Cytotoxicity and Genotoxicity Testing of Extracts of Snus Tobacco

Louise Neilson[1], Deborah Dillon[1], Helen Pearce[2], Victoria Stone[2] and Clive Meredith[1]
[1]British American Tobacco, Group Research & Development, Regents Park Road, Southampton, SO15 8TL, United Kingdom
[2]Covance Laboratories Ltd, Otley Road, Harrogate. HG3 1PY, United Kingdom
Corresponding Author: louise_neilson@bat.com

INTRODUCTION

In vitro methods for cytotoxicity and genotoxicity testing of tobacco smoke (particulate phase) are well defined and documented, however equivalent methods for smokeless tobacco products are yet to be established. In this preliminary study, we investigated the use of different solvents and extraction procedures to prepare extracts of Swedish-style snus and an American oral tobacco product for cytotoxicity and genotoxicity testing. Solvents tested were dimethyl sulphoxide (DMSO) with and without sonication, saline, and an artificial saliva preparation including enzymes. For each extract, nicotine, benzo(a)pyrene (B(a)P) and tobacco-specific nitrosamines (TSNAs) levels were measured. Compatibility of extraction solvents with *in vitro* test systems for cytotoxicity (Neutral Red Uptake (NRU)) and genotoxicity (Ames, *In Vitro* Micronucleus (IVMN) and Mouse Lymphoma Assay (MLA)) was determined. Finally we performed some preliminary *in vitro* assays using extracts of the test products to evaluate their biological activity in the four assays.

MATERIALS AND METHODS

Test Products

- Skoal Bandits Wintergreen (SB)
- Lucky Strike Brown snus (LS)

} Commercially available products

- Formulation 1 Swedish-style snus (Dark air cured tobacco)
- Formulation 2 Swedish-style snus (Dark air cured tobacco)

} Experimental products made in BAT

	Moisture (%)	Nicotine (% dry weight)	B(a)P (ppb dry weight)	TSNAs (ppm dry weight)
Skoal Bandits	54	2.9	22	15
Lucky Strike	51	1.9	1.1	1.8
Formulation 1	53	1.9	1.7	1.9
Formulation 2	38	2.5	1.7	1.9

Extraction Solvents

- DMSO - a solvent usually used for the toxicological testing of tobacco smoke particulates
- Physiological saline adjusted to pH 5
- Artificial saliva (AS) formulation used previously by Chou & Hee (1994) for work on toxicants in chewing tobacco. In initial studies the formulation was made with and without enzymes to assess its compatibility in the assays

1. Sodium chloride (1.4 mg/ml), potassium chloride (0.5 mg/ml), calcium chloride (0.1 mg/ml), sodium dihydrogenphosphate (0.15 mg/ml), magnesium chloride (0.025 mg/ml), urea (0.09 mg/ml), glucose (0.2 mg/ml)
2. Adjust to pH 7.0 (sodium hydroxide or hydrochloric acid)
3. Bovine mucin (2.7 mg/ml), α-amylase from human saliva (2.5 units/ml), lysozyme from chicken egg white (0.7 units/ml), acid phosphatase from potato (0.004 units/ml)
4. Stir for 2 hours to achieve homogeneity

Extraction Techniques

We investigated two extraction techniques (Covance), one included sonication and incubation for 21 hours (Rickert et al. (2009)) (SB only) and the second based on ISO 10993-12 for biological evaluation of insoluble medical devices: 24 hour extraction with no sonication.

1. Appropriate numbers of snus pouches were cut in half, weighed and mixed with appropriate volumes of extraction solvent to produce the required w/v concentration of 500 mg/ml (highest sample/solvent ratio to yield sufficient extract)*
2. Sonicated for 1 minute, if appropriate
3. Shaken for 21 or 24 hours at 37ºC
4. Centrifuged at 1,800 x 'g' for 30 minutes and the supernatant decanted from heavy particulates.
5. Supernatant centrifuged at 25,000 x 'g' for 30 minutes and the final supernatant decanted from fine particulates.
6. Final supernatant adjusted to pH 7.4 ± 0.2 with hydrochloric acid or sodium hydroxide (aqueous extracts only).
7. Resulting extracts were sterilised using 0.2 µm pore size filters.

* From the initial additions of 500mg of snus per ml of solvent, the extraction solvent recovery was approximately 20% resulting in the recovery of 1 ml per 2g tobacco

At least one batch of extract was made for each product and solvent. Samples of each extract were sent to BAT to measure the levels of nicotine, B(a)P and TSNAs using the following methods:

- Nicotine: Samples diluted 1:50 in methanol and analysed by GC-MS.
- B(a)P: A revised tobacco blend method was used. Samples were diluted 1:100 in acetonitrile and analysed by HPLC with fluorescence detection.
- TSNAs: Samples were diluted 50x with water and analysed by LC-MS/MS.

Extraction efficiencies were calculated by comparing the levels of analytes present in the extract compared to the level present in the blend (see above table), corrected for moisture content and pouch weight, expressed as a percentage.

In vitro assays

All assays were performed at Covance in accordance with GLP. The Ames, IVMN and MLA were performed to OECD guidelines with a reduced protocol design (as described below)

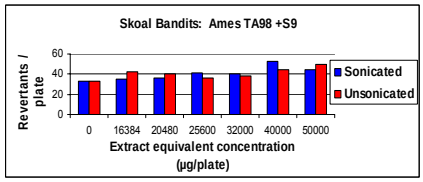
- Ames: One experiment, TA98, TA100, TA102 in the presence (+) and absence (-) of metabolic activation (S9).
- NRU: Balb/c 3T3 fibroblasts (based on ICCVAM protocol).
- IVMN: Chinese hamster V79 cells, 3 hours +S9, 24 hours –S9.
- MLA: Mouse lymphoma L5178Y cells, 24 hours –S9, 3 hours ±S9
- Maximum doses tested
  - Ames = 50,000 µg/ml equivalents for DMSO (0.1 ml/plate) and 250,000 µg/ml for aqueous extracts (0.5 ml/plate)
  - Mammalian cell assays = 5,000 µg/ml equivalents for DMSO (1% v/v) and 50,000 µg/ml for aqueous extracts (10% v/v)

RESULTS AND DISCUSSION

Effect of Sonication

- Sonication during DMSO extraction of Skoal Bandits or Lucky Strike did not appear to increase the extraction efficiency of toxicants or increase the activity of the extracts in all strains of the Ames assay (SB, TA98 +S9 data shown).

Treatment	Nicotine (mg/ml)/ (Extraction efficiency)	B(a)P (ng/ml)/ (Extraction efficiency)	TSNAs (ng/ml)/ (Extraction efficiency)
SB No Sonication	3.50 (59.1%)	5.23 (85.7%)	2342 (78.6%)
SB Sonication	3.80 (63.1%)	5.39 (83.7%)	2342 (78.1%)



Effect of extraction solvents (SB data only shown)

- DMSO was the only solvent which extracted B(a)P at a detectable level from Skoal Bandits, Formulation 1 and Formulation 2.
- Nicotine was generally more efficiently extracted by saline from SB (data shown) whereas artificial saliva was more efficient for LS (data not shown).
- Solvent extraction efficiency for TSNA's in SB and LS was ranked: DMSO > Artificial saliva > Saline, however only a small difference existed between the aqueous solvents' efficiencies (as shown)

Treatment	Nicotine (mg/ml)/ (Extraction efficiency)	B(a)P (ng/ml)/ (Extraction efficiency)	TSNAs (ng/ml)/ (Extraction efficiency)
SB DMSO (n=2)	3.99 (66.7%)	5.10 (88.4%)	2277 (76.2%)
SB Saline (n=2)	4.81 (80.2%)	<0.25*	1523 (50.8%)
SB AS (n=2)	4.09 (68.3%)	<0.25*	1591 (53%)

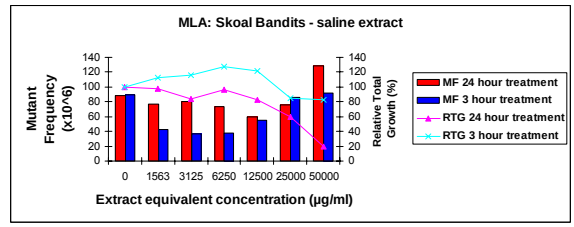
* Below limit of detection (<0.25ng/ml)

Extraction Solvents – Biological activity

- The addition of enzymes to saliva did not produce any increases in any of the measured genetic endpoints for each assay and there was no increase in cytotoxicity in the NRU assay (data not reported).

Skoal Bandits

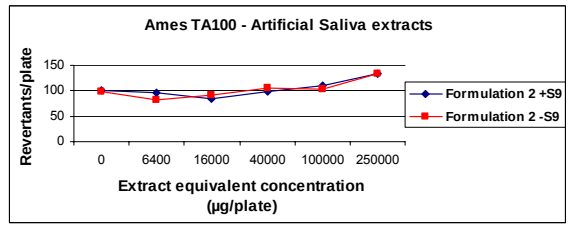
- There were no increases in any of the measured genetic endpoints for each assay and no increase in cytotoxicity in the NRU assay (data not reported).
- Observations:
  - Ames: DMSO extracts produced toxicity at the top dose (data not reported).
  - MLA: Decreases in relative total growth (RTG) at or close to the 10-20% limit were seen with the aqueous extracts following extended (24 hour) treatment only.



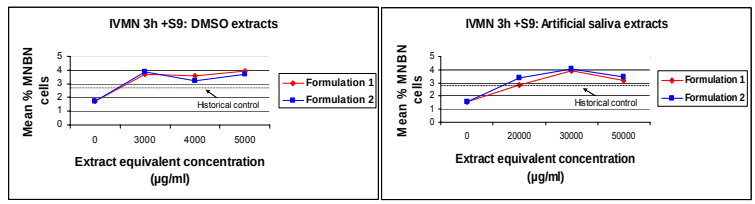
Swedish snus (Lucky Strike Brown, Formulation 1 and 2)

- There were no increases in mutant frequencies in the MLA and no increase in cytotoxicity in the NRU assay (data not reported)
- Observations:
  - MLA: A decrease in RTG was seen in one extract (Formulation 2 in AS).

- **Ames:** Occasionally, extracts produced positive increases in revertant numbers at the highest doses, however this was not replicated between the Swedish-style snus products (1 product/strain shown for clarity)



- **IVMN:** Evidence of weak genotoxicity was seen in Formulation 1 and 2 extracted in both artificial saliva and DMSO.



- Additional experiments with higher number of replicates will need to be performed in order to understand further the relevance of these weak genotoxic effects.

CONCLUSION

A snus extraction process based on ISO 10993-12, using an artificial saliva supplemented with enzymes, gives an opportunity to test up to maximum levels of extract inclusion in *in vitro* cytotoxicity and genotoxicity assays. The relevance of these weak genotoxic effects seen in our preliminary study must be further understood by the generation of more data and increased replicate experiments.

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