

Definitive Protocol

Study Title	Extracts of four Snus batches and Kentucky reference moist snuff: mutation at the thymidine kinase (<i>tk</i>) locus of mouse lymphoma L5178Y cells (MLA) using the Microtitre ^R fluctuation technique
Study Director	M Ballantyne
Sponsor	Swedish Match SE-11885 Stockholm SWEDEN
Study Monitor	David Johnson Swedish Match North America
Test Facility	Covance Laboratories Ltd Otley Road, Harrogate North Yorkshire HG3 1PY ENGLAND
Covance Study Number	1138/18
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OBJECTIVE

To evaluate the potential of Extracts of four Snus batches and Kentucky reference moist snuff to induce forward mutation at the thymidine kinase (*tk*) locus in L5178Y mouse lymphoma cells in the absence and presence of a rat liver metabolising system.

TEST SYSTEM

Many mammalian cell gene mutation assays are available but only four cell lines (V79, CHO, TK6 and L5178Y) and three genetic loci (*hprt*, *tk*, and the cell membrane Na^+/K^+ ATPase) are well-validated and widely used (1). Some of the mammalian gene mutation assays involving Chinese hamster cells are now considered to be insufficiently sensitive on statistical grounds due to the size of cell population that can be examined (2). Of all the available systems, the *tk* (thymidine kinase) locus in L5178Y cells has the advantage of detecting both gene mutations and chromosome aberrations.

The mouse lymphoma assay (MLA), employing the *tk* locus in L5178Y cells, is highly recommended and is the gene mutation assay of choice at Covance Laboratories Limited.

The mutation system works by placing treated cells under selective pressure so that only mutant cells are able to survive. The *tk* locus is autosomal and the L5178Y cell line is heterozygous ($tk^{+/-}$), producing the enzyme thymidine kinase. This enzyme is a salvage enzyme for nucleic acid breakdown products but if a toxic base analogue (5-trifluorothymidine) is present in the medium, the enzyme will incorporate the analogue into the cells. Thus the cells die unless the enzyme is rendered inactive, by mutation for example. Resistance to 5-trifluorothymidine (TFT) results from a lack of thymidine kinase (TK) activity. Thus, the mutants ($tk^{-/-}$) are unable to use the toxic analogue and survive in its presence.

Two types of TFT-resistant mutant colonies are selected and these are designated large colonies and small (slow-growing) colonies. Molecular analysis has indicated that the large colonies tend to represent events within the gene (base-pair substitutions and deletions) whereas small colony mutants often involve large genetic changes frequently visible as chromosome aberrations (3,4). Thus, in this system, gene mutations within the *tk* gene (11 to 13 kilobases) and chromosomal events involving the gene may be detected. The TK system has a high spontaneous mutant frequency

and because of the high numbers of cells that can be treated and sampled it is the most satisfactory mammalian cell mutation assay from the statistical point of view. Furthermore, a fluctuation protocol has been developed for use with the assay (1,5). The protocol described here is consistent with the consensus agreement regarding protocol issues discussed during the Mouse Lymphoma Workshops, Portland, Oregon in 1994 (6) and Plymouth, UK 2002 (10).

Swedish moist snuff (Snus) is a commercially available tobacco product which may be considered for use as a smoking cessation product, and is undergoing safety evaluation testing. As part of this process, testing is scheduled to be performed in a number of *in vitro* genotoxicity assay systems. As Snus is an insoluble solid, to enable exposure of this test material to the cells in the assay system, as is commonly used for *in vitro* testing of other smokeless tobacco products, an extract of the test material will be used. The extraction methodology and concentrations employed for this study have been demonstrated in previous experimentation (CLE study number 1138/16) to provide the highest practicable extraction concentration for these materials, and therefore provide the most stringent assessment of these materials possible in this assay system.

Protocol is designed to meet the known requirements of OECD Guideline 476 (adopted 21 July 1997), and the UKEMS Guidelines (1990) providing either a positive result is obtained, or a second experiment is performed.

MATERIALS

Test articles

The test articles will be Extracts of four Snus batches and Kentucky reference moist snuff.

Swedish Moist Snuff (Snus) G
Swedish Moist Snuff (Snus) CPS
Swedish Moist Snuff (Snus) CDM
Swedish Moist Snuff (Snus) CDM2
Kentucky Reference Moist Snuff, Batch 2S3

The sponsor will normally be expected to supply sufficient material to permit sufficient extract volumes to perform all testing required for this study. The sponsor

will also supply as much information as possible regarding characterisation of the Snus/moist snuff, such as composition analysis, expiry date, storage conditions etc.

All test articles are handled as though they were potential carcinogens and appropriate safety procedures are employed.

Determinations of the stability and characteristics of the test article, where defined in GLP regulations, are the responsibility of the sponsor. Archiving a sample of the test article will be the responsibility of the Sponsor.

Extracts will be performed based on the methodology evaluated and developed in CLE study number 1138/16, and conducted using sterile containers and solutions, in order to minimise any contamination from external sources:

- Appropriate numbers of pouches/sachets of moist snuff/Snus will be cut approximately in half, and both the contents and the packaging (pouches/sachets) weighed and mixed with appropriate volumes of sterile purified water to produce the required w/v concentration. If the tobacco is not finely divided then brief homogenisation may be performed.
- Extractions will be performed for 24 hours at 37°C, with shaking.
- At the end of the 24 hour extraction period, extracts will be centrifuged at approximately 1800 x 'g' for 30 minutes, and heavy particulates removed by decanting off the supernatant.
- The supernatant will then be centrifuged at 25,000 x 'g' for 30 minutes, and fine particulates removed by decanting off the supernatant.
- The final supernatant will be adjusted to pH to 7.4 ± 0.2 with Hydrochloric acid (water extracts only)
- The resulting extracts will be filter sterilised using a 0.2 µm pore size filters (pre-filtering using a larger pore size filter may be performed).
- Aliquots of extracts will be stored at approximately -80°C, and used for up to 6 months after the extraction process is performed.

All Snus/moist snuff batches will be extracted in purified water at a concentration of 500 mg/mL equivalent, and CDM2 extracts at this concentration in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) will also be prepared for use in this study. Based on experimentation conducted in CLE study number 1138/16, this concentration was the highest extraction concentration that could practicably be

extracted from material of this nature, and therefore gives the highest achievable extract concentration.

Where extracts are prepared in two or more separate flasks for a Snus/moist snuff batch, extracts for use in each experiment will be pooled prior to use in the assay.

Definition of top concentration

Vials of frozen extract to provide the volume of extract required will be thawed on the day of assay, and appropriate dilutions performed for use in the assay.

Test article additions to the assay will be made at 10% (water extracts), 1.5% (DMSO extract for 3 hour treatments) or 1% (DMSO extract for 24 hour treatments) of the final culture volume, these being the highest volume additions that are possible in this assay system. Osmolality of the medium may be measured, as fluctuations in osmolality of more than 50 mOsm/kg have been responsible for increased mutation (7,8). Similarly as extremes in pH should also be avoided, pH assessment of post-treatment medium may be made (7,8).

A minimum of five doses will be selected for the mutation experiment, ranging from non-toxic to toxic (approximately 10-20% relative total growth) where possible (selection of appropriate dose levels will be based on information provided in previous experimentation; CLE study number 1138/16). The mutation experiment will be performed both in the absence and presence of metabolic activation using a 3 hour treatment incubation period, and also in the absence of S-9 using a 24 hour treatment incubation period. Normally, a minimum of four doses will be carried through all stages of the assay. The mouse lymphoma cells grow in suspension culture and heavy precipitates can interfere with the assay. At the end of the treatment incubation, the cells are pelleted by centrifugation and the precipitate may pellet with the cells making the control of exposure impossible. Thus, normally the lowest precipitating concentration will be the highest dose tested.

Depending on the results of the first experiment a second experiment may be performed. Treatment conditions and doses for this and any subsequent experiments will be determined in conjunction with the Sponsor.

Toxicity will be measured by assessment of the Relative Total Growth (RTG). Other data relevant to toxicity will be generated during the conduct of the study (suspension growth and Day 2 plating efficiency) and may contribute to the interpretation of the data.

An example of the dose ranges that may be tested is provided in the following table:

Treatment	Concentration of extract (mg/mL equivalent)	Final concentration (µg/mL equivalent)	
		Water extracts ^a	DMSO extracts ^b
3 Hour - or + S-9	50.00	5,000	750
	100.00	10,000	1,500
	200.00	20,000	3,000
	300.00	30,000	4,500
	400.00	40,000	6,000
	500.00*	50,000	7,500
24 Hour - S-9	100.00	10,000	1,000
	200.00	20,000	2,000
	300.00	30,000	3,000
	400.00	40,000	4,000
	450.00	45,000	4,500
	500.00*	50,000	5,000

* Neat extract - all lower concentrations achieved by dilutions of this extract concentration

a Test article additions as 10% of final culture volume

b Test article additions as 1% of final culture volume (24 hour treatments) or 1.5% of final culture volume (3 hour treatments)

If alternative dose ranges are used, these will be noted in the raw data.

Nicotine content determination

The nicotine content of the test article extracts will be performed using HPLC analysis. For each test article (extract), nicotine determination will be performed on samples from up to five extraction flasks (if more than five flasks are used to generate extract, samples from only five representative flasks will be analysed), together with a sample from the pooled extract to be used in the mutation assay. Details of the analytical method will be provided by amendment to protocol. Results from these analyses will be presented in the report as achieved nicotine concentrations.

Controls

Where two or more test articles are tested in parallel, common control treatments may be shared between test articles. Negative controls will comprise treatments with blank extract solvent (extract solvent subjected to the extraction conditions, but in the absence of test material) diluted to the same extent as the test article solutions. The positive control chemicals are supplied and will be used as shown in the following table (100-fold dilution):

Chemical	Source*	Stock** concentration (µg/mL)	Final concentration (µg/mL)	S-9
4-nitroquinoline 1-oxide (NQO)	Aldrich Chemical Co, Gillingham, UK	15	0.15 [#]	-
		20	0.20 [#]	-
Benzo(a)pyrene (BP)	Aldrich Chemical Co, Gillingham, UK	200	2.00	+
		300	3.00	+

* Source of supply as indicated, or a preparation of equivalent standard

** All stock solutions are prepared in anhydrous analytical grade dimethyl sulphoxide (DMSO). NQO and BP solutions, if not used immediately, will be stored as frozen aliquots at -80°C in the dark

For 24 hour treatments, final NQO concentrations of 0.05 and 0.1 µg/mL will be tested

Metabolic activation system

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation is obtained from Molecular Toxicology Incorporated, USA, where it is prepared from male Sprague Dawley rats induced with Aroclor 1254. The batches of MolTox™ S-9 are stored frozen at -80°C prior to use. Each batch is checked by the manufacturer for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufin-O-dealkylase activities). The quality control statement, relating to the batch of S-9 preparation used, will be included in the report.

Treatment will be carried out both in the absence and presence of S-9, prepared in the following way.

Glucose-6-phosphate (180 mg/mL), NADP (25 mg/mL), 150 mM KCl and rat liver S-9 will be mixed in the ratio 1:1:1:2. For all cultures treated in the presence of S-9, a 1 mL aliquot of the mix will be added to each cell culture (19 mL) to give a total of 20 mL. The final concentration of the liver homogenate in the test system will be 2%. Cultures treated in the absence of S-9 will receive 1 mL 150 mM KCl (3 hour treatment only). Culture volumes may be reduced to 10 mL, in which case all volumes above would be reduced proportionately.

Growth media and conditions

Three types of RPMI 1640 medium will be prepared as follows:

	Final concentration in:		
	RPMI A	RPMI 10	RPMI 20
Horse serum (heat inactivated)	0% v/v	10% v/v	20% v/v
Penicillin / streptomycin	100 units/mL	100 units/mL	100 units/mL
Amphotericin B	100 µg/mL	100 µg/mL	100 µg/mL
Pluronic	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL
	0.5 mg/mL	0.5 mg/mL	-

Heat inactivated horse-serum will be used in order to eliminate a factor which degrades TFT.

Cell cultures

L5178Y TK+/- mouse lymphoma cells are stored as frozen stocks in liquid nitrogen, the original cultures were obtained from Dr Donald Clive, Burroughs Wellcome Co. Each batch of frozen cells is purged of TK- mutants, checked for spontaneous mutant frequency and that they are mycoplasma free. For each experiment, one or more vials will be thawed rapidly, the cells diluted in RPMI 10 and incubated in a humidified atmosphere of 5% (v/v) CO₂ in air. When cells are growing well, subcultures will be established in an appropriate number of flasks.

METHODS

The test system will be suitably labelled (using a colour-coded procedure) to clearly identify the study number, test article, positive and negative control groups.

Cytotoxicity range-finder

Based on experimentation performed in CLE study number 1138/16, no separate toxicity range-finder experiment was considered to be required in this study. Little or no dose-limiting toxic effects are expected with any extracts tested using a 3 hour treatment incubation period in the absence or presence of S-9. Cytotoxicity at or approaching 10-20% RTG may occur with 24 hour treatments.

Mutation assay

Treatment of cell cultures

At least 1×10^7 cells (3 hour treatment) or 4×10^6 cells (24 hour treatment) will be placed in a series of sterile disposable 50 mL centrifuge tubes (3 hour treatment) or tissue culture flasks (24 hour treatment) and the appropriate volume of vehicle, test article or positive control solution will be added. For 3 hour treatments, the medium will contain a reduced level (5% v/v) of horse serum and 0.5 mL of S-9-mix or 0.5 mL (150 mM) KCl solution for treatments in the presence and absence of S-9-mix, respectively. The final treatment culture volume will be 20 mL for 24 hour treatments and 10 mL for 3 hour treatments. Cultures will be in duplicate (single cultures only will be used for each dose of the positive control).

3 hour treatment: After 3 hours incubation at $37 \pm 1^\circ\text{C}$ with gentle agitation, cultures will be centrifuged (200 x g) for 5 minutes, washed and resuspended in 50 mL fresh RPMI 10 medium.

24 hour treatment: After static incubation at $37 \pm 1^\circ\text{C}$ for 24 hours, cultures will be centrifuged (200 x g) for 5 minutes, washed and resuspended in fresh RPMI 10 medium (20 mL). Cell densities will be determined using a Coulter counter or haemocytometer and, where sufficient cells survive, adjusted to 2×10^5 cells/mL.

Cells will be transferred to tissue culture flasks for growth throughout the expression period. The solubility of the test compound in culture will be assessed, by eye, at the beginning and end of treatment.

Expression period

Cultures will be maintained in flasks for a total of 2 days (possibly 3 days if high toxicity is experienced) during which time the TK⁻ mutation will be expressed. During the expression period, subculturing will be performed as required with the aim of not exceeding 1×10^6 cells per mL and, where possible, retaining a total of at least 1×10^7 cells/flask. From observations on recovery and growth of the cultures during the expression period, normally at least four test dose levels plus negative and positive controls will be selected to be plated for viability and 5-trifluorothymidine (TFT) resistance.

Plating for viability

At the end of the expression period the cell densities in the selected cultures will be determined using a Coulter counter or haemocytometer and adjusted to 1×10^4 /mL with RPMI 20 in readiness for plating for TFT resistance.

Samples from these will be diluted to 8 cells/mL as follows:

	Initial cell conc ⁿ	Dilution		Intermediate cell conc ⁿ	Dilution		Final cell conc ⁿ
	(A)	mL A	mL medium	(B)	mL B	mL RPMI 20	(C)
Viability	1 x 10 ⁴ /mL	0.5	9.5	5 x 10 ² /mL	0.8	50	8/mL

Using a multichannel pipette, 0.2 mL of the final concentration of each culture will be placed into each well of two 96-well microtitre plates (192 wells, averaging 1.6 cells per well). The plates will be incubated at 37 ± 1°C in a humidified incubator gassed with 5% (v/v) CO₂ in air for one to two weeks. Wells containing viable clones will be identified by eye using background illumination and counted.

Plating for 5-trifluorothymidine (TFT) resistance

At the end of the expression period, cell concentrations will be adjusted to give 1 x 10⁴/mL, TFT (300 µg/mL) will be diluted 100-fold into these suspensions to give a final concentration of 3 µg/mL. Using a multichannel pipette, 0.2 mL of each suspension will be placed into each well of four 96-well microtitre plates (384 wells at 2 x 10³ cells per well). Plates will be incubated at 37 ± 1°C in a humidified incubator gassed with 5% (v/v) CO₂ in air for one to two weeks and wells containing clones will be identified as above and counted. In addition, scoring of large and small colonies is recommended as the additional information obtained may contribute to an understanding of the mechanism of action of the test article (1). The number of wells containing large colonies and the number containing small colonies will be scored for the negative and positive controls and for doses of test article showing a significant increase in mutant frequency over the negative control. In this way small colony and large colony mutant frequencies may be estimated.

Following Experiment 1, results will be communicated to the Sponsor. At this point, a decision will be made in conjunction with the Sponsor on whether a second or further experiment is to be performed, and the conditions/methodology to be employed.

Analysis of results

All calculations will be performed either manually or by computer using validated software.

Suspension Growth (SG) is a measure of the growth in suspension during treatment and the expression period.

Suspension Growth (SG) is calculated as follows:

Suspension growth = a x b x c

$$\text{where } a = \left(\frac{D_0 \text{ post - treatment cell count}}{\text{Pre - treatment cell density}} \right)$$

$$\text{where } b = \left(\frac{D_1 \text{ cell count}}{\text{Cell count set up on } D_0 \text{ post - treatment}} \right)$$

$$\text{where } c = \left(\frac{D_2 \text{ cell count}}{\text{Cell count set up on } D_1} \right)$$

NB for three hour treatments a is assumed to equal 1

Usually the denominators for b and c are 2×10^5 cells/mL. However, if cytotoxicity causes the cell count to be lower than 2×10^5 cells/mL following treatment and/or if the cells do not grow during part of the expression period, it can be lower. In these cases, the respective cell count values will be entered into the calculation above.

Relative suspension growth (RSG) is a measure of the growth in suspension during treatment and the expression period relative to the mean control.

Relative suspension growth (RSG) is calculated as follows:

$$\text{RSG (\%)} = \left(\frac{\text{Individual SG value}}{\text{Mean control SG value}} \right) \times 100$$

Viability is the measure of the cells ability to clone i.e. Cloning efficiency (CE).

Cloning Efficiency (CE) is calculated as follows:

For microtitre plate tests, calculations are based on P(o), the proportion of wells in which a colony has not grown:

$$P(o) = \left(\frac{\text{Number of wells with no colony}}{\text{Total number of wells}} \right)$$

The Cloning Efficiency (CE) for each culture is calculated according to the following calculation:

$$CE = \left(\frac{-\ln P(o)}{\text{Number of cells per well}^*} \right) \times 100$$

* Number of cells per well is 1.6 cells per well on average on all viability plates

Relative Total Growth (RTG) is the measure of cytotoxicity relative to the control, that takes into account all cell growth and cell loss during the treatment period and the 2 day expression period (RSG), and the cells' ability to clone 2 days after treatment (viability).

Relative Total Growth is calculated as follows:

$$RTG = RSG \times \left(\frac{\text{Individual Viability Value}}{\text{Mean Control Viability Value}} \right)$$

Determination of mutant frequency

Mutant frequency is calculated as follows:

$$MF = \frac{-\ln P(0) \text{ for mutant plates}}{\text{Number of cells per well}^* \times (\text{viability}/100)}$$

* Number of cells per well is 2000 cells per well on average on all mutant plates.

Small and large colony mutant frequencies will be calculated in an identical manner, using the relevant number of empty wells for small and large colonies, as appropriate.

Assessment of statistical significance of mutant frequency

Statistical significance of mutant frequencies (total wells with clones) will be carried out according to the UKEMS guidelines (9). Thus the control log mutant

frequency (LMF) will be compared with the LMF from each treatment dose based on Dunnett's test for multiple comparisons, and secondly the data will be checked for a linear trend in mutant frequency with treatment dose using weighted regression. The test for linear trend is one-tailed, therefore negative trend will not be considered significant. These tests require the calculation of the heterogeneity factor to obtain a modified estimate of variance.

Acceptance criteria

The assay will be considered valid if all the following criteria are met:

1. the mean mutant frequencies in the negative (solvent) control cultures fall within the normal range (50 to 170 mutants per 10^6 viable cells)
2. at least one concentration of each of the positive control chemicals induces a clear increase in mutant frequency (the difference between the positive and negative control mutant frequencies is greater than half the historical mean value)
3. the mean plating efficiencies of the negative controls from the mutation experiments are between the range 65% to 120% on Day 2
4. The mean suspension growth of the negative controls from the mutation experiments is between the range 8 to 32 following 3 hour treatments
5. There should be no excessive heterogeneity between replicate cultures.

Evaluation criteria

A test article will be considered to be mutagenic in this assay if all the following criteria are met:

1. the assay is valid
2. the mutant frequency at one or more doses is significantly greater than that of the negative control ($p < 0.05$)
3. there is a significant dose-relationship as indicated by the linear trend analysis ($p < 0.05$)

4. If more than one experiment is performed, for a test article to be considered mutagenic the positive trends/effects described above should be reproducible.

Results which only partially satisfy the above criteria will be dealt with on a case-by-case basis. Similarly, positive responses seen only at high levels of cytotoxicity will require careful interpretation when assessing their biological significance. Indeed, extreme caution will be exercised with positive results obtained at levels of RTG lower than 10%.

GLP COMPLIANCE

The study will be performed in compliance with:

United Kingdom Good Laboratory Practice Regulations 1999, Statutory Instrument No. 3106 as amended by the Good Laboratory Practice (Codification Amendments Etc.) Regulations, 2004.

OECD Principles on Good Laboratory Practice (revised 1997, issued Jan 1998) ENV/MC/CHEM (98) 17.

All procedures will be performed in accordance with Covance Laboratories Limited standard operating procedures (SOPs). The study will be subject to Quality Assurance evaluation by the Covance Laboratories Limited Quality Assurance Unit (QAU) in accordance with SOPs. Where appropriate, any change to this protocol will be made by an amendment issued in agreement with the Sponsor.

In accordance with the requirements of the UK Medicines and Healthcare Products Regulatory Agency GLP Monitoring Authority, if no client comments are received within six months of issue, the report will be finalised. Any further changes after this time will be addressed as an amended final report, which may result in additional costs.

A list of study records to be maintained by Covance Laboratories Limited is detailed in Appendix 1.

PROPOSED TIME SCHEDULE

It is estimated that the study will start in June 2005 such that the experimental phase will be completed in August 2005 and a draft report will be issued to the sponsor in September 2005. If a second or subsequent experiments are performed, this time schedule will be amended accordingly. Following comments from the sponsor and from the Covance Laboratories Limited QA Unit a final report will be agreed. Three signed copies (two bound, one unbound) of the final report will be issued.

Should any unforeseen circumstance alter the proposed timescale, the sponsor will be informed immediately. Covance Laboratories Limited cannot guarantee to meet the proposed timescale unless this protocol is approved (by signature and return or by telefax) and the test article received before 27 June 2005.

Following receipt of the signed protocol approval and the test article, a detailed schedule confirming timings of the experimental phase and issue date of the draft report will be sent to the Sponsor.

ARCHIVE STATEMENT

All primary data, or authenticated copies thereof, specimens and the final report will be retained in the Covance Laboratories Limited archives for one year after issue of the final report. At this time the sponsor will be contacted to determine whether the data should be returned, retained or destroyed on their behalf. Sponsors will be notified of the financial implications of each of these options at that time.

Specimens requiring storage deep frozen are specifically excluded from the above. These will be retained for as long as the quality of the material permits evaluation, but for no longer than three months after issue of the final report. The Sponsor will be notified of the intent to destroy samples and any financial implications before specimens are destroyed on their behalf.

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Appendix 1
Study records to be maintained

Definitive protocol
Amendment(s)*
File note(s)*
Study schedule
Report schedule
Study correspondence
Client correspondence
Test article description
Test article formulation assessment*
Dispensary requests
Test article utilisation
Details of formulation preparation
Formulation storage and dispatch*
Analysis of formulations*
Metrology#
Records for reagents and stock solutions#
Culture records
Plate count data
Statistical analysis

some records held centrally

* where appropriate

Appendix 2
Responsible personnel

STUDY MANAGEMENT

Study Director¹

NAME

M Ballantyne

OPERATIONAL SUPERVISION²

Responsible Scientist, Nicotine Determination

A Battle

QUALITY ASSURANCE²

Director, Quality Systems

C Clare

1 = Any change documented by protocol amendment

2 = Any change documented in study records

DISTRIBUTION: Personnel above

PROTOCOL APPROVAL

Please sign both approval pages; return one to the Study Director and retain one for your records.

D Johnson
Study Monitor
Swedish Match

Date

MB

M Ballantyne
Study Director
Covance Laboratories Ltd

14 June 05
Date

J. Clements

J Clements
Head of Genetic and Molecular Toxicology
Covance Laboratories Ltd

14 June 2005
Date

Definitive Protocol

Study Title Extracts of four Snus batches and Kentucky reference moist snuff: Induction of micronuclei in cultured chinese hamster V79 cells

Study Director T S Kumaravel

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Covance Study Number 1138/19

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PROTOCOL APPROVAL

Please sign both approval pages; return one to the Study Director and retain one for your records.

D Johnson
Study Monitor
Swedish Match

M Bar

M Ballantyne
Study Director
Covance Laboratories Ltd

J. Clements

J Clements
Head of Genetic and Molecular Toxicology
Covance Laboratories Ltd

Date

14 June 05

Date

14 June 2005

Date

Appendix 2
Responsible personnel

STUDY MANAGEMENT¹

Study Director

NAME

M Ballantyne

OPERATIONAL SUPERVISION²

Responsible Scientist, Nicotine Determination

A Battle

QUALITY ASSURANCE²

Director, Quality Systems

C Clare

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Appendix 1 Study records to be maintained

Definitive protocol
Amendment(s) *
File note(s) *
Study schedule
Report schedule
Study correspondence
Client correspondence
Test article description
Test article formulation assessment *
Dispensary requests
Test article utilisation
Details of formulation preparation
Formulation storage and dispatch *
Analysis of formulations *
Metrology #
Records for reagents and stock solutions #
Culture records
Plate count data
Statistical analysis

some records held centrally

* where appropriate

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8. Mahon G A T, Green M H L, Middleton B, Mitchell I de G, Robinson W D and Tweats D J (1989) Analysis of data from microbial colony assays. In "Statistical Evaluation of Mutagenicity Test Data". Report of the UKEMS Sub-committee on Guidelines for Mutagenicity Testing, Part III. Ed D J Kirkland. Cambridge University Press, pp 26-65.

A list of study records to be maintained by Covance Laboratories Limited is detailed in Appendix 1.

PROPOSED TIME SCHEDULE

It is estimated that the study will start in July 2005 such that the experimental phase will be completed in August 2005 and a draft report will be issued to the sponsor in October 2005. If a second or subsequent experiments are performed, this time schedule will be amended accordingly. Following comments from the sponsor and from the Covance Laboratories Limited QA Unit a final report will be agreed. Three signed copies (two bound, one unbound) of the final report will be issued.

Should any unforeseen circumstance alter the proposed timescale, the sponsor will be informed immediately. Covance Laboratories Limited cannot guarantee to meet the proposed timescale unless this protocol is approved (by signature and return or by telefax) and the test article received before 27 June 2005.

Following receipt of the signed protocol approval and the test article, a detailed schedule confirming timings of the experimental phase and issue date of the draft report will be sent to the Sponsor.

ARCHIVE STATEMENT

All primary data, or authenticated copies thereof, specimens and the final report will be retained in the Covance Laboratories Limited archives for one year after issue of the final report. At this time the sponsor will be contacted to determine whether the data should be returned, retained or destroyed on their behalf. Sponsors will be notified of the financial implications of each of these options at that time.

Specimens requiring storage deep frozen are specifically excluded from the above. These will be retained for as long as the quality of the material permits evaluation, but for no longer than three months after issue of the final report. The Sponsor will be notified of the intent to destroy samples and any financial implications before specimens are destroyed on their behalf.

Evaluation criteria

A test article will be considered to be mutagenic in this assay if:

1. the assay is valid (see above)
2. Dunnett's test gives a significant response ($p \leq 0.01$) and the data set(s) shows a significant dose correlation
3. If more than one experiment is performed, for a test article to be considered mutagenic the positive trends/effects described above should be reproducible.

GLP COMPLIANCE

The study will be performed in compliance with:

United Kingdom Good Laboratory Practice Regulations 1999, Statutory Instrument No. 3106 as amended by the Good Laboratory Practice (Codification Amendments Etc.) Regulations, 2004.

OECD Principles of Good Laboratory Practice (revised 1997, issued Jan 1998) ENV/MC/CHEM (98) 17.

All procedures will be performed in accordance with Covance Laboratories Limited standard operating procedures (SOPs). The study will be subject to Quality Assurance evaluation by the Covance Laboratories Limited Quality Assurance Unit (QAU) in accordance with SOPs. Where appropriate, any change to this protocol will be made by an amendment issued in agreement with the Sponsor.

Following completion of the study a draft report will be issued. Client comments should be supplied for inclusion into a final document within six months of receipt of the draft document.

In accordance with the requirements of the UK Medicines and Healthcare Products Regulatory Agency GLP Monitoring Authority, if no client comments are received within six months of issue, the report will be finalised. Any further changes after this time will be addressed as an amended final report, which may result in additional costs.

Colony counting

Colonies are usually counted electronically using an appropriate automated colony counter, having inspected the background lawn for signs of toxicity. However, plates may be scored manually (for example those exhibiting precipitate or contamination). Plates bearing many microcolonies may have a representative number of these colonies streaked on to Vogel-Bonner E agar plates to check whether or not they are true revertants.

Analysis of results

Treatment of data

Individual plate counts from each extract in each experiment will be recorded separately and the mean and standard deviation of the plate counts for each treatment will be determined. Control counts will be compared with the accepted normal ranges for our laboratory for numbers of spontaneous revertants on solvent control plates and numbers of induced revertants on positive control plates. The ranges that will be quoted are based on a large volume of historical control data accumulated from experiments where the correct strain and assay functioning were considered to have been confirmed. Data for our laboratory are consistent with ranges of spontaneous revertants per plate considered acceptable elsewhere (6).

For evaluation of test article and positive control data there are many statistical methods in use, and several are acceptable (7,8). The m-statistic will be calculated to check that the data are Poisson-distributed, and the Dunnett's test will be used to compare the counts at each dose with the control. The presence or otherwise of a dose response will be checked by linear regression analysis (8).

Acceptance criteria

The assay will usually be considered valid if the following criteria are met:

1. the negative control counts fall within the normal ranges
2. the positive control chemicals induce clear increases in revertant numbers confirming discrimination between different strains, and an active S-9 preparation
3. no more than 5% of the plates are lost through contamination or some other unforeseen event.

METHODS

The test system will be suitably labelled (using a colour-coded procedure) to clearly identify the study number, test article, positive and negative control groups.

Toxicity range-finder experiment

Based on experimentation performed in CLE study number 1138/16, no separate toxicity range-finder experiment was considered to be required in this study, as little or no dose-limiting toxic effects are expected.

Mutation experiment

Extracts of four Snus batches and Kentucky reference moist snuff will be tested for mutation in five strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102) initially in a single experiment (although one or more further experiments may be conducted). Appropriate concentrations will be used, employing triplicate plates with or without S-9. Negative (solvent) and positive controls will be included in quintuplicate and triplicate respectively with and without S-9. These platings will be achieved by the following sequence of additions to pre-incubation tubes:

- 0.1 mL of bacterial culture
- 0.5 mL (water extract) or 0.1 mL (DMSO extract) of test article extract or control
- 0.5 mL of 10% S-9 mix or buffer solution
- 0.5 mL of sodium phosphate buffer (added for DMSO extract treatments only)

The pre-incubation mixtures will be incubated for 60 minutes, with shaking, at $37\pm 1^\circ\text{C}$. After this time either 2.5 mL of molten 0.9% agar (for water extracts) or 2 mL of molten 1.125% agar (for DMSO extracts) at $46\pm 1^\circ\text{C}$ is added to each tube, followed by rapid mixing and pouring on to Vogel-Bonner E agar plates. When set, the plates will be inverted and incubated at $37\pm 1^\circ\text{C}$ in the dark for 2 to 3 days. The plates will be examined for signs of toxicity (see Colony counting).

Following Experiment 1, results will be communicated to the Sponsor. At this point, a decision will be made in conjunction with the Sponsor on whether a second or further experiment is to be performed, and the conditions/methodology to be employed.

Treatments will be carried out both in the absence and presence of a 10% S-9 mix, according to the following table (per 100 mL mix):

Ingredient	Concentration	Quantity (mL)	
		10% S-9 mix	Buffer solution
Sodium phosphate buffer pH 7.4	500 mM	20	20
Glucose-6-phosphate (disodium)	180 mg/mL	0.845	!
NADP (disodium)	25 mg/mL	12.6	!
Magnesium chloride	250 mM	3.2	!
Potassium chloride	150 mM	22	!
L-histidine HCl (in 250 mM MgCl ₂)	1 mg/mL	4	4
d-biotin	1 mg/mL	4.88	4.88
S-9	as detailed above	10	!
Water	!	to volume	to volume

Initial experimentation will include a pre-incubation step, in which bacteria, test article or control solutions and buffer solution or S-9 mix will be incubated for 60 minutes, with shaking, in the absence of agar before plating. If the first experiment gives negative or equivocal results, a second experiment may be conducted following consultation and agreement with the Sponsor, which may either retain this pre-incubation methodology, or employ a plate incorporation methodology where bacteria, test article or control solutions and buffer solution or S-9 mix are mixed directly with agar and plated immediately.

Bacteria

Five strains of *Salmonella typhimurium* bacteria (TA98, TA100, TA1535, TA1537 and TA102) will be used in this study. All the tester strains, with the exception of strain TA102, were originally derived from cultures obtained from the UK NCTC. Strain TA102 was derived from a culture obtained from Glaxo Group Research Limited. For all assays, bacteria will be cultured for 10 hours in nutrient broth, containing ampicillin (TA98, TA100) or ampicillin and tetracycline (TA102) as appropriate. Incubation will be carried out in a shaking incubator, set to turn on using a timer switch. Treatments will commence within 2 hours of the end of the period of incubation. The inocula will be taken from master plates or vials of frozen cultures, which have been checked for strain characteristics (histidine dependence, *rfa* character, *uvrB* character and resistance to ampicillin or ampicillin plus tetracycline). Checks will be carried out according to Maron and Ames (3) and De Serres and Shelby (6).

Controls

Negative control treatments will comprise additions at the same volume per plate (0.5 mL for water extracts, 0.1 mL for DMSO extracts) as the test article solutions, and all positive control treatments will comprise 0.1 mL additions of the appropriate stock positive control solution. Where two or more test articles are tested in parallel, common control treatments may be shared between test articles. Negative controls will comprise treatments with blank extract solvent (extract solvent subjected to the extraction conditions, but in the absence of test material). The positive control chemicals are supplied and will be used as shown in the following table:

Chemical	Source *	Stock ** concentration (µg/mL)	Final concentration (µg/plate)	Use	
				Strain(s)	S-9
2-nitrofluorene (2NF)	Sigma-Aldrich Chemical Co, Poole, UK	50	5.0	TA98	-
Sodium azide (NaN ₃)	Sigma-Aldrich Chemical Co, Poole, UK	20	2.0	TA100, TA1535	-
9-aminoacridine (AAC)	Sigma-Aldrich Chemical Co, Poole, UK	500	50.0	TA1537	-
Mitomycin C (MMC)	Sigma-Aldrich Chemical Co, Poole, UK	2	0.2	TA102	-
Benzo[a]pyrene (B[a]P)	Sigma-Aldrich Chemical Co, Poole, UK	100	10.0	TA98	+
2-aminoanthracene (AAN)	Sigma-Aldrich Chemical Co, Poole, UK	50	5.0	TA100, TA1535, TA1537	+
		200	20.0	TA102	+

* Source of supply as indicated, or a preparation of equivalent standard

** All stock solutions will be prepared in water (NaN₃ and MMC), or in anhydrous analytical grade dimethyl sulphoxide (2NF, AAC, AAN and B[a]P). All stock solutions will be stored in aliquots at 1-10°C in the dark, with the exception of B[a]P and MMC which will be stored in aliquots at -80°C in the dark

Metabolic activation system

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation is obtained from Molecular Toxicology Incorporated, USA, where it is prepared from male Sprague Dawley rats induced with Aroclor 1254. Batches of MolTox™ S-9 are stored frozen in aliquots at -80°C prior to use (5). Each batch is checked by the manufacturer for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufin O-dealkylase activities). The quality control statement, relating to the batch of S-9 preparation used, will be included in the report.

Where extracts are prepared in two or more separate flasks for a Snus/moist snuff batch, extracts for use in each experiment will be pooled prior to use in the assay.

Vials of frozen extract to provide the volume of extract required will be thawed on the day of assay. 0.5 mL additions of water extracts will normally be used per plate, or 0.1 mL additions of DMSO extract. A series of at least five doses will usually be separated by five-fold intervals in Experiment 1, but will be in a closer range near to the top limit (toxicity, solubility or 5000 µg/plate) in Experiment 2 (if performed). The treatment concentrations that will be used for a freely soluble non-toxic test article, and means of achieving final concentrations are given in the following table:

Experiment	Concentration of extract (mg/mL equivalent)	Final concentration (µg/plate equivalent)	
		Water extracts ^a	DMSO extracts ^b
Experiment 1	0.16	80	16
	0.80	400	80
	4.00	2,000	400
	20.00	10,000	2,000
	100.00	50,000	10,000
	500.00*	250,000	50,000
Experiment 2	5.12	2,560	512
	12.80	6,400	1,280
	32.00	16,000	3,200
	80.00	40,000	8,000
	200.00	100,000	20,000
	500.00*	250,000	50,000

* Neat extract - all lower concentrations achieved by dilutions of this extract concentration

a extracts tested at 0.5 mL volume additions per plate

b extracts tested at 0.1 mL volume additions per plate

Solubility restrictions or toxicity to the test bacteria may result in alternative dose ranges being used, and these will be confirmed by a note to file.

Nicotine content determination

The nicotine content of the test article extracts will be performed using HPLC analysis. For each test article (extract), nicotine determination will be performed on samples from up to five extraction flasks (if more than five flasks are used to generate extract, samples from only five representative flasks will be analysed), together with a sample from the pooled extract to be used in the mutation assay. Details of the analytical method will be provided by amendment to protocol. Results from these analyses will be presented in the report as achieved nicotine concentrations.

All test articles are handled as though they were potential carcinogens and appropriate safety procedures are employed.

Determinations of the stability and characteristics of the test article, where defined in GLP regulations, are the responsibility of the sponsor. Archiving a sample of the test article will be the responsibility of the Sponsor.

Extracts will be performed based on the methodology evaluated and developed in CLE study number 1138/16, and conducted using sterile containers and solutions, in order to minimise any contamination from external sources:

- Appropriate numbers of pouches/sachets of moist snuff/Snus will be cut approximately in half, and both the contents and the packaging (pouches/sachets) weighed and mixed with appropriate volumes of sterile purified water to produce the required w/v concentration. If the tobacco is not finely divided then brief homogenisation may be performed.
- Extractions will be performed for 24 hours at 37°C, with shaking.
- At the end of the 24 hour extraction period, extracts will be centrifuged at approximately 1800 x 'g' for 30 minutes, and heavy particulates removed by decanting off the supernatant.
- The supernatant will then be centrifuged at 25,000 x 'g' for 30 minutes, and fine particulates removed by decanting off the supernatant.
- The final supernatant will be adjusted to pH to 7.4 ± 0.2 with Hydrochloric acid (water extracts only)
- The resulting extracts will be filter sterilised using a 0.2 µm pore size filters (pre-filtering using a larger pore size filter may be performed).
- Aliquots of extracts will be stored at approximately -80°C, and used for up to 6 months after the extraction process is performed.

All Snus/moist snuff batches will be extracted in purified water at a concentration of 500 mg/mL equivalent, and CDM2 extracts at this concentration in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) will also be prepared for use in this study. Based on experimentation conducted in CLE study number 1138/16, this concentration was the highest extraction concentration that could practicably be extracted from material of this nature, and therefore gives the highest achievable extract concentration.

histidine. Different tester strains are used because each is mutated by particular chemical classes of compound. A compound that is mutagenic in one strain need not be so in another (4).

Swedish moist snuff (Snus) is a commercially available tobacco product which may be considered for use as a smoking cessation product, and is undergoing safety evaluation testing. As part of this process, testing is scheduled to be performed in a number of *in vitro* genotoxicity assay systems. As Snus is an insoluble solid, to enable exposure of this test material to the cells in the assay system, as is commonly used for *in vitro* testing of other smokeless tobacco products, an extract of the test material will be used. The extraction methodology and concentrations employed for this study have been demonstrated in previous experimentation (CLE study number 1138/16) to provide the highest practicable extraction concentration for these materials, and therefore provide the most stringent assessment of these materials possible in this assay system.

This protocol is designed to meet the known requirements of OECD Guideline 471 (adopted 1997), EEC Annex V Tests B 13/14 (2000) and UKEMS Guidelines (1990), providing either a positive result is obtained, or a second experiment is performed. 2

MATERIALS

Test articles

The test articles will be Extracts of four Snus batches and Kentucky reference moist snuff.

Swedish Moist Snuff (Snus) G

Swedish Moist Snuff (Snus) CPS

Swedish Moist Snuff (Snus) CDM

Swedish Moist Snuff (Snus) CDM2

Kentucky Reference Moist Snuff, Batch 2S3

The sponsor will normally be expected to supply sufficient material to permit sufficient extract volumes to perform all testing required for this study. The sponsor will also supply as much information as possible regarding characterisation of the Snus/moist snuff, such as composition analysis, expiry date, storage conditions etc.

OBJECTIVE

To evaluate the mutagenic activity of Extracts of four Snus batches and Kentucky reference moist snuff by examining their ability to revert five histidine-requiring strains of *Salmonella typhimurium* in the absence and in the presence of a rat liver metabolising system (S-9).

TEST SYSTEM

The objective of the *Salmonella*/microsome assay is to evaluate the mutagenic potential of a test article by its effects on one or more histidine-requiring strains of *Salmonella typhimurium* in the absence and presence of a liver metabolising system (1). The assay is a rapid, reliable and economical method for screening compounds of potential genetic activity at the nucleotide level. A large database has been accumulated with this assay, confirming its ability to detect genetically active compounds of most chemical classes with around 80 to 90% sensitivity and specificity (2).

The following bacterial strains will be used in this study:

Organism	Strain	Type of mutation in the histidine gene
<i>S. typhimurium</i>	TA98	frame-shift
<i>S. typhimurium</i>	TA100	base-pair substitution
<i>S. typhimurium</i>	TA1535	base-pair substitution
<i>S. typhimurium</i>	TA1537	frame-shift
<i>S. typhimurium</i>	TA102	base-pair substitution

With the exception of strain TA102, these strains require biotin as well as histidine for growth. In strain TA102 the critical mutation in the histidine gene is located on a multicopy plasmid pAQ1. This strain is particularly sensitive to the activities of oxidative and cross-linking mutagens. The pKM101 plasmid derivatives TA98, TA100 and TA102 also have increased sensitivity to certain mutagens as the pKM101 plasmid codes for an error-prone DNA repair system (3).

When exposed to a mutagen, some of the bacteria in the treated population, through chemical interaction with the compound, undergo genetic changes which cause them to revert to a non-histidine-requiring state and thus grow in the absence of exogenous

Definitive Protocol

Study Title	Extracts of four Snus batches and Kentucky reference moist snuff: reverse mutation in five histidine-requiring strains of <i>Salmonella typhimurium</i>
Study Director	M Ballantyne
Sponsor	Swedish Match SE-11885 Stockholm SWEDEN
Study Monitor	David Johnson Swedish Match North America
Test Facility	Covance Laboratories Ltd Otley Road, Harrogate North Yorkshire HG3 1PY ENGLAND
Covance Study Number	1138/17
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OBJECTIVES

To evaluate the clastogenic and aneugenic potential of a test article by its effects on the frequency of micronuclei in cultured Chinese hamster V79 cells treated in the absence and presence of a rat liver metabolising system.

TEST SYSTEM

Assays for the detection of chromosome damage in mammalian cells cultured *in vitro* are recommended in regulatory guidelines as a complement to Ames tests in a genotoxicity test battery (1). Although there are a number of assays that detect genotoxic chemicals, there are relatively few that detect chemicals which either interfere with the process of mitosis or cause chromosome aberrations. No one assay has been extensively evaluated on the same compounds in several laboratories but there is a large database on the use of chromosomal assays for screening purposes (2). Experiments with V79 cells can also be performed in conjunction with a rat liver metabolising system since, for short incubation periods, no toxicity is induced by the liver homogenate itself.

An alternative to measuring structural aberrations in mitotic cells is to measure micronuclei. These are produced from whole chromosomes or acentric fragments that are unable to attach to the spindle at mitosis and appear during the next interphase as small darkly staining bodies adjacent to the main daughter nucleus. These are more easily counted than structural aberrations at mitosis and analysis can be performed rapidly on large numbers of cells (3). The technique as described suffers a disadvantage, however, because the production of a micronucleus is dependent on nuclear division thus the frequency of micronuclei will vary with the proportion of cells which divide. This problem is solved if cytochalasin B is added to the cultures (3, 4). Cytochalasin B inhibits cytokinesis (cell division) but not karyokinesis (nuclear division) resulting in the formation of binucleate cells. If micronuclei are only counted in binucleate cells then a true measurement of their induction can be obtained.

The micronucleus test also allows one to distinguish between aneugenic and clastogenic events by using fluorescence *in situ* hybridization (FISH) or immuno-staining with anti-kinetochore antibodies (5, 6). If an increase in the frequency of micronucleated cells is observed in this study then provision may be made to further investigate this effect by determining the relative proportions of

micronuclei formed by clastogenic and aneugenic events using anti-kinetochore antibodies. This identifies micronuclei which carry centromeres (thus comprise whole chromosomes) and the data can be used to evaluate any aneugenic potential. If this additional analysis is required, it will be addressed in a protocol amendment.

In the experiment, cells will be exposed to a test article both in the absence and presence of S-9 (Aroclor-induced) for 3 hours, and sampled at 20 hours after the beginning of treatment. This is equivalent to approximately one and a half times the average generation time of the V79 cells used in this laboratory. Additionally, as a number of chemicals have been reported as only exerting positive effects following prolonged treatment, a continuous treatment for 20 hours in the absence of S-9 will also be included.

In this study, the clastogenic and aneugenic potential of a test article will be assessed by its effects on the chromosomes of V79 cells, cultured *in vitro* and treated in the absence and presence of a rat liver metabolising system (S-9).

Swedish moist snuff (Snus), a commercially available smokeless tobacco product, may be considered for use as a smoking cessation product, and is undergoing safety evaluation testing. As part of this process, testing is scheduled to be performed in a number of *in vitro* genotoxicity assay systems. As Snus is an insoluble solid, to enable exposure of this test material to the cells in the assay system, as is commonly used for *in vitro* testing of other smokeless tobacco products, an extract of the test material will be used. The extraction methodology and concentrations employed for this study have been demonstrated in previous experimentation (CLE study number 1138/16) to provide the highest practicable extraction concentration for these materials, and therefore provide the most stringent assessment of these materials possible in this assay system.

Although no definitive regulatory guidelines exist for this assay, the test methodology is in accordance with current literature and accepted scientific/regulatory principles described in current guidelines for clastogenicity testing *in vitro* (7, 8, 9, 10).

MATERIALS

Test article

The test articles will be Extracts of four Snus batches and Kentucky reference moist snuff.

Swedish Moist Snuff (Snus) G

Swedish Moist Snuff (Snus) CPS

Swedish Moist Snuff (Snus) CDM

Swedish Moist Snuff (Snus) CDM2

Kentucky Reference Moist Snuff, Batch 2S3

The sponsor will normally be expected to supply sufficient material to permit sufficient extract volumes to perform all testing required for this study. The sponsor will also supply as much information as possible regarding characterisation of the Snus/moist snuff, such as composition analysis, expiry date, storage conditions etc.

All test articles are handled as though they were potential carcinogens and appropriate safety procedures are employed.

Determinations of the stability and characteristics of the test article, where defined in GLP regulations, are the responsibility of the sponsor. Archiving a sample of the test article will be the responsibility of the Sponsor.

Extracts will be performed based on the methodology evaluated and developed in CLE study number 1138/16, and conducted using sterile containers and solutions, in order to minimise any contamination from external sources:

- Appropriate numbers of pouches/sachets of moist snuff/Snus will be cut approximately in half, and both the contents and the packaging (pouches and sachets) weighed and mixed with appropriate volumes of sterile purified water or DMSO to produce the required w/v concentration. If the tobacco is not finely divided then brief homogenisation may be performed.
- Extractions will be performed for 24 hours at 37°C, with shaking
- At the end of the 24 hour extraction period, extracts will be centrifuged at approximately 1800g for 30 minutes, and heavy particulates removed by decanting off the supernatant

- The supernatant will then be centrifuged at 25,000g for 30 minutes, and fine particulates removed by decanting off the supernatant
- The final supernatant will be adjusted to pH 7.4±0.2 with Hydrochloric acid (water extracts only)
- The resulting extracts will be filter sterilised using a 0.2 µm pore size filter (pre-filtering using a larger pore size may be performed)
- Aliquots of extracts will be stored at approximately -80°C, and used for up to 6 months after the extraction process is performed.

All Snus/moist snuff batches will be extracted in purified water at a concentration of 500 mg/mL equivalent, and CDM2 extracts at this concentration in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) will also be prepared for use in this study. Based on experimentation conducted in CLE study number 1138/16, this concentration was the highest extraction concentration that could practicably be extracted from material of this nature, and therefore gives the highest achievable concentration.

Where extracts are prepared in two or more separate flasks for a Snus/moist snuff batch, extracts for use in each experiment will be pooled prior to use in the assay.

Vials of frozen extract to provide the volume of extract required will be thawed on the day of assay, and appropriate dilutions performed for use in the assay.

Test article additions to the assay will be made at 10% (water extracts), 1% (DMSO extracts) of the final culture volume, these being the highest volume additions that are possible in this assay system. Osmolality of the medium may be measured, as fluctuations in osmolality of more than 50 mOsm/kg have been responsible for increased mutation. Similarly as extremes in pH should also be avoided, pH assessment of post-treatment medium may be made.

Initially, a preliminary toxicity range-finder experiment will be performed using a series of doses separated by no more than two-fold intervals, ranging down from the upper limit.

An example of the dose ranges that may be tested is provided in the following table:

Treatment	Concentration of extract (mg/mL equivalent)	Final concentration (µg/mL equivalent)	
		Water extracts ^a	DMSO extracts ^b
3 +17 hour treatment - or + S-9	50.00	5,000	750
	100.00	10,000	1,500
	200.00	20,000	3,000
	300.00	30,000	4,500
	400.00	40,000	6,000
	500.00*	50,000	7,500
20+0 hour treatment - S-9	100.00	10,000	1,000
	200.00	20,000	2,000
	300.00	30,000	3,000
	400.00	40,000	4,000
	450.00	45,000	4,500
	500.00*	50,000	5,000

* Neat extract - all lower concentrations achieved by dilutions of this extract concentration

a Test article additions as 10% of final culture volume

b Test article additions as 1% of final culture volume

If alternative dose ranges are used, these will be noted in the raw data.

When possible, stock test solutions will be filter-sterilized.

Concentrations for the main experiment will be based on the toxicity data from the cytotoxicity range-finder experiment.

Controls

Negative controls will comprise treatments with the chosen solvent diluted to the same extent as the test article solutions.

Untreated controls will be included should the selected solvent control be one that is not commonly used in this laboratory.

The positive control chemicals will be prepared (for the main experiment only) in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) as shown in the following table:

Chemical	Supplier*	Concentration of treatment solution (mg/mL)	Final concentration** (µg/mL)	S-9
Cyclophosphamide (CPA)	Sigma Chemical Co, Gillingham UK	0.6000	6.000	+
		0.8000	8.000	+
4-nitroquinoline 1-oxide (NQO)	Sigma Chemical Co, Gillingham UK	0.0125	0.125	-
		0.0250	0.250	-

* Source of supply as indicated, or a preparation of equivalent standard

** The treatments giving satisfactory responses in terms of quality and quantity of mitoses and extent of chromosomal damage will be analysed

Positive controls will not be included for 20+0 hour –S-9 treatments unless these are performed on a separate occasion to the 3+17 hour –S-9 treatments.

Metabolic activation system

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation is obtained from Molecular Toxicology Incorporated, USA, where it is prepared from male Sprague Dawley rats induced with Aroclor 1254. The batches of MolTox™ S-9 are stored frozen in aliquots at -80°C prior to use. Each batch is checked by the manufacturer for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufin O-dealkylase activities). The quality control statement(s), relating to the batch(es) of S-9 preparation used, will be included in the report.

Treatment will be carried out both in the absence and presence of S-9, prepared in the following way.

Glucose-6-phosphate, 0.2 M (61 mg/mL), NADP, 0.04 M (32 mg/mL), KCl, 1 M (74.55 mg/mL), MgCl₂·6H₂O, 0.25 M (18.64 mg/mL), NaHPO₄, 0.2 M (24 mg/mL), water and rat liver S-9 will be mixed as in the example given in the table below to achieve a 10% S-9/Co-factor mix.

Chemical	Molarity (M)	Concentration (mg/mL)	Volume (mL)
Glucose-6-phosphate	0.2	61.00	0.3125
NADP	0.04	32.00	1.25
KCl ₁	1.0	74.55	0.4125
MgCl ₂ 6H ₂ O	0.25	18.64	0.40
NaHPO ₄	0.20	24.00	6.25
Water	-	-	2.625
S-9	-	-	1.25

An aliquot of the resulting S-9 mix will be added to each cell culture to achieve the required final concentration of the test article in a total of 10 mL. The final concentration of the liver homogenate in the test system will be 1%.

Cell cultures

V79 cells, supplied by Dr Eian Massey, BAT, Southampton, England, are maintained at Covance Laboratories Limited in tissue culture flasks containing DMEM medium including 10% (v/v) foetal calf serum (FCS), 1% glutamine and 0.52% Penicillin/Streptomycin. They will be subcultured regularly at low density, and before overgrowth occurs, to maintain low aberration frequencies. Stocks of cells preserved in liquid nitrogen will be reconstituted for each experiment so as to maintain karyotypic stability. The cells are routinely screened for mycoplasma contamination.

Cell sheets will be removed from stock cultures using Trypsin/EDTA solution, and subcultured at a low to medium density (approximately between $1-6 \times 10^5$ cells per flask) into 75 sq cm tissue culture flasks. Cells will be passaged at least once prior to treatment.

On the day prior to treatment cells will be removed from stock cultures using Trypsin/EDTA solution, and subcultured at a density of approximately $5-7 \times 10^5$ cells per flask into 25 sq cm tissue culture flasks. The final volume of culture medium in each flask (following completion of treatment) will be 10 mL. Pre-treatment volumes may vary dependent on the volume additions required following solubility assessment. Flasks will be gassed with 5% (v/v) CO₂ in air for approximately 10-20 seconds, sealed and incubated at 37°C until treatment. Cultures at a suitable level of confluence will be selected for treatment.

METHODS

Treatment

The test system will be suitably labelled (using a colour-coded procedure) to clearly identify the study number, experiment number, treatment time, test article concentration (if applicable), positive and negative controls. S-9 mix or KCl will be added (1.0 mL per culture).

In the cytotoxicity range-finder experiment, duplicate cultures will be treated with the solvent and single cultures treated with the test article at appropriate concentrations.

In the main experiment, quadruplicate cultures (A,B,C, and D) are treated with the solvent, and duplicate cultures treated with the test article at appropriate concentrations. Additional sets of duplicate cultures are treated with the positive control chemicals.

Final post-treatment volume will be 10 mL for all experiments.

Treatment will comprise a 3 hour (pulse) treatment + and - S-9 followed by a 17 hour recovery period (3+17) and a 20 hour (continuous) treatment in the absence of S-9.

Summary of treatment conditions

Treatment	S-9	Duration of treatment (hours)	Harvest time (hours after start of treatment)
Continuous	20+0	-	20
Pulse	3+17	-	20
	3+17	+	20

Cultures receiving continuous treatment will retain treatment medium through to harvest. Pulse treatments will be for 3 hours only. Treatment medium will then be removed, cell monolayers washed twice (at least) with HBSS (at room temperature). Cultures will then be re-fed with fresh DMEM medium containing heat inactivated FCS (10%), 0.52% Penicillin/Streptomycin and Cytochalasin B (at a final concentration of 3 µg/mL).

In addition, Cytochalasin B, formulated in DMSO will be added to all continuous 20+0 hour treatment flasks (0.1 mL) to give a final concentration of 3 µg/mL.

All culture flasks will then be gassed with 5% (v/v) CO₂ in air for approximately 10-20 seconds prior to returning to an incubator at 37°C.

Harvesting

At the defined sampling time, the monolayers of these cultures will be removed using Trypsin/EDTA.

The suspension from each flask will be transferred to a plastic centrifuge tube containing fresh medium and an aliquot will be taken for determination of cell number using a Coulter Counter. The remaining suspension will be stored at room temperature prior to slide preparation.

Preparation of microscope slides

A further aliquot of the cell suspension will be removed for slide preparation and the cell concentration adjusted to approximately 6×10^4 cells/mL. Slides labelled with appropriate study details (eg study number, experiment number, treatment, replicate and date of preparation) will be loaded into a Cytospin[®] cytocentrifuge and 250 µL of cell suspension from the corresponding culture will be used per slide (where double funnel slides are used, 250 µL per funnel). One (range-finder experiment) or two (main study experiment) slides will be prepared per culture. The samples will be spun at 1000 rpm (approximately 110 x 'g') for 5 minutes at high acceleration. When the cycle is complete the slides are removed and allowed to air dry. Slides will then be fixed by immersion into a bath of ice-cold 90% methanol for 9 minutes. Slides will then be air-dried and stored either at -20°C (if using for anti-kinetochore staining) or at room temperature (if acridine orange staining).

Slides will then be stained by immersion in 125 µg/mL Acridine Orange in phosphate buffered saline (PBS), pH 6.8 for approximately 10 seconds. Slides will then be washed with PBS (with agitation) for a few seconds before transfer and immersion in a second container of PBS for approximately 8-10 minutes. The quality of the stain will then be checked. If slides appear over-stained (leaching of orange colouration), slides will be returned to the PBS bath for a further 10 minutes and the check repeated. Slides will then be air-dried and stored in the dark at room temperature prior to analysis.

Prior to analysis a minimal volume of PBS will be added to the slides before mounting with glass coverslips.

Selection of doses for main experiment

Slides (from the cytotoxicity range-finding experiment) will initially be examined, uncoded, for proportions of mono-, bi- and multinucleate cells, to a minimum of 200 cells per concentration. From these data the cytokinesis-block proliferation index (CBPI) will be determined using the formula described below. A suitable range of concentrations will be selected for the main experiment based on these toxicity data.

Selection of doses for micronucleus analysis

Slides from the main study experiments will be examined, uncoded, for proportions of mono-, bi- and multinucleate cells, to a minimum of 500 cells per culture. From these data the replication index (RI) will be determined using the following formula:

Replication Index (RI) indicates the relative number of nuclei compared with controls:

$$RI = \frac{\text{number binucleate cells} + 2 (\text{number multinucleate cells}) / \text{total number of cells counted in treated cultures}}{\text{number binucleate cells} + 2 (\text{number multinucleate cells}) / \text{total number of cells counted in controls}} \times 100$$

This indicates a value relative to the control. Expressed as a percentage cytotoxicity, the value is: $100 - RI = \% \text{Cytotoxicity}$

A selection of random fields will be observed from enough treatments to determine whether chemically induced cell cycle delay or cytotoxicity has occurred.

Doses for analysis will be selected based on RI determinations. The highest dose for micronucleus analysis from cultures sampled at 24 hours should be one at which at least 60% (approximately) cytotoxicity has occurred or should be the highest dose tested as illustrated in the Test article section. Analysis of slides from highly cytotoxic concentrations will be avoided, if possible. Slides from cultures treated with heavily precipitating doses will be checked to confirm that the presence of precipitate does not preclude analysis. Slides from the highest selected dose and a minimum of two lower doses, such that a range of toxicity from maximum to little or none is covered, will be taken for microscope analysis.

The rationale for the limit of approximately 60% cytotoxicity is based on limited data (discussed by the IWGTP (14)) which show that in some cases (i.e. some aneugens), a very steep toxicity curve is observed and very closely spaced doses in the range of 50-60% toxicity need to be evaluated. Data from certain validation experiments have demonstrated that the lowest observed effective dose (LOEDs) for the aneugens

diethylstilbestrol and vincristine showed a relative cell count (RCC) of 42% and 43% respectively; this corresponding to a toxicity of approximately 60%. These compounds might not have been found to be micronucleus inducers if they had not been tested up to the 60% toxicity level.

For each treatment regime, two solvent control cultures will initially be analysed for micronuclei. Slides from the remaining solvent control cultures (C and D) will only be analysed for micronuclei if necessary to help resolve an equivocal result or increased frequencies of micronuclei are observed in solvent controls, in either case the Sponsor will be contacted.

Slides from any untreated control cultures will only be analysed if solvent control cultures exhibit frequencies of micronuclei which exceed the historical negative control range.

A single positive control dose level, which gives satisfactory responses in terms of quality and quantity of binucleate cells and extent of micronucleated binucleate cells, will be analysed (see Controls section).

Slide analysis

Acceptance criteria

Binucleate cells will only be included in the analysis if all of the following criteria are met:

1. the cytoplasm has remained essentially intact, and
2. the daughter nuclei are of approximately equal size, and
3. any micronuclei present are separate from the main nucleus (micronuclei may be touching the main nucleus but must have clear boundaries).

Procedure

Slides from the positive control treated cultures will initially be examined for micronuclei to ensure that the system has responded satisfactorily. Slides from the selected treatments and from solvent and positive controls will then be coded, using randomly generated letters, by a person(s) not connected with the scoring of the slides. Labels will be used to cover any treatment details on the slides, so that the analyst(s) can only see the study reference number, experiment number and the code.

Analysis will be performed using fluorescence microscopy. Nuclei and micronuclei will appear as a bright orange/yellow colour and the cytoplasm as a dull red colour.

Where possible, one thousand binucleate cells from each replicate (2000 per dose level) will be analysed for micronuclei. Observations will be recorded on raw data sheets and the microscope stage co-ordinates of the first six cells containing one or more micronuclei will be recorded.

Slide analysis will be performed by analysts trained in accordance with Covance Laboratories Limited Standard Operating Procedures. Details of the analysts will be recorded in the raw data and included in the final report. All slides and raw data will be returned to Covance Laboratories Limited for archiving in accordance with the Archive statement.

Analysis of results

Treatment of data

After completion of scoring and decoding of slides, the numbers of binucleate cells with micronuclei in each culture will be obtained and tabulated in the report.

The proportions of micronucleated cells in each replicate will be used to establish acceptable homogeneity between replicates by means of a binomial dispersion test (13).

The proportion of cells with micronuclei for each treatment condition will be compared with the proportion in negative controls by using Fisher's exact test (13). Probability values of $p \leq 0.05$ will be accepted as significant.

Acceptance criteria

The assay will usually be considered valid if the following criteria are met:

1. the binomial dispersion test demonstrates acceptable heterogeneity between replicate cultures, particularly where no positive responses are seen, and
2. the positive control chemicals induce statistically significant increases in the proportion of cells with micronuclei.

3. Negative control cultures should exhibit a proportion of binucleate cells which is at least 50% of the total cell population.

Acceptance under any other criteria would be discussed in the report.

Evaluation criteria

A test article will be considered as positive in this assay if:

1. A statistically significant increase in the proportion of cells with micronuclei occurs at one or more concentrations.
2. The proportion of micronucleated binucleate cells (MNBN) at these concentrations exceeds the current historical negative control (normal) range in both replicate cultures.

Good scientific judgement will be used in the interpretation of the results.

Confirmation of positive results may be discussed with the Sponsor prior to completion of the study.

GLP COMPLIANCE

The study will be performed in compliance with:

United Kingdom Good Laboratory Practice Regulations 1999, Statutory Instrument No. 3106 as amended by the Good Laboratory Practice (Codification Amendments Etc.) Regulations, 2004.

OECD Principles on Good Laboratory Practice (revised 1997, issued Jan 1998) ENV/MC/CHEM (98) 17.

All procedures will be performed in accordance with Covance Laboratories Limited standard operating procedures (SOPs). The study will be subject to Quality Assurance evaluation by the Covance Laboratories Limited Quality Assurance Unit (QAU) in accordance with SOPs. Where appropriate, any change to this protocol will be made by an amendment issued in agreement with the Sponsor.

In accordance with the requirements of the UK Medicines and Healthcare Products Regulatory Agency GLP Monitoring Authority, if no client comments are received within six months of issue, the report will be finalised. Any further changes after this time will be addressed as an amended final report, which may result in additional costs.

A list of study records to be maintained by Covance Laboratories Limited is detailed in Appendix 1.

PROPOSED TIME SCHEDULE

It is estimated that the study will start in June 2005 such that the experimental phase will be completed in August 2005 and a draft report will be issued to the sponsor in September 2005. If a second or subsequent experiments are performed, this time schedule will be amended accordingly. Following comments from the sponsor and from the Covance Laboratories Limited QA Unit a final report will be agreed. Three signed copies (two bound, one unbound) of the final report will be issued.

Should any unforeseen circumstance alter the proposed timescale, the sponsor will be informed immediately. Covance Laboratories Limited cannot guarantee to meet the proposed timescale unless this protocol is approved (by signature and return or by telefax) and the test article received before 27 June 2005.

Following receipt of the signed protocol approval and the test article, a detailed schedule confirming timings of the experimental phase and issue date of the draft report will be sent to the Sponsor.

ARCHIVE STATEMENT

All primary data, or authenticated copies thereof, specimens and the final report will be retained in the Covance Laboratories Limited archives for **one** year after issue of the final report. At this time the Sponsor will be contacted to determine whether the data should be returned, retained or destroyed on their behalf. Sponsors will be notified of the financial implications of each of these options at that time.

Specimens or samples requiring frozen storage at Covance are specifically excluded from the above. These will be retained for as long as the material permits further evaluation, up to three months after issue of the draft report. At this time, the Sponsor will be contacted to determine whether samples should be returned, retained or destroyed on their behalf. Any financial implications of these options will also be notified at this time. Samples will not be destroyed without prior approval of the Study Director

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Appendix 1
Study records to be maintained

Definitive study plan (general study plan and study specific supplement)
Amendment(s)*
File note(s)*
Study schedule
Report schedule
Study correspondence
Client correspondence
Test article description
Test article formulation assessment*
Dispensary requests
Test article utilisation
Details of formulation preparation
Formulation storage and dispatch*
Analysis of formulations*
Metrology#
Records for reagents and stock solutions#
Culture records
Slide preparation*
Cytogenetic data*
Statistical analysis

some records held centrally

* where appropriate

Appendix 2

Responsible personnel

STUDY MANAGEMENT¹

Study Director

NAME

T Kumaravel

QUALITY ASSURANCE²

Director, Quality Systems

C Clare

1 = Details will be provided in the study specific supplements. Any subsequent change will be documented by protocol amendment

2 = Any change documented in study records

DISTRIBUTION: Personnel above

PROTOCOL APPROVAL

Please sign both approval pages; return one to Covance Laboratories Ltd and retain one for your records.

D Johnson
Study Monitor
Swedish Match

Date

T S Kumaravel

T S Kumaravel
Study Director
Covance Laboratories Ltd

17 June 2005

Date

J. Clements

J Clements
CLE Management
Covance Laboratories Ltd

17 June 2005

Date

Definitive Protocol

Study Title	Extracts of four Snus batches and Kentucky reference moist snuff: evaluation of <i>in vitro</i> cytotoxicity in Balb/c 3T3 cells using the Neutral Red Uptake assay
Study Director	M Johnson
Sponsor	Swedish Match SE-11885 Stockholm SWEDEN
Study Monitor	David Johnson Swedish Match North America
Test Facility	Covance Laboratories Ltd Otley Road Harrogate North Yorkshire HG3 1PY ENGLAND
Covance Study Number	1138/20
Page Number	1 of 14

OBJECTIVE

To evaluate the *in vitro* cytotoxicity of Extracts of four Snus batches and Kentucky reference moist snuff in Balb/c 3T3 mouse fibroblast cells using the Neutral Red Uptake assay, and if possible to perform a statistical evaluation of any potency differences between the extracts.

INTRODUCTION

The Neutral Red Uptake assay is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind Neutral Red, a supravital dye. Neutral Red is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes (1,2). Thus, the Neutral Red Uptake assay examines the cellular membrane integrity as well as the cellular energy status since Neutral Red is taken up into lysosomes by an energy dependent process. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of Neutral Red. It is thus possible to distinguish between viable and damaged or dead cells, which is the basis of this assay.

Healthy Balb/c 3T3 cells, when maintained in culture, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in a reduction of the growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration-dependent reduction of the uptake of Neutral Red after 24 hours of chemical exposure, thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

TEST SYSTEM

Balb/c 3T3 cells are seeded into 96-well plates and maintained in culture for approximately 24 hours to form a semi-confluent monolayer. They are then exposed to the test article over a range of eight concentrations. After a 24 hour exposure, Neutral Red Uptake is determined for each treatment concentration and compared to that determined in control cultures. For each concentration of the test article the percent inhibition of growth is calculated. The IC₅₀ (the concentration producing 50% reduction of Neutral Red uptake) is calculated from the concentration-response.

Swedish moist snuff (Snus) is a commercially available tobacco product which may be considered as a smoking cessation product, and is undergoing safety evaluation testing. As part of this process, testing is scheduled to be performed in a number of *in vitro* assay systems. As Snus is an insoluble solid, to enable exposure of this test material to the cells in the assay system, as is commonly used for *in vitro* testing of other smokeless tobacco products, an extract of the test material will be used. The extraction methodology and concentrations employed for this study have been demonstrated in previous experimentation (CLE Study Number 1138/16) to provide the highest practical extraction concentration for these materials, and therefore provide the most stringent assessment of these materials possible in this assay system.

There are no testing guidelines for this type of *in vitro* assay.

MATERIALS

Cell line

Species of origin	Mouse
Source	American Type Culture Collection
Identification	ATCC-CCL 163 (Balb/c 3T3 clone A31)
Maintenance	Frozen stocks are stored in liquid nitrogen. Laboratory stock cultures are maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% newborn calf serum, 4 mM glutamine and penicillin/streptomycin (DMEM complete) at 37±1°C in a humidified atmosphere of 5% CO ₂ in air. Cells are passaged regularly to avoid overgrowth.

Test articles

The test articles will be Extracts of four Snus batches and Kentucky reference moist snuff:

- Swedish Moist Snuff (Snus) G
- Swedish Match Moist Snuff (Snus) CPS
- Swedish Moist Snuff (Snus) CDM
- Swedish Moist Snuff (Snus) CDM2
- Kentucky Reference Moist Snuff, Batch 2S3

The Sponsor will normally be expected to supply sufficient material to permit sufficient extract volumes to perform all testing required for this study. The Sponsor will also supply as much information as possible regarding characterisation of the Snus/moist snuff, such as compositional analysis, expiry date, storage conditions, etc.

All test articles will be handled as though they are potential cytotoxic / carcinogenic compounds and appropriate safety procedures will be employed.

Determinations of the stability and characteristics of the test article, where defined in GLP regulations, are the responsibility of the Sponsor. Archiving a sample of the test article will be the responsibility of the Sponsor.

Extracts will be performed based on the methodology evaluated and developed in CLE study number 1138/16, and conducted using sterile containers and solutions, in order to minimise any contamination from external sources:

- Appropriate numbers of pouches/sachets of moist snuff/Snus will be cut approximately in half, and both the contents and the packaging (pouches and sachets) weighed and mixed with appropriate volumes of sterile purified water or DMSO to produce the required w/v concentration. If the tobacco is not finely divided then brief homogenisation may be performed.
- Extractions will be performed for 24 hours at 37°C, with shaking
- At the end of the 24 hour extraction period, extracts will be centrifuged at approximately 1800g for 30 minutes, and heavy particulates removed by decanting off the supernatant
- The supernatant will then be centrifuged at 25,000g for 30 minutes, and fine particulates removed by decanting off the supernatant
- The final supernatant will be adjusted to pH 7.4±0.2 with Hydrochloric acid (water extracts only)
- The resulting extracts will be filter sterilised using a 0.2 µm pore size filter (pre-filtering using a larger pore size may be performed)
- Aliquots of extracts will be stored at approximately -80°C, and used for up to 6 months after the extraction process is performed.

All Snus/moist snuff batches will be extracted in purified water at a concentration of 500 mg/mL equivalent, and CDM2 extracts at this concentration in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) will also be prepared for use in this study. Based on experimentation conducted in CLE study number 1138/16, this concentration was the highest extraction concentration that could practicably be extracted from material of this nature, and therefore gives the highest achievable concentration.

Where extracts are prepared in two or more separate flasks for a Snus/moist snuff batch, extracts for use in each experiment will be pooled prior to use in the assay.

Vials of frozen extract to provide the volume of extract required will be thawed on the day of assay. For extracts formulated in DMSO, a hundred-fold dilution into DMEM complete will be used for treatment. For extracts formulated in water, a ten-fold dilution into DMEM complete will be used for treatment. If volumes of the organic solvent exceeding 1% (v/v) or the aqueous solvent exceeding 10% (v/v) need to be used, the effects on viability may need to be checked. A series of eight doses will be separated by two-fold intervals in Experiment 1, but will be in a closer range in

Experiment 2 (if performed). The treatment concentrations that will be used and means of achieving the final concentrations for Experiment 1 are given in the following table:

Experiment	Concentration of extract (mg/mL equivalent)	Final concentration (mg/mL equivalent)	
		DMSO extracts ^a	Water extracts ^b
Experiment 1	3.906	0.0390625	0.390625
	7.813	0.078125	0.78125
	15.63	0.15625	1.5625
	31.25	0.3125	3.125
	62.50	0.625	6.25
	125.00	1.25	12.50
	250.00	2.50	25.00
	500.00*	5.00	50.00

* Neat extract - all lower concentrations achieved by dilutions of this extract concentration

a extracts tested at 1:100 (v/v) in DMEM complete

b extracts tested at 1:10 (v/v) in DMEM complete

Controls

Negative controls will comprise treatments with blank extract solvent (extract solvent subjected to the extraction conditions but in the absence of test material) diluted 1:10 (v/v) for water extracts or 1:100 (v/v) for DMSO extracts in DMEM complete. The positive control will be 100 µg/mL sodium dodecyl sulphate (SDS) in DMEM complete. Negative and positive control treatments will be performed for each extract.

Nicotine content determination

The nicotine content of the test article extracts will be performed using HPLC analysis. For each test article (extract), nicotine determination will be performed on samples from up to five extraction flasks (if more than five flasks are used to generate extract, samples from only five representative flasks will be analysed), together with a sample from the pooled extract to be used in the assay. Details of the analytical method will be included in a protocol amendment. Results from these analyses will be presented in the report as achieved nicotine concentrations.

METHODS

Treatment plates will be labelled with date, study number, test article treatment concentrations, positive and negative controls.

Cell culture preparation

Balb/c 3T3 mouse fibroblasts will be cultured at $37\pm 1^{\circ}\text{C}$ in a humidified atmosphere of 5% CO_2 in air. Cultures will be refed with DMEM complete and subcultured whenever required.

Day 1 procedure

Near confluent cultures will be trypsinized and resuspended in DMEM complete. The cell number in the suspension prepared will be determined using a haemocytometer. Aliquots (100 μL) of DMEM complete only will be dispensed into all peripheral wells (blanks) of an appropriate number of 96-well plates. The cell suspension will be diluted to give a final concentration of 1×10^5 cells/mL and 100 μL pipetted into the appropriate number of wells (i.e. 10^4 cells/well). Six wells will be identified for negative control, positive control and each test article concentration. Plates will be incubated at $37\pm 1^{\circ}\text{C}$ for approximately 24 hours in a humidified atmosphere of 5% CO_2 in air to achieve approximately half-confluent monolayers.

Day 2 procedure

The culture medium will be removed from the plates and wells washed with phosphate buffered saline (PBS). Aliquots (100 μL) of DMEM complete containing concentrations of the test article, positive control or negative control will be applied to the plates. The plates will be incubated at $37\pm 1^{\circ}\text{C}$ for approximately 24 hours in a humidified atmosphere of 5% CO_2 in air.

Day 3 procedure

Following incubation, the cells will be examined microscopically in order to provide a visual assessment of toxicity.

Immediately following the visual assessment of toxicity, the treatment medium will be removed and the cells in each well washed with 150 μ L PBS. This will be removed, and 100 μ L Neutral Red solution (50 μ g/mL) will be added. The plates will be incubated at $37\pm 1^\circ\text{C}$ for approximately 3 hours in a humidified atmosphere of 5% CO_2 in air. Following the incubation, the Neutral Red solution will be removed and the cells washed at least once with 150 μ L PBS per well which will be removed before adding 100 μ L Neutral Red destain solution (ethanol:acetic acid:distilled water 50:1:49 (v/v/v)). Plates will be shaken on a plate shaker until it is considered that all Neutral Red has been extracted from the cells.

Analysis will be performed using a THERMOmaxTM plate reader at a wavelength setting of 540 nm. Neutral Red absorbances will be expressed in terms of absolute optical density (OD_{540}).

DATA ANALYSIS

A calculation of cell viability expressed as Neutral Red Uptake is made for each concentration of the test article, the negative and positive control, by using the mean Neutral Red absorbance of the six replicate values. The mean OD_{540} values for each test article treatment dose and the positive control will then be compared to the mean OD_{540} value for the negative control. Relative cell viability is then expressed as a percentage of the negative control. If achievable, the eight concentrations of test article should span the range of no effect up to total inhibition of cell viability.

If possible, the concentration of the test substance inducing a 50% inhibition of the Neutral Red Uptake (IC_{50} value) will be estimated, along with 95% Fiducial limits for the estimates, using Probit analysis. The Fiducial limits will be compared across extracts; where the Fiducial limits for two extracts overlap, they will be considered as "not significantly different." The IC_{10} and IC_{90} values will also be estimated using Probit analysis.

Following Experiment 1, results will be communicated to the Sponsor. At this point, a decision will be made in conjunction with the Sponsor on whether a second or further experiment is to be performed, and the conditions/methodology to be employed.

Acceptance criteria

The assay will be considered valid if the following criteria are met:

1. For all treatments, there is a low variability in OD₅₄₀ values between all treatment replicates (coefficient of variance < 15%).
2. The positive control treatment causes > 50% decreases in Neutral Red Uptake relative to the negative control.
3. The mean OD₅₄₀ of the negative control is > 0.2.

If these conditions are not met, the acceptability of the results may be discussed on a case-by case-basis. If equivocal results are obtained additional treatments may be required.

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GLP COMPLIANCE

The study will be performed in compliance with:

United Kingdom Good Laboratory Practice Regulations 1999, Statutory Instrument No. 3106 as amended by the Good Laboratory Practice (Codification Amendments Etc.) Regulations 2004.

OECD Principles of Good Laboratory Practice (revised 1997, issued Jan 1998) ENV/MC/CHEM (98) 17.

All procedures will be performed in accordance with Covance Laboratories Limited (CLE) standard operating procedures (SOPs). The study will be subject to Quality Assurance evaluation by the CLE Quality Assurance Unit (QAU) in accordance with SOPs. Where appropriate, any change to this protocol will be made by an amendment issued in agreement with the Sponsor.

Following completion of the study a draft report will be issued. Client comments should be supplied for inclusion into a final document within six months of receipt of the draft document.

In accordance with the requirements of the UK Medicines Control Agency GLP Monitoring Authority, if no client comments are received within six months of issue, the report will be finalised. Any further changes after this time will be addressed as an amended final report, which may result in additional costs.

A list of study records to be maintained by Covance Laboratories Ltd is detailed in Appendix 1.

PROPOSED TIME SCHEDULE

It is estimated that the study will start in early July 2005 such that the experimental phase will be completed by early August 2005 and a draft report will be issued to the Sponsor in September 2005. If a second or subsequent experiments are performed, this schedule will be amended accordingly. Following comments from the Sponsor and from Covance Laboratories Ltd QA unit a final report will be agreed. Three signed copies (two bound, one unbound) of the final report will be issued.

Should any unforeseen circumstances alter the proposed timescale, the Sponsor will be informed immediately. Covance Laboratories Ltd cannot guarantee to meet the proposed timescale unless this protocol is approved (by signature and return or by telefax) and the test articles received before 20 June 2005.

Following receipt of the signed protocol approval and the test article, a detailed schedule confirming timings of the experimental phase and issue date of the draft report will be sent to the Sponsor.

ARCHIVE STATEMENT

All primary data, or authenticated copies thereof, specimens and the final report will be retained in Covance Laboratories Ltd archives for one year after issue of the final report. At this time the Sponsor will be contacted to determine whether the data should be returned, retained or destroyed on their behalf. Sponsors will be notified of the financial implications of each of these options at that time.

Specimens requiring storage deep frozen are specifically excluded from the above. These will be retained for as long as the quality of the material permits evaluation, but for no longer than three months after issue of the final report. The Sponsor will be notified of the intent to destroy samples and any financial implications before specimens are destroyed on their behalf.

Appendix 1 Study records to be maintained

Definitive protocol
Amendment(s)*
File note(s)*
Study schedule
Report schedule
Study correspondence
Client correspondence
Test article description
Test article formulation assessment*
Dispensary requests
Test article utilisation
Details of formulation preparation
Formulation storage and dispatch*
Analysis of formulations*
Metrology#
Records for reagents and stock solutions#
Culture records
Plate count data
Statistical analysis

some records held centrally

* where appropriate

Appendix 2
Responsible personnel

STUDY MANAGEMENT¹

Study Director

NAME

M Johnson

OPERATIONAL SUPERVISION¹

Responsible Scientist, Nicotine Determination

A Battle

QUALITY ASSURANCE²

Director, Quality Systems

C Clare

1 = Any change documented by protocol amendment

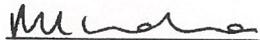
2 = Any change documented in study records

DISTRIBUTION: Personnel above

PROTOCOL APPROVAL

David Johnson
Study Monitor
Swedish Match

Date



M Johnson
Study Director
Covance Laboratories Ltd

9 June 2005

Date



N Dawkes
CLE Management
Covance Laboratories Ltd

9 June 2005

Date

Curvall, Margareta (STO)

Från: Dawkes, Neil [Neil.Dawkes@covance.com]
Skickat: torsdag 26 maj 2005 14:40
Till: Johnson, David (OWE)
Kopia: Ballantyne, Mark; Riley, Susan; Clements, Julie; Curvall, Margareta (STO)
Ämne: SNUS extract study prices

Dear David

Following on from last weeks project meeting at Covance, please find below a breakdown of study price estimates for the project.

1. Validation study (1138/16)

The total quoted study price was ?41,600. However, following discussions with Julie and Mark after Friday's meeting we acknowledge that we made an error in the initial study quote. The nicotine analysis component of the study was omitted in error from the first quotation for ?20,800. The price for this nicotine analysis was then included in the second quotation. Due to the mistake made in our costing process we propose that the nicotine analysis component of the validation is only charged to you at cost price.

This reduces the total price of this study to ?36,600. Please let me know if this is acceptable.

2. New studies on the 6 snus extracts

The study prices below are based on the protocol designs discussed in last weeks meeting. The prices are for a single experiment.

Ames	study price ?1500 per extract	Total study price ?9000
In vitro micronucleus	study price ?10000 per extract	Total study price ?60000
Mouse Lymphoma	study price ?9500 per extract	Total study price ?57000
Neutral Red	study price ?1100 per extract	Total study price ?6600

The above prices are dependent on final protocol design.

If a second experiment is required an additional 85% of the above would be added to the study price.

Unfortunately, we do not currently have the price for the nicotine analysis component of these studies so this is not included in the above study prices. I will forward this to you as soon as it is available.

Please don't hesitate to contact Mark or myself if you require any further information.

Best regards

Neil Dawkes
Operations Manager
Genetic and Molecular Toxicology

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