Evaluation of the candidate modified risk tobacco product THS2.2 in a battery of *in vitro* genotoxicity and cytotoxicity tests

D. McHugh, G. Cudazzo, D. Smart, D. Keller and P. Vanscheeuwijck
Philip Morris International R&D, Philip Morris Products S.A., 2000 Neuchâtel, Switzerland
(part of the Philip Morris group of companies)

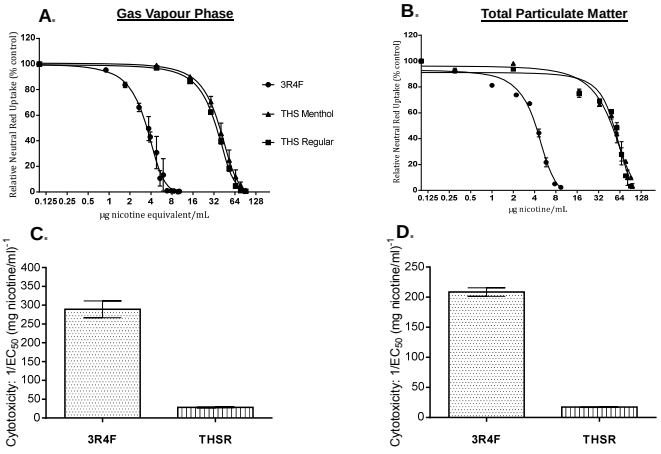
Introduction

- THS2.2 is a candidate modified risk tobacco product in which the tobacco is heated to a maximum of 350°C, leading to a significant reduction in the formation of harmful and potentially harmful constituents (HPHC) in the aerosol generated, in comparison to the HPHCs generated during combustion of a conventional cigarette.
- For this evaluation, we have tested the mainstream THS2.2 aerosols from two product variants, regular and menthol, in a battery of regulatory *in vitro* genotoxicity and cytotoxicity tests, namely the Ames and mouse lymphoma assays (MLA) and the neutral red uptake (NRU) assay.

Materials and Methods

- All studies were performed in full compliance with GLP.
- **Neutral Red Uptake Assay**: The mouse embryonic fibroblast cell line Balb/c 3T3 (clone A31) was obtained from the European Collection of Authenticated Cell Cultures (Salisbury, UK), and was used to perform the NRU cytotoxicity assay according to INVITTOX protocol 3a (INVITTOX, 1990), with some modifications. Sodium dodecyl sulfate was used as the positive control. The EC_{50} was determined with the SAS® Enterprise guide® 4.3 (SAS 9.2) software program (SAS, Cary, NC, USA).
- **Mouse Lymphoma Assay:** The L5178Y *tk*$^{+/-}$ cell line (sub-clone 3.7.2C (IVGT) was obtained from Public Health England (Salisbury, UK) and used for the performance of the microwell version of the MLA according to the OECD TG 490 guideline (OECD, 2015). The cells were treated for 4 hours in the presence (+S9; (Moltox, Boone, NC, USA) and absence (-S9) of metabolic activation and 24 h in the absence of metabolic activation (−S9) treatment conditions. *Tk* mutants were detected following culture in trifluorothymidine (TFT)-containing growth medium (Sigma–Aldrich, St. Louis, MO, USA) for typically 14 days and with the mutation frequencies calculated according to published methods (Clements, 2000).
- **Bacterial reversion tests:** Mutagenic activity was evaluated by using the *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535, and TA1537 with and without an S9 enzymatic metabolizing fraction (Moltox ,Boone, NC, USA), by following a pre-incubation method (Maron and Ames, 1983) and the OECD 471 test guideline. The S9 metabolising fraction was obtained from Aroclor 1254-induced male Sprague–Dawley rat liver (Moltox, NC, USA). His$^+$ revertant colonies were counted using an automatic colony counter (Sorcerer, Perceptive Instruments, Bury Saint Edmunds, UK). The mutagens used as positive controls in the S9- group were 4-nitrophenylenediamine (10 µg/plate) for TA98 and TA100, sodium azide (1.25 µg/plate) for TA1535 and TA1537, and cumene hydroperoxide (3 µg/plate) for TA102. For the S9+ group, benzo[a]pyrene (1 µg/plate) was used for TA98, and 2-aminoanthracene (2.5 µg/plate) was used for TA100, TA102, TA1535, and TA1537. DMSO (50 µl/plate) served as the solvent control. All positive control chemicals were obtained from either Sigma-Aldrich (St. Louis, MO, USA) or Moltox (Boone, NC, USA).
- **Sample generation:** 3R4F research cigarettes and THS2.2 tobacco sticks were conditioned for at least 48 h at 22 ± 1°C and 60 ± 3% relative humidity (ISO standard 3402) prior to being used for aerosol generation. Following conditioning, aerosols were generated using an RMB20 smoking machine (Burghart, Tabaktechnik GmbH, Wedel, Germany) according to the Health Canada Intense (HCI) smoking regimen (Health Canada, 2000). The generated aerosol and smoke were trapped to analyze the aerosols.

Results: Neutral Red Uptake: Cytotoxicity



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	THS2.2		3R4F		THS2.2 M		3R4F	
	TPM	GVP	TPM	GVP	TPM	GVP	TPM	GVP
Mean	17.34	28.40	208.55	289.06	19.73	26.07	239.51	276.21
SEM	0.52	1.20	6.92	22.38	0.87	1.78	6.07	22.51
N	3	3	3	3	3	3	3	3
Relative cytotoxicity (%)	8.3	9.8	100	100	8.2	9.4	100	100

Relative cytotoxicity (%) = (cytotoxicity of THS2.2/3R4F) × 100

Figure 1: The cytotoxicity responses induced by aerosol fractions derived from THS2.2 and 3R4F in the NRU assay over three independent tests expressed on a per-mg nicotine basis. A.C. Gas Vapor Phase fraction. B.D. Total Particulate Matter fraction. E. Cytotoxicity of TPM and GVP, expressed as 1/EC_{50} (ml/mg nicotine).

Results: Bacterial Reversion Test

Salmonella typhimurium Strain		THS2.2		3R4F[A]		Solvent Control		Positive[B] Control		THS2.2 M		3R4F[A]		Solvent Control		Positive[B] Control	
		Mean*	SD	Mean*	SD	Mean*	SD	Mean*	SD	Mean*	SD	Mean*	SD	Mean*	SD	Mean*	SD
	TA98	22	4	658	89	21	1	109	7	21	2	636	24	25	2	97	17
+S9	TA100	94	21	428	25	87	2	481	22	93	12	440	20	90	6	471	67
	TA102	358	12	409	15	272	22	1005	29	290	15	399	16	265	23	968	25
	TA1535	9	3	17	6	6	1	70	8	15	6	15	6	10	3	113	11
	TA1537	8	5	98	9	6	2	50	5	15	3	94	9	7	2	35	5
	TA98	16	4	17	5	23	6	81	6.2	22	3	10[C]	6	26	3	93	10
-S9	TA100	61	8	87	13	66	3	195	25	81	11	96	21	62	8	187	17
	TA102	291	15	282	12	267	21	267	21	230	56	264	4	258	26	620	8
	TA1535	12	3	7	3	6	2	37	8	9	4	16	6	12	2	51	6
	TA1537	6	5	3	2	6	3	84	5	15	5	17	5	7	2	83	6

A: These samples were generated and tested concurrently with the respective THS variant
B: Details of dose and substance are provided in the Ames methods section
C: Toxicity was detected at this dose
*: Each mean and SD value was derived from 3 plates and the values were rounded

Figure 2: Revertant colonies obtained following exposure to the TPM (1 mg per plate) from THS2.2, THS2.2 M, or 3R4F. The concentration of S9 was fixed at 10%. All doses were tested in triplicate and on two independent test occasions. The maximum dose tested of THS2.2 was 10 mg and 5mg for THS2.2 M. No biologically relevant mutagenicity was detected up to these doses.

Results: Mouse Lymphoma Assay

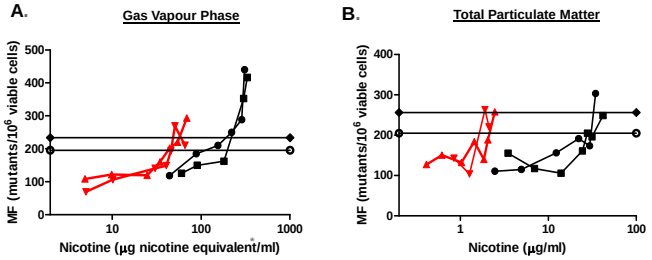

Figure 3: The mutagenic responses induced by aerosol fractions derived from THS2.2 and 3R4F in the 4 h +S9 treatment condition in two independent tests expressed on a per-mg nicotine basis. **A**. TPM. MFs for the DMSO-treated controls in tests #1 and #2 were 129.77 ± 22.57 and 78.73 ± 1.55 mutants/10^6 viable cells, respectively. ●THS2.2R #1; ■THS2.2 D2 #2; ▲3R4F #1; ▼3R4F #2; ♦GEF+DMSO MF #1; ✪ GEF+DMSO MF #2. **B**. GVP. MFs for the PBS-treated controls in tests #1 and #2 were 107.73 ± 10.40 and 69.44 ± 3.10 mutants/10^6 viable cells, respectively. ●THS2.2 D2 #1; ■THS2.2R #2; ▲3R4F #1; ▼3R4F #2; ♦GEF+PBS MF #1; ✪ GEF+PBS MF #2. The mutagenic responses were determined within the test cytotoxicity limits (10-20% Relative Total Growth).

Analytical Results

		THS2.2		THS2.2 M		3R4F	
Parameter	Unit	mean ± $CI_{95\%}$	N	mean ± $CI_{95\%}$	N	mean ± $CI_{95\%}$	N
TPM	mg/stick	54.1 ± 2.4	4	53.8 ± 3.6	4	46.3 ± 2.9	4
Water	mg/stick	39.4 ± 4.6	4	39.1 ± 3.6	4	13.3 ± 1.6	4
Nicotine	mg/stick	1.26 ± 0.24	4	1.32 ± 0.11	4	2.09 ± 0.14	4
NFDPM	mg/stick	13.4 ± 2.8	4	13.4 ± 0.6	4	30.9 ± 1.9	4
Carbon monoxide	mg/stick	0.598 ± 0.072	4	0.620 ± 0	4	30.7 ± 3.0	4
Benzo[a]pyrene	ng/stick	1.19 ± 0.08	4	1.08 ± 0.09	3	13.7 ± 0.8	4
Puff Count	/stick	12 ± 0	4	12 ± 0	4	10.7 ± 0.7	4
Menthol	mg/stick	n.a.		2.98 ± 0.21	4	n.a.	
Glycerin	mg/stick	4.1 ± 1.07	4	4.59 ± 0.47	4	2.39 ± 0.15	4
1-aminonaphthalene	ng/stick	0.063 ± 0.006	4	<0.061	4	19.7 ± 1.6	4
2-aminonaphthalene	ng/stick	<0.035	4	<0.035	4	14.8 ± 1.9	4
3-aminobiphenyl	ng/stick	<0.013	4	<0.013	4	3.90 ± 0.42	4
4-aminobiphenyl	ng/stick	<0.021	4	n.a.		3.13 ± 0.60	4
Acetaldehyde	µg/stick	213 ± 19	4	220 ± 22	4	1589 ± 76	4
Acetone	µg/stick	33.8 ± 6.4	4	42.6 ± 8.1	4	729 ± 36	4
Acrolein	µg/stick	9.44 ± 0.87	4	10.91 ± 2.98	4	193 ± 21	4
Butyraldehyde	µg/stick	25.3 ± 2.7	4	26.4 ± 0.9	4	103.9 ± 8.3	4
Crotonaldehyde	µg/stick	3.75 ± 0.34	4	4.15 ± 0.64	4	92.1 ± 13.2	4
Formaldehyde	µg/stick	5.22 ± 0.24	4	6.19 ± 2.00	4	68.7 ± 7.8	4
Methyl ethyl ketone	µg/stick	7.94 ± 0.75	4	10.19 ± 2.23	4	241 ± 16	4
Propionaldehyde	µg/stick	13.6 ± 1.5	4	15.9 ± 2.2	4	147 ± 8	4
Acrylonitrile	µg/stick	0.186 ± 0.028	4	0.196 ± 0.016	4	31.6 ± 2.3	4
1,3-butadiene	µg/stick	0.319 ± 0.073	4	0.411 ± 0.093	4	91.8 ± 11.0	4
Benzene	µg/stick	0.575 ± 0.072	4	0.628 ± 0.073	4	100.4 ± 2.8	4
Isoprene	µg/stick	2.44 ± 0.50	4	2.63 ± 0.60	4	869 ± 50	4
Pyridine	µg/stick	9.38 ± 0.95	4	10.08 ± 0.46	4	51.8 ± 7.5	4
Quinoline	µg/stick	0.014 ± 0.002	4	0.010 ± 0.003	4	0.390 ± 0.101	4
Styrene	µg/stick	0.672 ± 0.063	4	0.632 ± 0.079	4	28.9 ± 2.2	4
Toluene	µg/stick	1.61 ± 0.17	4	1.67 ± 0.37	4	198.8 ± 10.9	4
Catechol	µg/stick	16.4 ± 0.6	4	12.8 ± 1.3	4	88.7 ± 2.6	4

		THS2.2		THS2.2 M		3R4F	
Parameter	Unit	mean ± $CI_{95\%}$	N	mean ± $CI_{95\%}$	N	mean ± $CI_{95\%}$	N
o-cresol	µg/stick	0.105 ± 0.017	4	0.059 ± 0.007	4	4.86 ± 0.50	4
m-cresol	µg/stick	0.042 ± 0.006	4	0.032 ± 0.005	4	3.71 ± 0.34	4
p-cresol	µg/stick	0.073 ± 0.009	4	0.042 ± 0.007	4	8.50 ± 0.78	4
Hydroquinone	µg/stick	7.86 ± 0.63	4	6.21 ± 0.86	4	84.1 ± 3.3	4
Phenol	µg/stick	1.51 ± 0.23	4	1.00 ± 0.17	4	13.2 ± 0.9	4
Resorcinol	µg/stick	0.055 ± 0.013	4	0.036 ± 0.005	4	1.95 ± 0.55	4
NAB	ng/stick	3.52 ± 0.48	4	3.27 ± 0.15	4	34.1 ± 3.0	4
NAT	ng/stick	22.3 ± 1.6	4	18.6 ± 2.9	4	300 ± 53	4
NNK	ng/stick	10.1 ± 0.4	4	7.9 ± 1.1	4	257 ± 39	4
NNN	ng/stick	10.3 ± 0.4	4	7.7 ± 1.0	4	268 ± 50	4
Ammonia	µg/stick	15.6 ± 1.1	4	13.9 ± 1.1	4	39.2 ± 4.1	4
Hydrogen cyanide	µg/stick	3.78 ± 0.44	4	5.57 ± 0.35	4	451 ± 47	4
Nitric oxide	µg/stick	21.0 ± 8.1	3	18.4 ± 3.6	4	501 ± 33	3
Nitrogen oxides	µg/stick	22.6 ± 8.8	3	19.4 ± 4.0	4	541 ± 74	3
Arsenic	ng/stick	<1.13	4	<1.13	4	6.56 ± 0.46	4
Cadmium	ng/stick	<0.350	4	<0.350	4	122 ± 12	4
Chromium	ng/stick	<0.17	4	0.44	4	2.70*	2
Lead	ng/stick	<3.35	4	<3.35	4	25.1 ± 2.1	4
Mercury	ng/stick	1.02 ± 0.05	4	1.12 ± 0.19	4	4.17 ± 0.74	4
Nickel	ng/stick	<0.55	4	0.88	4	1.30*	2
Selenium	ng/stick	<0.550	4	<0.550	4	1.43 ± 0.15	4
Pyrene	ng/stick	7.93 ± 0.78	4	7.71 ± 0.63	3	87.3 ± 4.1	4
o-toluidine	ng/stick	1.204 ± 0.149	4	0.868 ± 0.087	4	90.5 ± 3.1	4
Acetamide	µg/stick	4.13 ± 0.21	4	3.43 ± 0.17	4	13.7 ± 0.7	4
Acrylamide	µg/stick	2.27 ± 0.28	4	1.90 ± 0.12	4	5.3 ± 0.4	4
Ethylene oxide	µg/stick	0.314 ± 0.011	4	0.273 ± 0.036	4	34.2 ± 3.6	4
Nitrobenzene	ng/stick	0.092 ± 0.008	4	0.155 ± 0.004	8	0.55 ± 0.04	4
Propylene oxide	µg/stick	0.175 ± 0.03	4	0.14 ± 0.019	4	1.72 ± 0.16	4
Vinyl chloride	ng/stick	<3.47	4	<3.47	4	95.3 ± 12.3	4
Benz[a]anthracene	ng/stick	2.58 ± 0.17	4	2.50 ± 0.06	3	26.6 ± 1.7	4
Dibenz[a,h]anthracene	ng/stick	<0.100	4	<0.100	4	1.79 ± 0.14	4

Figure 4: Analyte yields from THS2.2, THS2.2 M, and 3R4F obtained under HCI machine-smoking conditions and expressed on a per-cigarette/*tobacco stick* basis.

Summary and Conclusion

- The mutagenic and cytotoxic potencies of the mainstream aerosol fractions from THS2.2, when evaluated by the mouse lymphoma, and NRU assays were reduced by at least 85%–95% compared with the mainstream smoke aerosol of 3R4F. The Ames assay yielded no biologically relevant mutagenicity.
- The low operating temperature of THS2.2 results in significantly lower concentrations of HPHCs in the mainstream aerosol compared with the mainstream smoke of the 3R4F reference cigarette when expressed on either a per-Tobacco Stick/cigarette or a per-mg nicotine basis.
- While a conclusion underlying the mechanism(s) of these in vitro results cannot be definitively made on the basis of these data, it is reasonable to suggest that the overall reduction in the burden of toxicants present in the THS2.2 aerosols may play a role in the manifestation of the reduced cytotoxic and mutagenic potency *in vitro*.

References

Clements, J., 2000. The Mouse Lymphoma Assay. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 455, 97-110.

Health Canada (2000), Health Canada - Tobacco Products Information Regulations SOR/2000-273, Schedule 2.

International Organization for Standardization: International Standard ISO 3402, Tobacco and tobacco products - Atmosphere for conditioning and testing, 4th ed., 1999.

INVITTOX Protocol No. 3a, 1990, INVITTOX: THE ERGATT/FRAME Data Bank of In Vitro Techniques in Toxicology.

Organisation for Economic Co-operation and Development, 1997. Test No. 471: Bacterial Reverse Mutation Test. OECD Publishing.

Organisation for Economic Co-operation and Development, 2015. Test No. 490: In Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene. OECD Publishing.

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Philip Morris International Research & Development, Quai Jeanrenaud 5, 2000 Neuchâtel, Switzerland
T: +41 58 242 21 11, **F**: +41 58 242 28 11, **W**: www.philipmorrisinternational.com