

INTRODUCTION

In vitro genotoxicity assays have been widely used to study the biological activity of tobacco smoke and tobacco smoke condensate (DeMarini, 1983; Lee, 1987; Bombick, 1998). A significant portion of the mutagenic activity of cigarette smoke condensate appears to be due to products of pyrolysis (Mizusaki et al, 1977; Doolittle et al, 1990). Thus cigarette smoke condensate from a cigarette that burns only a small amount of tobacco should have reduced genotoxic and cytotoxic potential compared with cigarette smoke condensate from cigarettes that burn tobacco. The Eclipse cigarette developed by R.J. Reynolds Tobacco primarily heats, rather than burns, tobacco.

In this study we investigated whether the genotoxic or cytotoxic activity of tobacco-burning and tobacco-heating cigarettes was affected by the addition of menthol. Eclipse non-menthol and menthol cigarettes were compared to each other, and compared to tobacco-burning menthol and non-menthol cigarettes as well. Kentucky Reference 1R4F (Sullivan, 1984) was included as an additional Reference cigarette. We evaluated the cytotoxic and genotoxic potential of these cigarette smoke condensates (CSC) in the Ames *Salmonella*/microsome assay, Sister Chromatid Exchange and the Neutral Red cytotoxicity assays.

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187

DESCRIPTION OF CIGARETTES

Cigarette	Description	mg TPM/ cigarette*	# Puffs/ cigarette	mg menthol in smoke/ cigarette
Eclipse non-menthol	Non-menthol cigarette that primarily heats tobacco	4.1 ± 0.27	15	0
Eclipse menthol	Menthol cigarette that primarily heats tobacco	4.7 ± 0.25	15	0.15
TOB-Non-menthol	Ultra-low 'tar' tobacco-burning cigarette	4.8 ± 0.18	9.0 ± 0.16	0
TOB-menthol	Ultra-low 'tar' tobacco-burning menthol cigarette	4.2 ± 0.16	9.0 ± 0.09	0.42
1R4F	Kentucky Reference low 'tar' cigarette	11.6 ± 0.23	9.2 ± 0.16	0

* Total particulate matter, obtained by FTC smoking regimen (see materials and methods)

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187

MATERIALS AND METHODS

Preparation of cigarette smoke condensate (CSC):

Cigarette smoke condensate was prepared by smoking the cigarettes on a smoking machine under a standard Federal Trade Commission (FTC) smoking regimen (35-ml puff volume, 2-sec duration, once per minute). Cigarette total particulate matter (TPM) was collected onto a Cambridge filter pad, and extracted with dimethylsulfoxide (DMSO) to yield a 10 mg TPM/ml stock solution. The stock solution was further diluted to provide dosing solutions.

Ames assay:

- Standard procedure of Maron and Ames (1983), with pre-incubation modification (Yahagi, 1975).
- Tester strains: TA98, TA100, TA1535, TA1537, TA1538
- S9 activation: rat liver S9 (Aroclor 1254-induced), in the incubation medium at a concentration of 5%, NADP, Glucose-6-phosphate, potassium chloride, magnesium chloride & phosphate buffer.

Assay Positive Controls

	TA98	TA100	TA1535	TA1537	TA1538
Activation	2AA[a] (0.5 µg) 1R4F 25-250 µg	2AA (0.5 µg) 1R4F 25-250 ug	2AA (1.0 µg)	2AA (1.0 µg)	2AA (0.5 µg)
Non-activation	2 NF[b] (4.0 µg)	Na Azide (1.0 µg)	Na Azide (1.0 µg)	9 AA (100 µg)	2 NF (4 µg)

[a] 2-aminoanthracene [b] 2 nitrofluorene

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187

Sister Chromatid Exchange

- Standard Procedure of Perry and Wolff (1974); Galloway *et al.*. (1985).
- Cell line: CHO, WBL
- S9 activation: Rat liver S9 (15 ul/ml, from Aroclor 1254-induced rats, Mol-Tox, Boone, NC); NADP and isocitric acid in media without serum.

Assay Conditions	Treatment duration	Harvest time	Positive controls
Activation	2 hours	27-31 hours after treatment initiation	Cyclophosphamide 1.5 ug/ml media
Non-activation	26 hours	27-31 hours after treatment initiation	Mitomycin C 0.005 ug/ml media

Neutral Red Cytotoxicity Assay

- Standard procedure of Borenfreund *et al.* 1984, modified by Bombick D. *et al.*, 1995.
- Cell line: CHO, WBL
- Treatment duration: Activation: not tested
Nonactivation: 24 hours
- Positive control: Kentucky Reference 1R4F in-house standard

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187

AMES ASSAY RESULTS

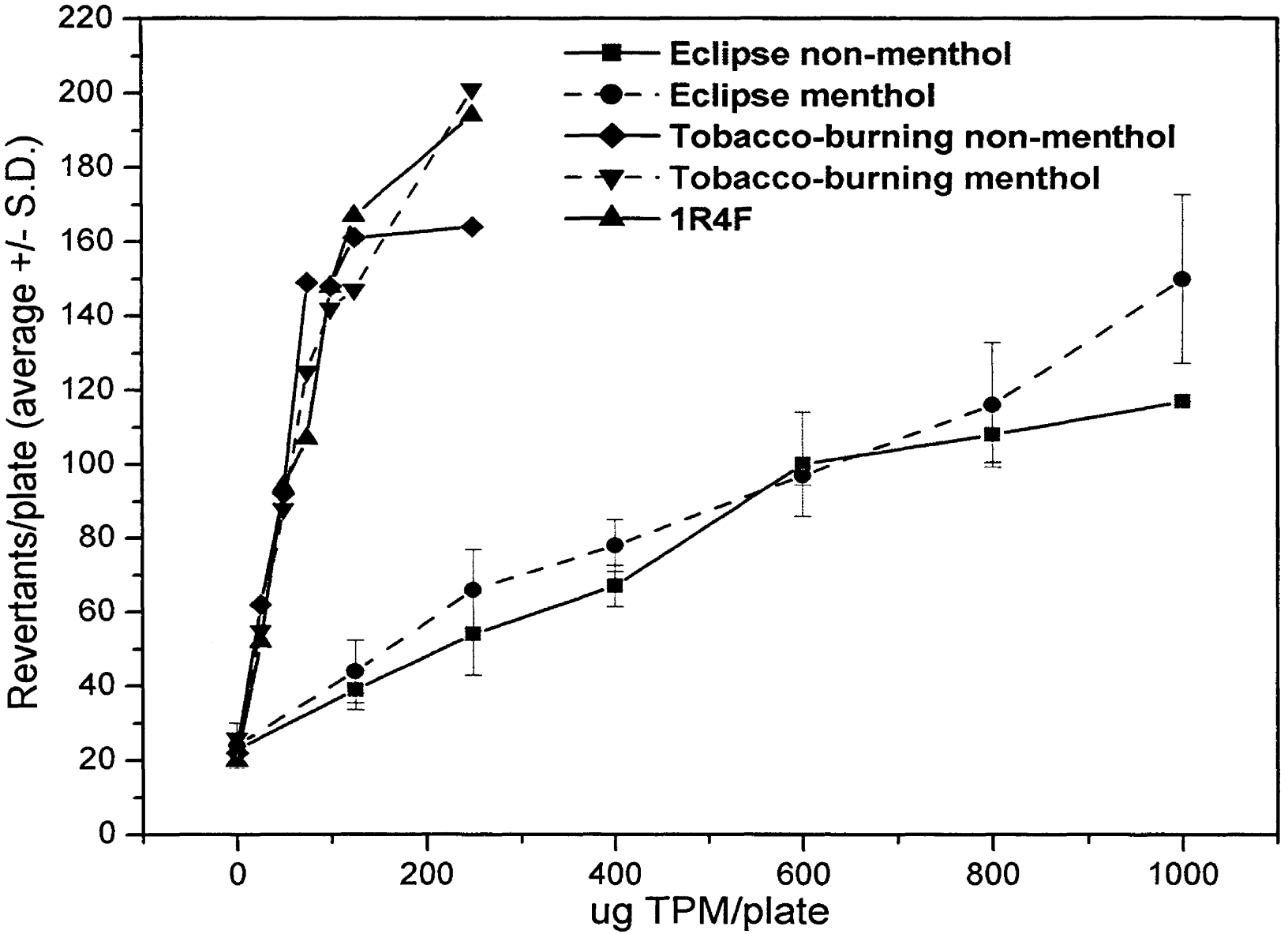
Sample	% S9	Revertants/mg total particulate matter (TPM)[1]				
		TA98	TA100	TA1535	TA1537	TA1538
Eclipse Non-menthol	0	Negative[2]	Negative[2]	Negative[2]	Negative[2]	Negative[2]
	5	**123**	Negative[2]	Negative[2]	Negative[2]	**85**
Eclipse menthol	0	Negative[2]	Negative[2]	Negative[2]	Negative[2]	Negative[2]
	5	**120**	Negative[2]	Negative[2]	Negative[2]	**115**
TOB-Non-menthol	0	**194**	Negative[2]	Negative[2]	Negative[2]	Negative[2]
	5	**1,592**	**637**	Negative[2]	**107**	**1024**
TOB-Menthol	0	**194**	Negative[2]	Negative[2]	Negative[2]	Negative[2]
	5	**1,280**	**738**	Negative[2]	**77**	**1175**
1R4F	0	**172**	Negative[2]	Negative[2]	Negative[2]	Negative[2]
	5	**1,276**	**780**	Negative[2]	**92**	**952**

[1] CSC samples from Eclipse cigarettes were assayed at concentrations ranging from 0-1000 ug/plate; CSC from tobacco-burning cigarettes were assayed at concentrations ranging from 0-250 ug/plate. The linear portion of the dose-response curve was used to calculate a slope value (Bernstein, 1982).

[2] No significant dose-related increase; response not double background.

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187

Ames Assay TA98 with S9 activation



53030 1907

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187

SCE Assay Results

Average slope values[a]

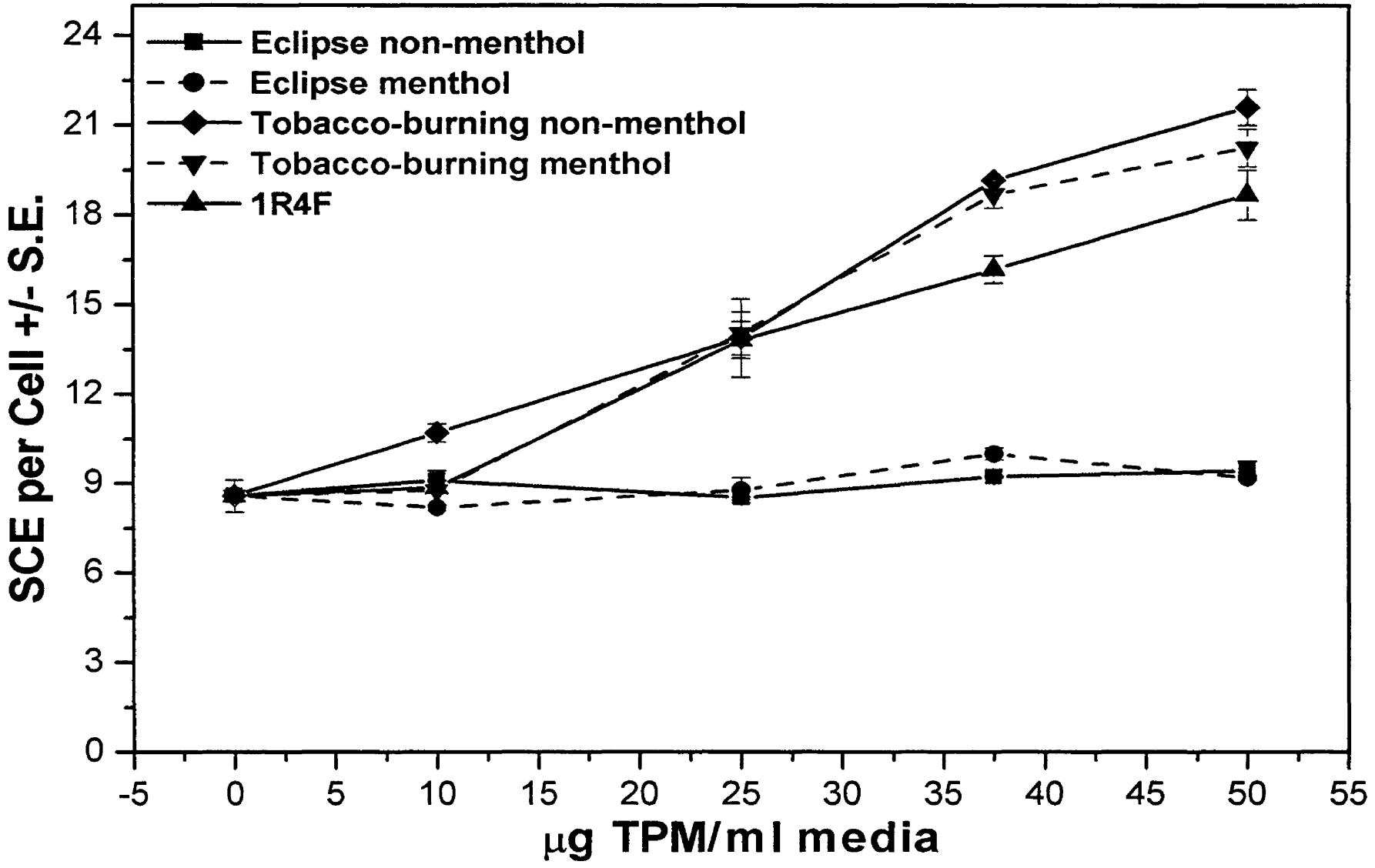
Sample	With S9 ± S.E.	Without S9 ± S.E.
Eclipse Non-menthol	-0.00038 ± 0.00038	0.0047 ± 0.0018*
Eclipse Menthol	-0.00043 ± 0.00038	0.0052 ± 0.0018*
TOB-Non-menthol	0.00422 ± 0.0054*	0.0378 ± 0.0018*
TOB-Menthol	0.00375 ± 0.0046*	0.0352 ± 0.0018*
Kentucky Reference 1R4F	0.00322 ± 0.00038*	0.0306 ± 0.0018*

[a] from regression lines relating square root of SCE counts to concentration

* positive ($p<0.05$)

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187

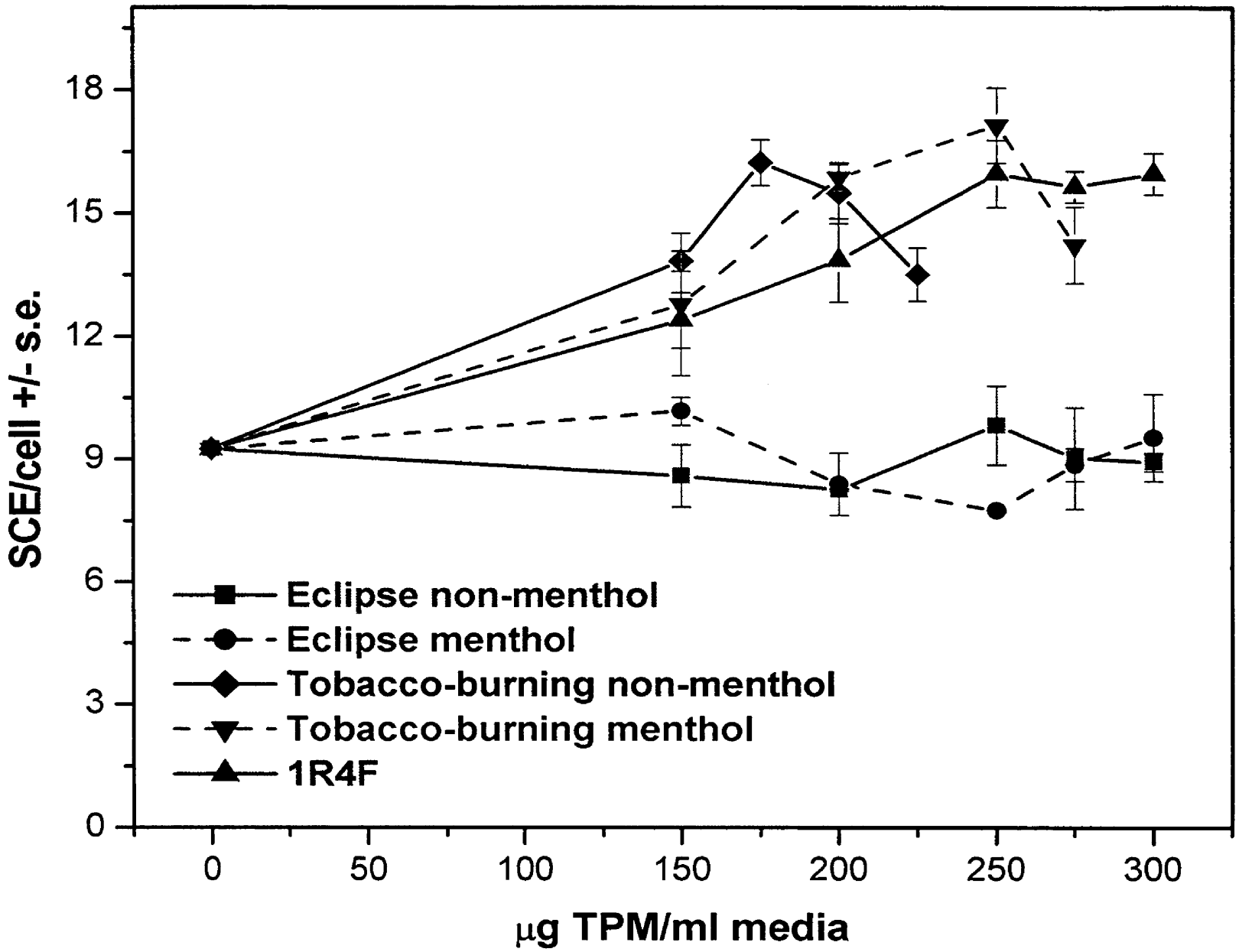
Sister Chromatid Exchange Assay without S9 metabolic activation



530301909

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187

Sister Chromatid Exchange Assay with S9 metabolic activation



53030 1910

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187

Neutral Red Cytotoxicity

Sample	EC_{50} value (ug/ml)[a]	Initial concentration where cytotoxicity is observed (ug/ml)	Slope of dose-response relationship
Eclipse non-menthol	> 150[b]	> 150[b]	- 0.145[c]
Eclipse menthol	> 150[b]	> 150[b]	- 0.027[c]
Tobacco-burning non-menthol	47.0	25	-0.848
Tobacco-burning menthol	58.8	25	-0.806
Kentucky Reference 1R4F	59.3	25	-0.853

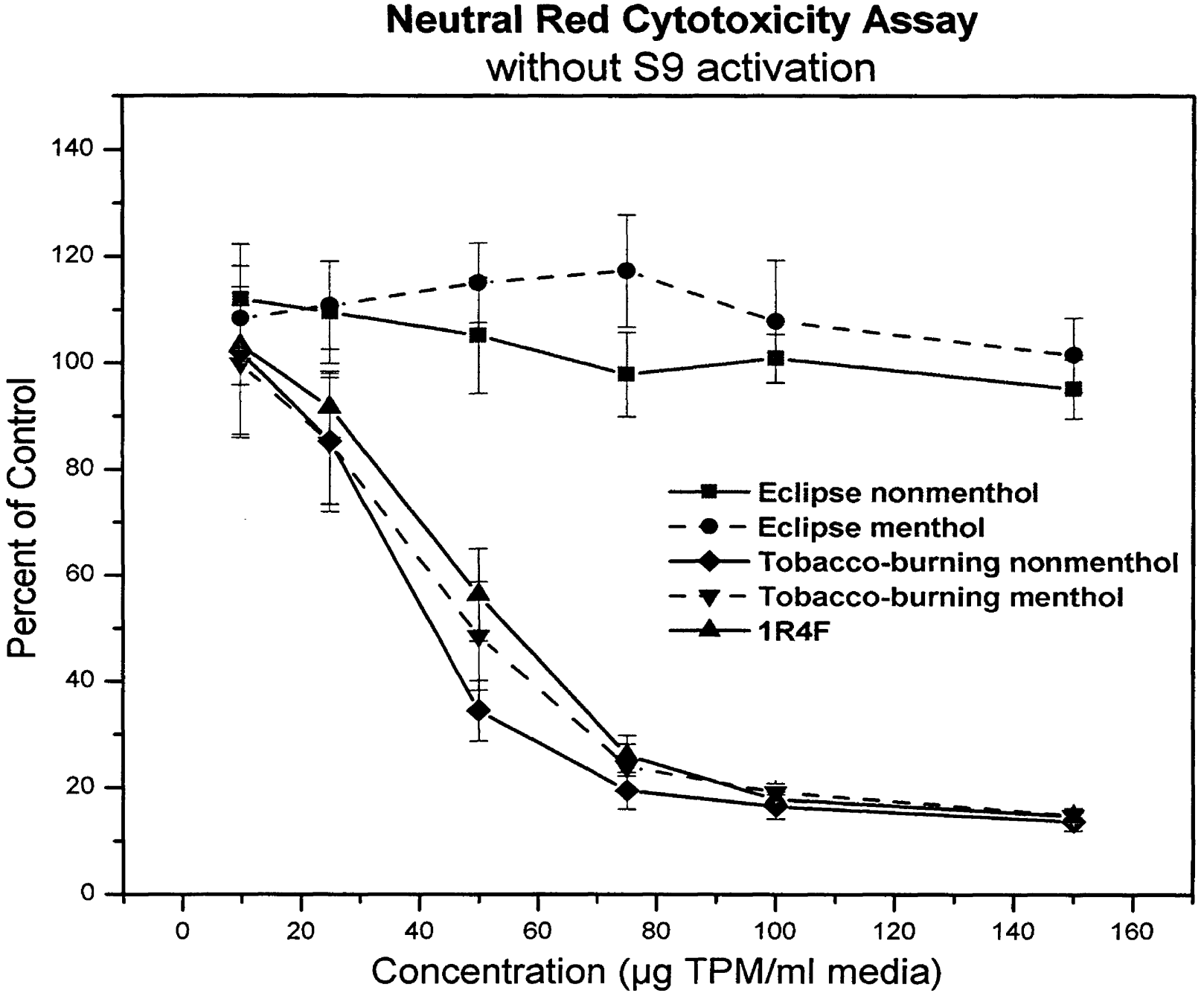
[a] Effective concentration of cigarette smoke condensate (ug/ml) to cause 50% reduction in growth of cell population

[b] No dose-response relationship observed up to 150 ug/ml CSC.

[c] Slope not significantly different from zero (at $p<0.05$).

53030 1911

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187



53030 1912

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187

Summary

Cigarette	Ames		SCE		Neutral Red
	- S9	+ S9	- S9	+ S9	- S9
Eclipse Non-menthol	Negative	+	+	Negative	Negative
Eclipse Menthol	Negative	+	+	Negative	Negative
TOB-Non-menthol	+	++	++	++	++
TOB-Menthol	+	++	++	++	++
1R4F	+	++	++	++	++

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187

CONCLUSIONS

- CSC from Eclipse menthol cigarettes is not significantly different than CSC from Eclipse nonmenthol cigarettes (at $p<0.05$).
- CSC from tobacco-burning menthol cigarettes is not significantly different than CSC from tobacco-burning nonmenthol cigarettes (at $p<0.05$).
- CSC from Eclipse menthol cigarettes is significantly lower than CSC from Tobacco-burning menthol cigarettes (at $p<0.05$).
- CSC from Eclipse nonmenthol cigarettes is significantly lower than CSC from Tobacco-burning nonmenthol cigarettes (at $p<0.05$).

The use of menthol did not significantly increase the genotoxic or cytotoxic potential of CSC as measured in the Ames, SCE, and Neutral Red assays

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187

REFERENCES

Bernstein, L., J. Kaldor, J. McCann, and M.C. Pike. An empirical approach to the statistical analysis of mutagenesis data from the Salmonella test. Mutat Res 97, 267-281, 1982.

Bombick, B. R., H. Murli, J.T. Avalos, D.W. Bombick, W.T. Morgan, K. P. Putnam, and D.J. Doolittle. Chemical and Biological Studies of new cigarette that primarily heats tobacco. Part 2. *In vitro* toxicology of mainstream smoke condensate. Food Chem Tox 36, 183-190, 1998.

Bombick D.W., and D. J. Doolittle. The role of chemical structure and cell type in the cytotoxicity of low molecular weight aldehydes and pyridine. In vitro Tox 8 (4) 349-356, 1995.

Borenfreund, E. and J.A. Puerner. A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90). J Tissue Culture Methods 65:55-63, 1985.

DeMarini, D M. Genotoxicity of tobacco smoke and tobacco smoke condensate. Mutat Res 114: 59-89, 1983.

Doolittle, D.J., C.K. Lee, J.L. Ivett, J.C. Mirsalis, E. Riccio, C.J. Rudd, G.T. Burger, and A.W. Hayes. Comparative studies on the genotoxic activity of mainstream smoke condensate from cigarettes which burn or only heat tobacco. Environ Mol Mutagen 15, 93-105, 1990.

Galloway, S.M., A.D. Bloom, M. Resnick, B.H. Margolin, F. Nakamura, P. Archer, and E. Zeiger. Development of a standard protocol for *in vitro* cytogenetic testing with Chinese hamster ovary cells: comparison of results for 22 compounds in 22 laboratories. Environ Mutagen 7: 1-61, 1985.

Lee, C.K., D.J. Doolittle, D.A. Lee, and J.R. Hayes. Genotoxic profile of 1R4F reference cigarette smoke condensate in an *in vitro* test battery. The Toxicologist 7(1), 262, 1987.

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187

Maron, D.M. and B.N. Ames. Revised methods for the Salmonella mutagenicity test. Mutat Res 113: 247-256, 1983.

Mizusaki S., H. Okamoto, A. Akiyama, and Y. Fukuhara. Relationship between chemical constituents of tobacco and mutagenic activity of cigarette smoke condensate. Mutat Res 48: 319-326, 1977.

Perry, P. and S. Wolff. New Giemsa method for the differential staining of sister chromatids. Nature 251: 156-158, 1974.

Sullivan, S. The Reference and Research Cigarette Series. Lexington, KY: University of Kentucky Printing Services, 1984.

Yahagi, T., M. Degawa, Y. Seino, J. Matsushima, M. Nagao, T. Sugimura, and Y. Hashimoto. Mutagenicity of carcinogenic azo dyes and their derivatives. Cancer Lett 1:91-97; 1975.

Source: https://www.industrydocuments.ucsf.edu/docs/kxjm0187