Genetic And Cell Toxicology In Vitro Assessment Of Potentially Reduced Risk Products For Smokers.

Damian McHugh, Daniel Smart, Gianluca Cudazzo and Patrick Vanscheeuwijck

PMI R&D, Philip Morris Products S.A., Quai Jeanrenaud 5, CH-2000 Neuchâtel, Switzerland (Part of Philip Morris International group of companies)

Abstract and Objectives

Smoking causes serious diseases. Tobacco harm reduction strategies can complement efforts to prevent the initiation and encourage the cessation of tobacco smoking. Central to the tobacco harm reduction principle are products that present less risk of harm compared with continued cigarette smoking while, at the same time, being acceptable to adult smokers thereby facilitating switching. The genotoxic and cytotoxic potential of novel products is evaluated *in vitro* early in their development using a standard battery of toxicology assays composed of the Ames test, neutral red uptake, mouse lymphoma and *in vitro* micronucleus assays executed under Good Laboratory Practice conditions. Results from a recent assessment of aerosols derived from a potentially reduced risk product indicate that their genotoxic and cytotoxic potencies are decreased by at least 90-95% compared to smoke from the 3R4F reference cigarette. Importantly, these data corroborate chemistry findings on the levels of harmful and potentially harmful constituents present in the aerosols. In conclusion, we believe that the assessment approach deployed allows the reduced risk potential of novel products to be evaluated.

- 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 bacterial version assay and mouse lymphoma assays (MLA) and the neutral red uptake (NRU) assay.

Methods

- All studies were performed in full accordance with the principles of Good Laboratory Practice.

- **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).

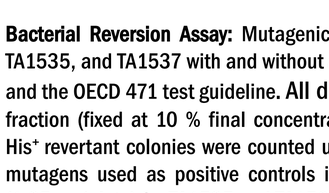
- **Bacterial Reversion Assay:** Mutagenic activity was evaluated by using the *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535, and TA1537 with and without an S9 enzymatic metabolizing fraction by following a pre-incubation method (Maron and Ames, 1983) and the OECD 471 test guideline. **All doses were tested in triplicate and on two independent test occasions.** The S9 metabolising fraction (fixed at 10 % final concentration) 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 via the Health Canada Intensive regimen 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 Assay: 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.B. Gas Vapor Phase fraction. C.D. Total Particulate and Aerosol Collected matter fraction.

A.



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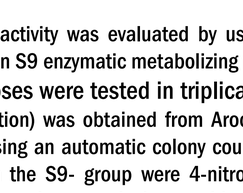

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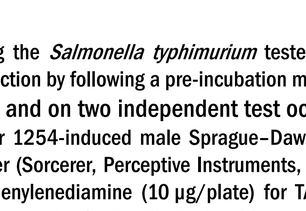

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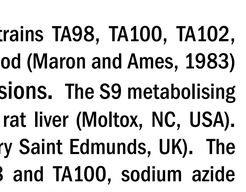
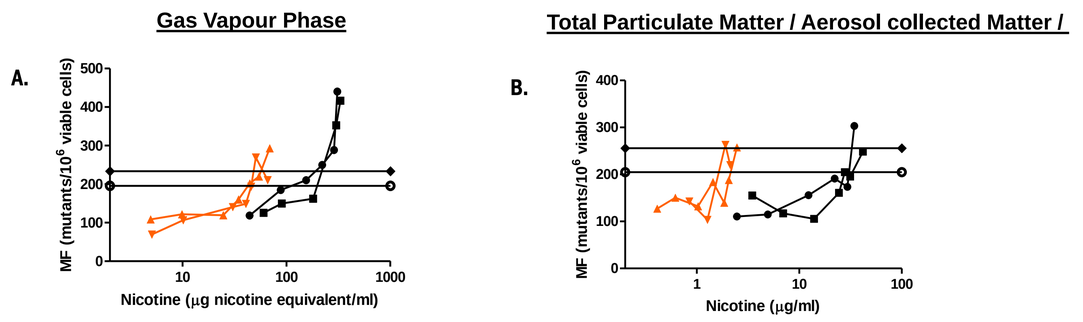


E. Cytotoxicity of TPM, ACM and GVP, expressed as $1/EC_{50}$ (ml/mg nicotine).

	THS2.2		3R4F		THS2.2 M		3R4F	
	ACM	GVP	TPM	GVP	ACM	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

Results

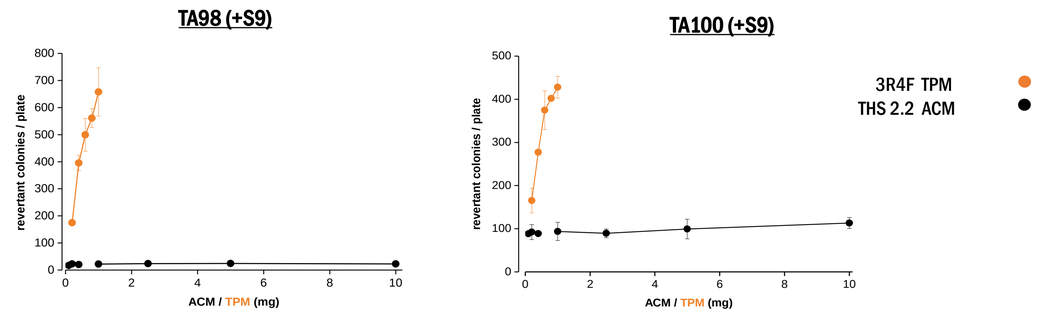
Mouse Lymphoma Assay: The mutagenic responses induced by aerosol fractions derived from THS2.2 and 3R4F.



Data from 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).

Bacterial Reversion Assay: The mutagenic responses induced by Total particulate and aerosol derived matter from THS2.2 and 3R4F.

The mean ± standard deviation of the revertant colonies obtained following exposure to the ACM or TPM from THS2.2 or 3R4F in two of the Salmonella *typhimurium* tester strains on one test occasion (three plates per dose). No biologically relevant mutagenicity was detected up to these doses.



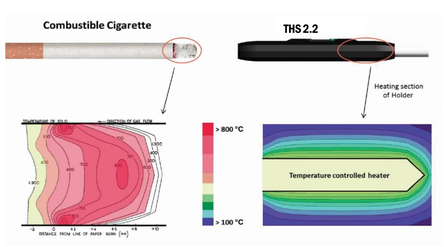
Revertant colonies obtained following exposure to the ACM or TPM (1 mg per plate) from THS2.2, THS2.2 M, or 3R4F No biologically relevant mutagenicity was detected up to these doses.

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

Conclusions

- 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.




cigarette smoke


THS 2.2 aerosol

Photographs of the Cambridge filter pads after the collection of:

- 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.

- 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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