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The effect of long term storage on tobacco smoke particulate matter in *in vitro* genotoxicity and cytotoxicity assays

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ABSTRACT

Particulate matter (PM) collected from mainstream tobacco smoke is a test article commonly used for *in vitro* genotoxicity and cytotoxicity testing of combustible tobacco products. However, little published data exists concerning the stability of PM. We completed a 2 year study to quantify the effect of PM storage at −80 °C, on the genotoxicity and cytotoxicity of PM generated from 3R4F and M4A reference cigarettes. The Ames test, Micronucleus assay (MNvit), Mouse Lymphoma assay (MLA) and the Neutral Red Uptake assay (NRU) were used. The majority of M4A and 3R4F PMs were genotoxic and cytotoxic at the timepoints tested. Some minor but statistically significant differences were observed for stored versus freshly prepared PM, but the magnitude of changes were within the variability observed for repeat testing.

1. Introduction

Tobacco smoke is a complex and dynamic aerosol that consists of a particulate matter (PM) phase and a gas-vapour phase (GVP) (Scian et al., 2009). Routine toxicological assessment of tobacco smoke commonly uses the PM fraction of the smoke aerosol. PM is easily collected from mainstream tobacco smoke by a variety of methods (Wan et al., 2009) and produces consistent and reproducible responses *in vitro*. In addition there is a history of use of PM in a variety of *in vitro* assays including the Neutral Red Uptake assay (NRU), the *in vitro* micronucleus (MNvit) and the Ames test extending over 30 years and more recently, the Mouse Lymphoma assay (MLA) (Bakland et al., 2005; CORESTA, 2004; Wan et al., 2009).

Several guidelines such as those developed by the International Conference on Harmonisation (ICH, 2011) and Committee on Mutagenicity (COM, 2011) suggest using a battery of core *in vitro* assays for the detection of mutagenicity and potential for carcinogenicity. In the case of tobacco products, Health Canada require the Ames test, the MNvit and NRU data (Health Canada, 2005). In the absence of specific regulatory guidelines, the CORESTA *in vitro* Toxicology Taskforce (CORESTA, 2004) recommend (i) a bacterial mutagenicity assay (Ames Salmonella mutagenicity assay); (ii) a mammalian cell assay for cytogenetics/mutation (MNvit, chromosome aberration assay or the MLA); and (iii) a cytotoxicity assay (NRU).

The OECD principles of GLP and compliance monitoring (OECD, 1998) section 6 specifies that the Sponsor must detail the expiry date, specific storage instructions and stability of the test article. For PM, limited information is available regarding these criteria, with the exception of a 1 month expiry date for PM when stored at −75 °C and tested in the Ames test (Roemer et al., 2002). The CORESTA *in vitro* Toxicology Task Force (CORESTA, 2004) recommend: (i) PM extracts should be stored at −70 °C within one hour of extraction; (ii) extracts can be stored at −70 °C for up to 4 years; (iii) extracts should not be refrozen once thawed. The scientific rationale to support these recommendations, however, has not been published.

Abbreviations: ANCOVA, analysis of covariance; BAT, British American Tobacco; COM, Committee on Mutagenicity; CORESTA, Cooperation Centre for Scientific Research Relative to Tobacco; DMSO, dimethyl sulfoxide; GLP, Good Laboratory Practice; GVP, gas vapour phase; IC_{50}, the concentration causing 50% toxicity in the Neutral Red Uptake assay; ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods; ICH, International Conference on Harmonisation; ISO, International Standards Organisation; MF, mutant frequency; MLA, Mouse Lymphoma assay; MNBN, micronucleated binucleate cells; MNvit, *in vitro* Micronucleus assay; NRU, Neutral Red Uptake assay; NS, not significant; OECD, Organisation of Economic Cooperation and Development; PM, particulate matter; S9, post-mitochondrial supernatant; SAS, Statistical Analysis System; US, United States.

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Table 1
Specification of 3R4F and M4A reference cigarettes.

	3R4F	M4A
Tobacco blend	USB* tobacco	Flue-cured tobacco
Filter type	Cellulose acetate	Cellulose acetate
PM (mg/cig)	11.0	13.6
Tar (mg/cig)	9.4	11.3
Nicotine (mg/cig)	0.73	0.95

* United States blended.

In order to characterise the effect of storage, we have commissioned a study on PM prepared from two reference cigarettes, 3R4F and M4A (Table 1). The PMs were stored at −80 °C for approximately 2 years and tested at defined intervals in the Ames test, MNvit, MLA and NRU assay. Freshly prepared PM was generated and tested simultaneously for comparison. The outcome of this study provides scientific data to support a recommendation for the maximum storage time of PM at −80 °C, i.e. an expiry date.

2. Materials and methods

2.1. Cigarettes

Two reference cigarettes were evaluated in this study. M4A, a 100% flue cured tobacco product (British American Tobacco's historical control) and 3R4F, a 'US style' blended product (University of Kentucky). Cigarette parameters are detailed in Table 1.

2.2. Particulate matter preparation

All PMs were generated (according to ISO puffing parameters; 35 mL puff volume, taken over 2 s, every 60 s) and extracted in DMSO at British American Tobacco, Southampton, as previously described in McAdam et al., 2011. All extracts were frozen at −80 °C and transported to Covance Laboratories Ltd. UK on dry ice where upon receipt they were stored at −80 °C. The choice of storage temperature was dictated by the transportation of PMs on dry ice (−78.5 °C) and the need to avoid further fluctuations in storage temperature.

For the determination of storage effects, sufficient PM from 3R4F and M4A cigarettes were prepared at the beginning of the study to provide sufficient test article for the 2 year period. PMs were divided into 1 mL aliquots to avoid freeze–thawing. Fresh 3R4F and M4A PMs were prepared at 1 (T_1), 3 (T_3), 6 (T_6), 12 (T_{12}), 18 (T_{18}) and 24 (T_{24}) months, transported to Covance Laboratories Ltd. UK and stored as above. At each timepoint, 'fresh' PMs were subsequently tested within 10 days with the respective 'stored' PM.

2.3. In vitro toxicology testing

All *in vitro* toxicology testing was conducted at Covance Laboratories Ltd. UK. Appropriate positive and negative controls were used in each assay. Treatment conditions were selected based on responsiveness to tobacco products as described in Combes et al., 2012. Where specified, the mammalian liver post-mitochondrial fraction (S9) (Mol Tox™) was used for metabolic activation.

2.4. Ames test

The Ames test was performed according to the principles of OECD Test Guideline 471 (OECD, 1997a). Pre-incubation was used with tester strains TA98, TA100 and TA1537, in the presence of 10% S9 mix, with three replicates per concentration.

Table 2
Results from Analysis of Covariance for stored and fresh 3R4F and M4A PMs.

Assay	Treatment condition	Concentration	Analysis of Covariance	
			Stored v Fresh M4A	Stored v Fresh 3R4F
Ames	TA98 + S9	100 μg/plate	NS	NS
		200 μg/plate	NS	NS
		300 μg/plate	*	NS
	TA100 + S9	100 μg/plate	NS	NS
		200 μg/plate	NS	NS
		300 μg/plate	NS	NS
	TA1537 + S9	100 μg/plate	NS	NS
		200 μg/plate	NS	NS
		300 μg/plate	NS	NS
MLA	24 h-S9	20 μg/mL	NS	NS
		25 μg/mL	NS	NS
		30 μg/mL	†	*
MNvit	24 h-S9	30 μg/mL	NS	†
		35 μg/mL	†	NS

NS, not significant; †, not analysed.
* $p \leqslant 0.05$

2.5. MLA

The MLA used L5178Y cells, using a Microtitre® fluctuation technique, according to the principles of OECD Test Guideline 476 (OECD, 1997b). Two replicates per concentration were assayed for 24 h.

2.6. MNvit

For the MNvit, PMs were tested according to the principles of OECD Test Guideline 487 (OECD, 2010) in V79 cells for 24 h, four replicates per concentration.

2.7. NRU

For the NRU assay, PMs were tested in Balb/c 3T3 cells, six replicates per concentration, based on guidance described in ICCVAM "*in vitro* cytotoxicity methods for estimating starting doses for acute oral systemic toxicity testing" (ICCVAM, 1996). The PM concentration resulting in 50% toxicity in the NRU test (IC_{50}) were derived by Phototox Version 2 (OECD, 2004).

2.8. Statistics

2.8.1. Ames, MLA, MNvit

For each stored and fresh PM (M4A and 3R4F), assay, treatment condition and common concentration (on the linear part of the dose response curve), separate regression lines were fitted across time (T_0, T_1, T_3, T_6, T_{12}, T_{18} and T_{24} months) to test whether there was a significant response across time (i.e. a significant slope). An analysis of covariance (ANCOVA) model was then applied to test whether the response with each stored PM compared to that with each fresh PM, by time interaction was significant (i.e. significantly non-parallel slopes). Data analysis were performed with SAS, Version 9.2 (SAS Institute Inc.: Cary, North Carolina, USA 2002–2008).

The resolving power of the assays used in this study have been evaluated to have a resolving power of 80% to detect a 30% difference with 3 and 4 replicates in the Ames test and MNvit, respectively, and between 18 to 85% difference in the MLA with 2 replicates. The NRU is able to detect 18–49% difference between PMs (Oldham et al., 2012).