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The *in vitro* cytotoxicity and genotoxicity of cigarette smoke particulate matter with reduced toxicant yields

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ABSTRACT

Tobacco smoke contains more than 5600 constituents, of which approximately 150 are toxicants. This paper describes the activities in the Neutral Red uptake (NRU) assay, the *Salmonella* mutagenicity test (SAL), the mouse lymphoma mammalian cell mutation assay (MLA) and the *in vitro* micronucleus test (IVMNT) of Particulate Matter (PM) obtained from experimental cigarettes (ECs), designed to produce reduced levels of toxicants. The designs included tobacco substitute sheet (TSS) containing glycerol, which dilutes toxicants in smoke, or the incorporation of blend-treated (BT) tobacco to reduce the levels of nitrogenous toxicant precursors and some polyphenols. All samples were cytotoxic in the NRU, however TSS reduced PM cytotoxicity in this assay. All PMs were mutagenic in the SAL, MLA and IVMNT. Reductions in bacterial mutagenicity were observed in the SAL, for cigarettes with BT tobacco, compared with their respective controls. The quantitative changes in bacterial mutagenicity could be explained by analytical chemistry data on smoke generated from the ECs used in the study. These observations, and the absence of consistent qualitative differences in the activities of the experimental, control and reference cigarettes, suggest that reduced toxicity cigarettes, as measured by the tests described in this paper, may be developed without introducing any additional cytotoxic or genotoxic hazards, but the impact of this on human health risks remains unknown.

1. Introduction

Tobacco smoke is a complex, dynamic, mixture of more than 5600 identified constituents (Perfetti and Rodgman, 2011), of which approximately 150 have been documented as toxicants (Fowles and Dybing, 2003).

In recent papers we have described four different individual technological approaches to the reduction of toxicants in cigarette smoke, two of which modified the tobacco blend (McAdam et al., 2011; Liu et al., 2011), and two of which modified the cigarette filter (Branton et al., 2011a, 2011b).

This paper describes an assessment of the *in vitro* toxicities of experimental cigarettes (ECs) incorporating these technologies. The designs include the use of tobacco substitute sheet (TSS), which has the dual function afforded by containing glycerol to dilute toxicants in smoke, and by the provision of a low organic content material in order to reduce the amount of tobacco burnt in the cigarette, and hence smoke yields. Another cigarette includes blend-treated (BT) tobacco to reduce the levels of toxicant precursors in tobacco and hence of toxicants in the tobacco smoke generated from them.

TSS is a new material, which, in addition to high levels of glycerol, also contains calcium carbonate (ca. 75%) to minimise the organic content of the TSS and hence smoke yields. The glycerol (ca. 12%) distils into the smoke to dilute the tar, by contributing to the total amount of particulate smoke, measured as nicotine-free dry particulate matter (NFDPM) (also known as 'tar'). As most cigarettes are designed to meet a specific International Standards Organisation (ISO) NFDPM yield value, incorporation of glycerol into the smoke stream effectively results in a reduced contribution of the tobacco combustion products to the overall NFDPM value, a process termed 'dilution'. Confirmation of the dilution effect through the incorporation of TSS into cigarettes, has been achieved by showing reductions in a wide range of smoke constituents, after smoking TSS containing cigarettes (McAdam et al., 2011).

Cigarettes with BT tobacco have also been developed. BT tobacco has reduced levels of soluble and insoluble proteins, and amino acids in tobacco, which act as toxicant precursors, as well as reduced levels of water soluble polyphenols, such as chlorogenic acid, rutin and scopoletin. The BT process is based on the fact that proteins and amino acids have been reported to be precursors for a number of potentially toxic constituents of tobacco smoke, including 2-aminonaphthalene and 4-aminobiphenyl (Torikaiu et al., 2005) and mutagenic heterocyclic amines (Mizusaki et al., 1977; Matsumoto and Yoshida, 1981; Clapp et al., 1999), the latter being implicated as a primary source of genotoxicity of cigarette smoke condensates (CSCs) (DeMarini, 2004).

The BT process is carried out on cut, flue-cured tobacco, and involves the sequential extraction of the tobacco with water,

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followed by treatment of the tobacco solids and aqueous extract with an aqueous protease enzyme solution, and by further treatment of the resulting solution with adsorbents, followed by reapplication of the remaining soluble materials to the extracted tobacco. The treated tobacco retains the structure of the original tobacco, and 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).

Most cigarette filters contain cellulose acetate, however, adsorbant filters were developed containing CR20L ion-exchange resin. CR20L is an amine-functionalised resin bead material which can be incorporated into cigarette filters (Diainon, Mitsubishi Chemical Corporation, Tokyo). The characterisation and use of CR20L in cigarette filters were described in detail by Branton et al. (2011b). In comparison to filters containing conventional activated carbon, CR20L offers superior reductions for hydrogen cyanide (HCN), formaldehyde and acetaldehyde.

Activated carbon is more efficient than CR20L in removing other volatile constituents from the smoke stream. One of the filters in the study contained a polymer-derived activated carbon, with a modified pore structure, to increase adsorption of a range of volatile smoke constituents (Branton et al., 2009). Two of the filters contained both CR20L and polymer-derived activated carbon.

Full details of the design and performance of these experimental cigarettes are described elsewhere (McAdam et al., 2012).

The results are described of subjecting cigarette smoke particulate matter (PM) samples, generated under ISO machine-smoking conditions (ISO 3308:1977) from reference, control and reduced toxicant cigarettes, to four *in vitro* toxicity assays. These assays – Neutral Red uptake (NRU), *Salmonella* mutagenicity test (SAL), the mouse lymphoma mammalian cell mutation assay (MLA) and *in vitro* micronucleus test (IVMNT) – have been accepted by regulatory agencies and the Organisation of Economic Cooperation and Development (OECD) (Aardema et al., 2006; Anon, 2006; Corvi et al., 2008; Honma et al., 1999a; Garriott et al., 2002; Kirsch-Volders et al., 2003; Matsushima et al., 1999; Phelps et al., 2002). The methods were also recommended by the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) (Anon, 2010).

2. Materials and methods

2.1. Cigarettes

Cigarette specifications are detailed in Table 1. 3R4F and M4A were reference cigarettes, providing historical control data. CC1 and CC6 were the control cigarettes, with ISO tar yields of 1 mg and 6 mg per cigarette respectively (ISO, 2000). CC1 and CC6 contained conventional cigarette materials. TSS6, TSS1 and BT1 were the test cigarettes, to evaluate the new materials. CC1 was the control for TSS1 and BT1. CC6 was the control for TSS6.

2.2. PMs

Tobacco preparation, cigarette manufacture and PM production were undertaken at BAT Group Research and Development, Southampton, United Kingdom (UK). To generate cigarette smoke PM, cigarettes were conditioned according to the ISO method, for a minimum of 48 h at 22 ± 1 °C and 60 ± 3% relative humidity (ISO, 1999) and smoked on a RM20CSR smoking machine (Borgwalt-KC, Hamburg, Germany) using ISO standard puffing parameters (35 ml puff volume taken over 2 s once per minute) (ISO, 2000). An appropriate number of cigarettes were smoked to obtain approximately 220 mg PM on a 44 mm Cambridge filter pad (Whatman, Maidstone, UK). The pads were weighed before and after smoking to determine the weight of particulate matter deposited, and the PM was then eluted in dimethyl sulphoxide (DMSO) to a concentration of 24 mg/ml and stored protected from light in single-use aliquots at −80 °C.

Samples were shipped at −80 °C to an independent laboratory for *in vitro* tests, where they were stored at −80 °C, and used within 1 month. Crooks et al., 2013 have demonstrated stability of PM when stored at −80 °C for up to 2 years.

Test material was tested at different concentrations calculated as μg/ml of PM. At the end of scoring, the concentration data were corrected to give the NFDPM concentration. This was achieved by gas chromatographic analysis of PM from each smoke run to determine nicotine and water contents. The resulting information was used to compute an NFDPM correction factor to convert the concentration of PM tested to NFDPM in μg/ml for each test material. The correction factor was applied to the experimental data to convert the test concentrations from μg PM/ml to μg NFDPM/ml, by using the formula:

$$\text{NFDPM concentration} = (\text{PM dose} \times \text{NFDPM factor})$$

NFDPM concentrations were used to compare the toxicities of the different PMs.

2.3. In vitro toxicology testing

All *in vitro* tests were performed in an independent GLP laboratory. Aroclor 1254 induced rat liver post-mitochondrial supernatant (S9) mix provided metabolic activation in the genotoxicity assays.

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

PM/cigarette code	Blend	Filter
CC6	United States (US)-style blend	Cellulose acetate[c]
TSS6	US-style blend (80%)[a]/TSS[b] (20%)	Two stage filter of cellulose acetate[c]/modified charcoal[d] (80 mg)
CC1	US-style blend	Cellulose acetate[c]
TSS1	US-style blend (80%)[a]/TSS[b] (20%)	Three stage filter of cellulose acetate[c]/modified charcoal[d] (60 mg)/CR20L (20 mg)[e]
BT1	US-style blend[a] (25%)/BT tobacco[f] (75%)	Three stage filter of cellulose acetate[c]/cavity with modified charcoal[d] (60 mg)/CR20L (20 mg)[e]
M4A[g]	Flue-cured blend	Cellulose acetate[c]
3R4F[h]	US-style blend	Cellulose acetate[c]

[a] Leaf tobacco.
[b] Tobacco substitute sheet.
[c] Cellulose acetate as found in conventional cigarettes.
[d] Polymer-derived activated carbon to adsorb a broad range of volatile components.
[e] Ion exchange resin filter additive to selectively adsorb volatile acidic and carbonyl smoke constituents.
[f] Tobacco treated to remove some of the protein and phenols by the method described.
[g] M4A is the historical reference cigarette used by British American Tobacco (BAT).
[h] 3R4F reference cigarettes used to provide historical control data (originally obtained from the University of Kentucky).