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The effect of a novel tobacco process on the *in vitro* cytotoxicity and genotoxicity of cigarette smoke particulate matter

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ABSTRACT

Some of the toxic effects of smoking have been attributed to the combustion of nitrogenous protein in tobacco. The effects of a treatment which reduces tobacco's protein nitrogen level, on the *in vitro* cytotoxicity and genotoxicity of cigarette smoke particulate matter (PM), were measured. PMs were tested in the Neutral Red Uptake (NRU) test; the *Salmonella* mutagenicity assay (SAL); the mouse lymphoma mammalian cell mutation assay (MLA) and the *in vitro* micronucleus test (IVMNT). PMs from all of the cigarettes were cytotoxic and genotoxic. PM obtained from smoking treated tobacco, showed a small, consistent and statistically significant reduced mutagenicity (revertants/μg) in TA98 with post-mitochondrial supernatant (S9). No consistent quantitative or qualitative differences were detected in the other tests. The data are discussed in relation to published information on smoke chemistry obtained from cigarettes made of tobacco treated using this technique. The observations confirm that the method did not give rise to any new qualitative or quantitative cytotoxic or genotoxic effects, and may have reduced PM's bacterial mutagenicity in TA98 with S9. Further toxicity testing is warranted, to investigate the effects of the tobacco treatment in more detail and add to the data already obtained.

1. Introduction

Proteins and amino acids have been reported to be precursors for a number of potentially toxic constituents of tobacco smoke, including aromatic amines (2-aminonaphthalene and 4-aminobiphenyl) (Torikaiu et al., 2005) and mutagenic heterocyclic amines (Clapp et al., 1999; Matsumoto and Yoshida, 1981; Mizusaki et al., 1977), the latter being implicated as a primary source of PM genotoxicity (DeMarini et al., 2008).

This paper describes an investigation into the *in vitro* assay responses of cigarette smoke PM from cigarettes containing tobacco which had been subject to a novel tobacco blend treatment (BT) (Liu et al., 2011). The effect of the blend treatment process is to reduce levels of soluble and insoluble proteins, amino acids and water soluble polyphenols, such as chlorogenic acid, rutin and scopoletin in tobacco.

The BT process is carried out on cut tobacco, and involves the sequential extraction of the tobacco with water and an aqueous protease enzyme solution, followed by addition to the resulting solution of adsorbents and then reapplication of the soluble materials to the extracted tobacco. The treated tobacco retains the structure of original tobacco, is designed to be used with an adsorbent filter, to create a cigarette with a conventional appearance, usage, and smoking experience (Liu et al., 2011). The effect of the BT process on the yields of mainstream and sidestream smoke toxicants from cigarettes made with this tobacco and smoked under International Standards Organisation (ISO) smoking conditions (ISO 3308:1977) are described elsewhere (Liu et al., 2011). The smoke composition of the BT cigarettes compared in this study demonstrated reduced levels of a range of smoke constituents, including ammonia, hydrogen cyanide, aromatic amines and some phenols; consistent with the aims of the BT process.

Abbreviations: ANOVA, analysis of variance; BAT, British American Tobacco; BT, novel tobacco blend treatment; CORESTA, Cooperation Centre for Scientific Research Relative to Tobacco; DMEM, Dulbecco's minimal essential medium; DMSO, dimethyl sulphoxide; IC_{50}, the concentration causing 50% toxicity in the NRU test; ICH, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; ISO, International Standards Organisation; IVMNT, *in vitro* micronucleus test; MF, mutation frequency; MLA, mouse lymphoma mammalian cell mutation assay; MnBn, micronucleated binucleate cells; NIH, National Institutes of Health; NFDPM, nicotine-free dry particulate matter; NRU, Neutral Red Uptake; NT, not tested; OECD, Organisation of Economic Cooperation and Development; PM, particulate matter; RPMI, Roswell Park Memorial Institute medium; SAL, *Salmonella* mutagenicity assay; SEM, standard error of measurement; SD, standard deviation; S9, post-mitochondrial supernatant; TFT, trifluoro-thymidine; TK, thymidine kinase; US, United States.

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This paper presents the results of subjecting cigarette smoke PM samples, from cigarettes containing BT flue-cured tobacco, to four *in vitro* toxicity assays. These assays – the Neutral Red Uptake (NRU) assay, the *Salmonella* mutagenicity assay (SAL), the mouse lymphoma mammalian cell mutation assay (MLA) and the *in vitro* micronucleus test (IVMNT) – have been validated and accepted by regulatory agencies, including the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and the Organisation of Economic Cooperation and Development (OECD) (Aardema et al., 2006; Anon., 2006, 1990; Corvi et al., 2008; Garriott et al., 2002; Gatehouse et al., 1990; ICH, 1995, 1997; Kirsch-Volders et al., 2003; Matsushima et al., 1999; OECD, 2010, 1997a,b; Phelps et al., 2002); and recommended by the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) Task Force *In Vitro* Toxicity Testing of Tobacco Smoke (CORESTA, 2010; 2003).

2. Materials and methods

2.1. Test materials

PM preparation was as described by McAdam et al. (2011). Briefly, cigarettes were conditioned according to ISO 3402 (1999), then smoked on a RM20CSR smoking machine (Borgwalt-KC, Hamburg, Germany) according to ISO 3308 (2000). An appropriate number of cigarettes were smoked to obtain approximately 300 mg PM on a 44 mm Cambridge filter pad. PM was eluted in dimethyl sulphoxide (DMSO) to a concentration of 24 mg/ml and stored protected from light in single-use aliquots at −80 °C. Fresh samples of PM were prepared for each study.

For the external laboratory providing the MLA, PM was delivered frozen (−80 °C).

PMs were tested under code, as follows: W860, W861, W862, W863, W864, M4A and 2R4F (Table 1). The series W860–W864 represents different cigarette designs. Any effects of BT tobacco would be seen in general comparisons of PMs with (W862, W863 and W864) and without BT tobacco (W860 and W861), and also the specific comparison of W862 (80% BT tobacco) and its control, W861 (no BT tobacco).

2.2. In vitro toxicology testing

Post-mitochondrial supernatant (S9), prepared from male Sprague Dawley rats, induced with Aroclor 1254, was used for metabolic activation.

The NRU cytotoxicity assay was performed as described by McAdam et al. (2011). Briefly, V79 cells were maintained in tissue culture in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat inactivated foetal calf serum and penicillin/streptomycin (0.52% v/v). Cytotoxicity was expressed as a reduction in the uptake of Neutral Red dye into the lysosomes of cells after 48 h culture, measured by absorbance at 450 nm. Serial dilutions of PM were made to determine concentration-dependent inhibition of V79 cell growth. Four separate assays were performed for each test substance and the concentration causing 50% toxicity in the NRU test (IC_{50}) values were derived by software analysis of the dose–response curves obtained. This protocol conforms to the guidelines issued by the National Institutes of Health (NIH) (NIH, 2001). A higher IC_{50} value represents a lower cytotoxicity. One-way analysis of variance (ANOVA) was used to detect any significant differences between IC_{50} concentrations for the different test materials.

The SAL mutagenicity test was performed as described by McAdam et al. (2011), using five *Salmonella typhimurium* strains: TA98, TA100, TA102, TA1535 and TA1537, in the presence and absence of S9. All tests were performed in duplicate. A further two tests using strains TA98, TA100 and TA1537 in the presence of S9 were carried out over the linear portion of the dose–response curve. All data were analysed using Dunnett's test for significant differences between solvent control plates and those treated with PM. The numbers of revertants per μg PM were calculated using data from the linear part of the dose–response curve. Subsequently, Tukey's statistic was used to compare specific activities of the PMs. This protocol complied with OECD guideline 471 (OECD, 1997a) and ICH guidelines (ICH, 1995, 1997).

The IVMNT was performed as described by McAdam et al. (2011). Briefly, duplicate V79 cell cultures in DMEM supplemented with 10% foetal calf serum, were pulsed with test or control samples for 3 h followed by a 17 h recovery, with and without S9, or for 20 h without S9. At least six dose levels for each PM were scored for cytotoxicity and for micronucleus formation in bi-nucleate cells, on duplicate slides. Differences between micronucleated binucleated cells (MnBn) at the different test concentrations and the solvent controls were subjected to paired *t*-tests. This method complied with OECD draft guideline 487 (OECD, 2004).

The MLA was performed as described by McAdam et al. (2011), using L5178Y thymidine kinase (tk) +/- cells cultured in Roswell Park Memorial medium (RPMI). There were two independent experiments using a 3 h exposure with S9; and two independent experiments used 3 and 24 h exposures without S9; each with duplicate treatment cultures. After a two day expression period, cells were grown for eight days, and then trifluoro-thymidine

Table 1
Composition of different cigarettes smoked to yield PM test products.

PM/cigarette code	Blend	Filter
W860	Untreated BT tobacco[a] (80%)/Stem[b] (20%)	Cellulose acetate[c]
W861	Untreated BT tobacco[a] (80%)/Stem[b] (20%)	Cellulose acetate[c]/cavity with CR20[d] (40 mg) and charcoal[e] (20 mg)
W862	BT Tobacco[f] (80%)/Stem[b] (20%)	Cellulose acetate[c]/cavity with CR20[d] (40 mg) and charcoal[e](20 mg)
W863	BT Tobacco[f] (80%)/Stem[b] (20%)	Cellulose acetate[c]/cavity with charcoal[e] (60 mg)
W864	Flue-cured lamina[a] (40%)/BT Tobacco[f] (40%)/Stem[b] (20%)	Cellulose acetate[c]/cavity with charcoal[e] (60 mg)
M4A[g]	Flue-cured blend	Cellulose acetate[c]
2R4F[h]	United States (US)-style blend	Cellulose acetate[c]

[a] Leaf tobacco.
[b] Mid-rib of leaf.
[c] Cellulose acetate as found in conventional cigarettes.
[d] Ion exchange resin filter additive to selectively adsorb some smoke constituents.
[e] Coconut shell derived activated carbon to adsorb some volatile components.
[f] Tobacco treated to remove some of the protein and phenols by the method described.
[g] M4A is British American Tobacco's (BAT's) historical control.
[h] Reference cigarettes to provide historical control data (2R4F was originally obtained from the University of Kentucky).