ASSESSMENT OF THE IRRITATION POTENTIAL OF SWEDISH SNUS INGREDIENTS USING THE EPIORAL™ TISSUE MODEL

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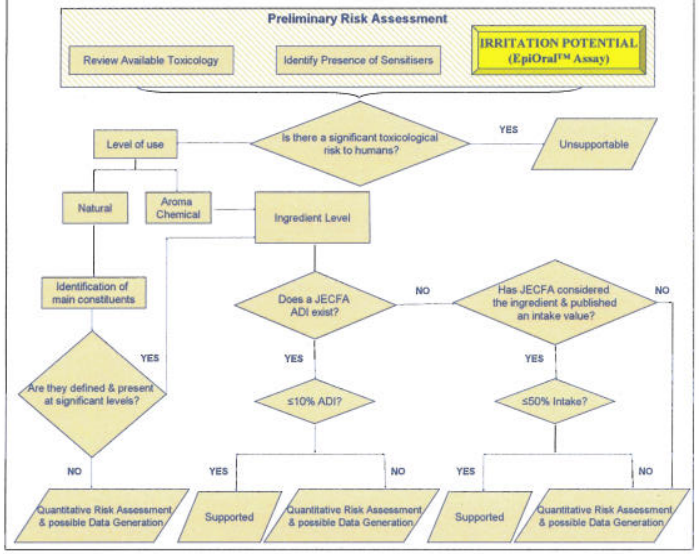
INTRODUCTION

The risk assessment for ingredients in Swedish style pouched snus differs significantly from that used for traditional combusted product. Swedish style pouched snus comprises finally ground moist tobacco and flavourings encased within a porous pouch that is placed in the oral cavity, most commonly under the upper lip. Prolonged contact of the product with the oral mucosa may allow certain ingredients to exert an irritant potential on the mucosal tissue. We conducted a survey in 2007 to investigate the behaviour and consumption patterns of Swedish snus users and found that the median residence time in the mouth for each snus pouch was 60 minutes, however there was evidence that some individuals had a constant presence of snus in the oral cavity during waking hours(1). As part of our risk assessment for pouched snus (see below), we consider the irritation potential of ingredients. This has been aided by the utilisation of an *in vitro* model to screen potential irritancy to define non-irritant levels of ingredients for use in snus products.

POUCHED SNUS RISK ASSESSMENT PARADIGM



IRRITATION POTENTIAL

During the preliminary risk assessment Material Safety Data Sheets (MSDS) are reviewed to identify any ingredients with irritancy potential, classified by "Xi" and/or relevant Risk Phrases (R36-38). These ingredients were then prioritized, based on concentration, for *in vitro* testing using the EpiOral™ tissue model. The irritation potential of an ingredient is dependent both on concentration of the ingredient and duration of exposure. By studying these factors within the EpiOral™ tissue model, we defined a level of use for each ingredient that does not cause an irritancy concern.

REFERENCES

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(4) Coquette, A., Berna, N., Vandenbosch, A., Rosdy, M., De Wever, B., Poumay, Y., 2003. Analysis of interleukin-1α (IL-1α) and interleukin-8 (IL-8) expression and release in *in vitro* reconstructed human epidermis for the prediction of *in vivo* skin irritation and/or sensitization. *Toxicology in vitro*, 17, pp.311-321

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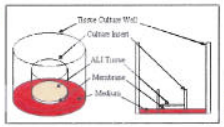
in vitro IRRITANCY SCREEN

The EpiOral™ Tissue Model, developed by MatTek Corporation, consists of cultured human epidermal keratinocytes forming a multilayered, highly differentiated model of human oral epithelium with a buccal phenotype.

The EpiOral™ inserts were placed in wells containing media supplied by MatTek (Figure 1) and the test ingredients were applied topically.

Figure 1

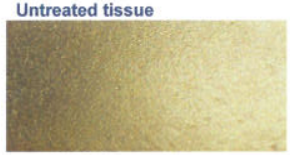


Taken from http://www.mattek.com/pages/in_vitro_basics.

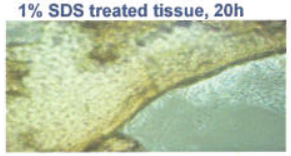
The maximum concentration for each of the test ingredients was 5000 µg/ml in DMSO (which in some cases was the limit of solubility). This concentration is significantly higher than that suggested in the OECD guidelines for the neutral red cytotoxicity test(2). Assays were conducted in duplicate using two different incubation times of 1h and 20h. These time points were chosen to model exposure to either a single snus pouch (1h) or sustained exposure during waking hours (20h). Triton X-100 (1%) and sodium dodecyl sulphate (SDS 1%) were included as positive controls. Both positive controls were also included at 0.5% to demonstrate the dose dependency of irritant effect. Dimethyl sulphoxide (DMSO 0.5%) was used as a negative control.

EpiOral™ tissue (x100 magnification)

Untreated tissue



1% SDS treated tissue, 20h



Irritant (cytotoxic) effects to the tissue were measured by using the Methyl Thiazoyl Tetrazolium (MTT) assay. Viable tissues convert the MTT (yellow) to formazan (purple) which is measured spectrophotometrically at OD_{570}.

Irritant effect of a test ingredient at a defined concentration was confirmed if a reduction of ≥25% tissue viability relative to the negative controls was demonstrated at either time point. This approach differs from the MatTek standard test protocol that identifies an exposure time at a fixed concentration that reduces the tissue viability to 50% (ET_{50})(3). This modification to the protocol reflects our need to define concentrations of ingredient without irritant potential.

In some experiments, media samples were removed following treatments and were analysed for the release of the cytokine Interleukin-1α (IL-1α) using a specific ELISA kit, as described in the manufacturer's instructions (Invitrogen, Paisley UK).

RESULTS

Positive Control Data (Table 1)

Test Substance	Concentration (µg/ml)	Average Tissue Viability Relative to Controls (%)		Irritant (Yes/No)	
		1h	20h	1h	20h
SDS	5000	107.0	15.5	No	Yes
SDS	10000	36.2	7.3	Yes	Yes
Triton X-100	5000	75.7	8.5	No	Yes
Triton X-100	10000	43.7	7.1	Yes	Yes

Negative Control Data

Mean OD_{570} for 0.5% DMSO: 1.77 ± 0.2 (1h, n=32), 1.54 ± 0.2 (20h, n=32)

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RESULTS

Table 2. Tissue viabilities of each test substance over both time points and confirmation of irritant effect.

Test Substance	Concentration (µg/ml)	Average Tissue Viability Relative to Controls (%)		Irritant (Yes/No)	
		1h	20h	1h	20h
Benzyl carbinol	5000	118.6	114.5	No	No
Cinnamaldehyde	5000	99.1	93.3	No	No
Citronellol	5000	124.2	118.4	No	No
Geranium oil	5000	100.3	110.8	No	No
Linalyl acetate	5000	102.9	105.8	No	No
Menthol	5000	121.3	122.7	No	No

Table 3. Effects of positive controls on the release of IL-1α after 1h and 20h exposure

Test Substance (time)	Concentration (µg/ml)	IL-1α (pg/ml)	Tissue Viability (%)
SDS (1h)	5000	31.1	107.0
SDS (20h)	5000	230.1	15.5
Triton X-100 (1h)	10000	41.2	43.4
Triton X-100 (20h)	10000	240.6	11.6

DISCUSSION

Our results showed that at the maximum concentration of 5000 µg/ml, none of the test ingredients listed in Table 2 displayed an irritant effect in the EpiOral™ tissue model at either 1h or 20h exposure time. The positive controls (SDS and Triton X-100), at a concentration of 1%, were defined as irritants after both 1h and 20h. However SDS and Triton X-100, at a concentration of 0.5%, were only defined as irritants after the 20h exposure, demonstrating that exposure time is an important factor.

Our preliminary data on IL-1α release suggest that this assay may also have a utility in prediction of irritation potential. Differences between the levels of IL-1α seen after either 1h or 20h exposure presumably reflect the time taken for the cells to respond to the irritant insult with the production and release of the cytokine into the supernatant. At this stage we are unable to propose an absolute level of release of the cytokine IL-1α into the supernatant that could be used to define irritant potential, but our preliminary results suggest that it might be of the same order of magnitude as the levels seen in the extracellular medium of reconstructed human epidermis exposed to irritant chemicals(4).

Based on these findings, we propose that the 20h exposure time is optimal to identify ingredients with irritant effects at concentrations up to 5000 µg/ml. This exposure time reflects a worst-case scenario for predicting possible irritant effects of snus ingredients in the population who use snus continually in waking hours. It may be possible to develop a tiered testing strategy using MTT in association with IL-1α, such as that proposed previously for the EpiDerm™ (5) skin model.

CONCLUSION

The EpiOral™ tissue model has proved to be a useful screen to predict the irritation potential of candidate ingredients for use in pouched snus products. Within our Risk Assessment paradigm, those ingredients that have been identified as potential irritants from Material Safety Data Sheets can be tested in the EpiOral™ tissue model at concentrations up to 5000 µg/ml for 1h or 20h. Those ingredients that demonstrate a lack of irritation potential within this assay at 20h can move forward within the Risk Assessment paradigm for potential inclusion in pouched snus product, subject to further toxicological assessments.