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SUMMARY OF LABSTAT PROJECT M100:

NEUTRAL RED UPTAKE ASSAYS OF SMOKELESS TOBACCO SAMPLES

OBJECTIVE:

To summarize data and conclusions from Neutral Red Uptake (NRU) assays of smokeless tobacco samples and Kentucky reference cigarette 2R4F conducted at Labstat International ULC.

SUMMARY:

Seven smokeless tobacco samples were submitted to Labstat International ULC (Kitchener, Canada) for Neutral Red Uptake (NRU) testing: 2S3 Research Moist Smokeless Tobacco, Camel Snus Frost, Camel Fresh Orbs, Camel Fresh Strips, Camel Mellow Sticks, Copenhagen Long Cut, and Ariva Wintergreen. Kentucky Reference cigarettes 2R4F were also evaluated. Smokeless tobacco samples were extracted in dimethyl sulfoxide (DMSO) for 21 hours at 37 °C. 2R4F cigarettes were smoked using ISO smoking regimen 35/60/2 with no vent blocking and TPM was extracted from Cambridge filter pads with DMSO. Testing was conducted in triplicate. Smokeless tobacco samples were compared on a DMSO-extracted smokeless tobacco basis, DMSO-extracted moisture-corrected smokeless tobacco basis and DMSO-extracted nicotine basis. Smokeless tobacco samples were also compared to 2R4F on extracted nicotine basis. Results were summarized in the Labstat Report "Toxicology of Smokeless Tobacco Products: Neutral Red Cytotoxicity Project Code M100" Revision 7.

In many cases weak or non-cytotoxic responses from the smokeless samples resulted in minimum relative absorbance of greater than 50%. In assays where 50% reduction relative to control was not achieved, IC₅₀ values were not calculated since in these instances it is not possible to determine an IC₅₀ value with any degree of confidence. For all replicates of Camel Fresh Strips, Camel Mellow Sticks, Copenhagen Long Cut, and Camel Fresh Orbs, plus one replicate of Ariva Wintergreen and two replicates for each of Camel Snus Frost and 2S3, a 50% reduction in relative absorbance was not achieved at any concentration and an IC₅₀ value was not calculated. (Only Ariva and

2R4F had IC_{50} values from more than one replicate). Therefore, statistical comparisons were conducted on only the three smokeless samples (Camel Snus Frost, 2S3, and Ariva Wintergreen) for which at least one replicate IC_{50} was calculated.

Camel Snus Frost, 2S3 and Ariva Wintergreen were compared on “DMSO-extracted smokeless tobacco” basis, “DMSO-extracted moisture-corrected smokeless tobacco” basis and “DMSO-extracted nicotine” basis. There were no statistically significant differences observed between these three smokeless samples in any comparison. Although Ariva Wintergreen was more cytotoxic than Camel Snus Frost and 2S3 on moisture-corrected and nicotine bases, the difference was not statistically significant.

Camel Snus Frost, 2S3 and Ariva Wintergreen were compared to 2R4F on “DMSO-extracted nicotine” basis. 2R4F was significantly more cytotoxic than these three smokeless samples on an extracted nicotine basis.

IC_{50} values could not be calculated for Camel Mellow Sticks, Camel Fresh Strips, Camel Fresh Orbs and Copenhagen Long Cut due to lack of appreciable cytotoxicity. Therefore, these samples were not compared to 2R4F.

In summary, smokeless samples elicited weak or non-cytotoxic responses, with no statistically significant differences between the three smokeless samples where IC_{50} values could be calculated for at least one replicate; TPM from Kentucky Reference 2R4F was statistically significantly more cytotoxic than these three smokeless samples.

STATUS:

This work is complete.

KEYWORDS:

Smokeless tobacco, dissolvable, Camel Snus Frost, 2S3, Camel Fresh Strips, Camel Mellow Sticks, Camel Fresh Orbs, Copenhagen Long Cut, Ariva Wintergreen, Neutral Red Uptake, Cytotoxicity, Labstat Project M100, Labstat Project M97, Labstat Project M125

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SUMMARY OF LABSTAT PROJECT M100:
NEUTRAL RED UPTAKE ASSAYS OF SMOKELESS TOBACCO SAMPLES

Test Facility: Labstat International ULC
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Kitchener, ON Canada N2C 1L3

Labstat project # M100
Study initiated: Labstat received samples on September 16, 2008 and October 21, 2008
Study completed: February 4, 2011 – date of final Labstat report (Revision 7)
Study monitor: Suzana Theophilus
Study reviewers: Ryan Potts (RJRT), Betsy Bombick (RJRT), Kathy Fowler (RJRT), Walter Morgan (RJRT)
Study director: Amit Trivedi (Labstat International ULC)
Study personnel: Labstat personnel
Statistician: Wendy Wagstaff (Labstat International ULC)

OBJECTIVE:

To summarize data and conclusions from Neutral Red Uptake (NRU) assays of smokeless tobacco samples and Kentucky reference cigarette 2R4F conducted at Labstat International ULC.

EXPERIMENTAL DESIGN:

This study was conducted to evaluate the potential of seven smokeless tobacco samples and one cigarette to induce cytotoxicity in the Neutral Red Uptake assay.

Seven smokeless tobacco samples and one cigarette were submitted to Labstat International ULC, Kitchener, ON Canada for Neutral Red Uptake cytotoxicity testing. The Labstat project was identified as Project M100. The samples tested were coded as follows:

Sample	Labstat Code	Sample	Labstat Code
Camel Snus Frost	084394	2S3 research moist smokeless tobacco	084395
Fresh Strips	084454	Mellow Sticks	084455
Copenhagen Long Cut	084456	Ariva Wintergreen	084457
Fresh Orbs	084458	Kentucky Reference 2R4F	084396

2R4F cigarettes were smoked using ISO smoking regimen 35/60/2 with no vent blocking. Total particulate matter (TPM) was extracted from Cambridge filter pads with DMSO.

Smokeless tobacco samples were extracted with DMSO using the following methodology:

- dispersion in DMSO (1:9 w/v) using an ultrasonic homogenizer
- incubation at 37°C for 21 hours followed by centrifugation & ultra-filtration
- storage at -80°C prior to assay

Assays were conducted on a “DMSO-extracted smokeless tobacco” basis. All smokeless samples were tested up to 2.22 mg smokeless tobacco/mL on this basis. Results from moisture and nicotine determinations (from Labstat Project M97 – Ames mutagenicity assays of the same samples) were then used to calculate response on a DMSO-extracted moisture-corrected smokeless tobacco and DMSO-extracted nicotine basis. Kentucky Reference 2R4F TPM was tested up to 0.2 mg TPM/mL and nicotine determination used as above for further analysis.

The NRU testing was conducted according to Health Canada Method T-502 with the exception that vapor phase testing was not performed for the 2R4F cigarette.

Labstat issued its first report January 9, 2009. Subsequent revisions were submitted November 3, 2009, December 17, 2009, July 29, 2010, December 22, 2010, January 10, 2011, and February 4, 2011. Revisions were required due to requests for additional or revised procedures for statistical analysis of the data. This RDM is based on results provided in Labstat’s report “Toxicology of Smokeless Tobacco Products: Neutral Red Cytotoxicity” Project Code M100 (revision 7, dated February 4, 2011).

RESULTS:

Key results are summarized below. Detailed results and data are available in the Labstat M100 report for Neutral Red Cytotoxicity, Revision 7.

Analysis of Response

Raw absorbance values were blank-corrected and expressed relative to the negative control absorbance value. The IC_{50} value, the concentration of test article which results in 50% reduction in cell viability, was estimated by fitting the concentration-response data to a non-linear regression model. In assays where 50% reduction relative to control was not achieved, IC_{50} values were not calculated since in these instances it is not possible to determine an IC_{50} value with any degree of confidence. For all replicates of Camel Fresh Strips, Camel Mellow Sticks, Copenhagen Long Cut, and Camel Fresh Orbs, plus replicate 1 of Ariva Wintergreen, replicates 1 and 3 of Camel Snus Frost, and replicates 2 and 3 of 2S3, a 50% reduction in relative absorbance was not achieved and the non-linear regression model was not fit.

Comparison of Results

For each assay where 50% reduction in relative absorbance was achieved, a separate IC_{50} value was calculated and samples were compared using analysis of variance (ANOVA) to compare means of the log-transformed IC_{50} values. P-values were adjusted

according to the Bonferroni method to control for multiple comparisons with p-values less than 0.05 after adjustment considered statistically significant. Only Ariva and 2R4F had IC_{50} values from more than one replicate; IC_{50} values were obtained for one replicate at most for the other smokeless samples. Since no smokeless sample produced triplicate IC_{50} values, it was not possible to evaluate the data for inconsistent variation among the samples, therefore, only ANOVA-based comparisons assuming equal variance were applied.

Only four of the 21 total replicate assays of smokeless samples had calculated IC_{50} estimated from the non-linear model. Therefore, statistical comparisons were conducted on only three smokeless samples (Camel Snus Frost, 2S3, and Ariva Wintergreen). These three smokeless tobacco samples were compared on the following basis:

- DMSO-extracted smokeless tobacco (as-is)
- DMSO-extracted moisture-corrected smokeless tobacco (dry weight)
- DMSO-extracted nicotine

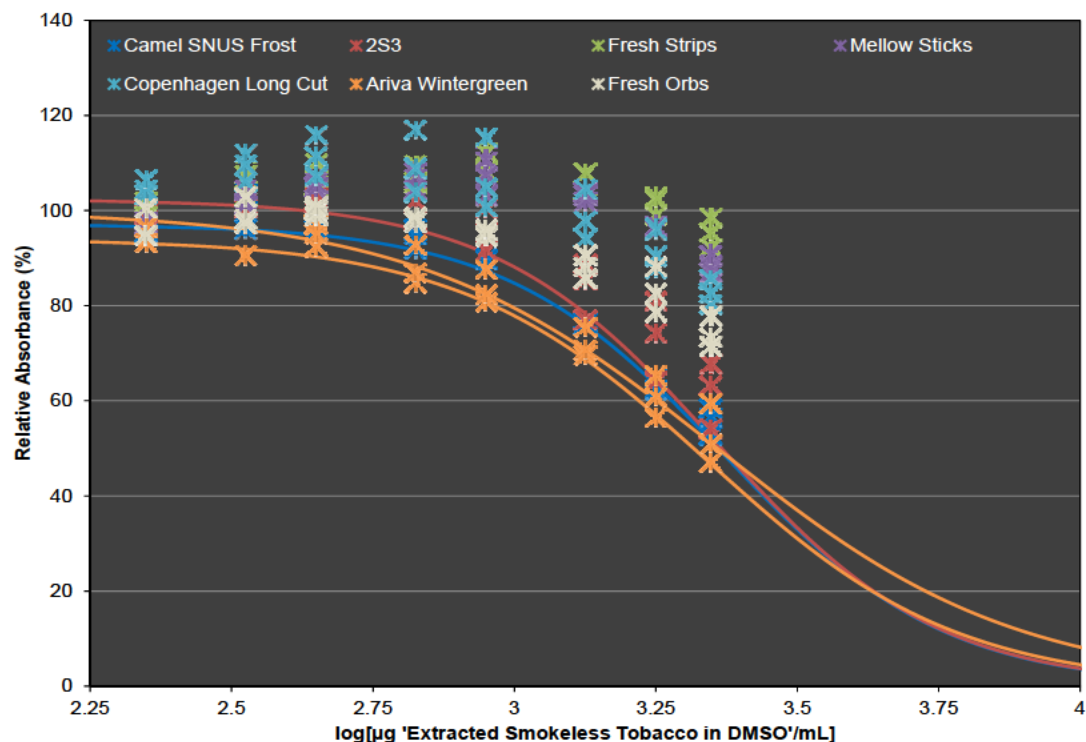
Camel Snus Frost, 2S3 and Ariva Wintergreen were also compared to 2R4F on extracted nicotine basis.

A. Results on DMSO-extracted smokeless tobacco (as-is) basis

Sample	IC ₅₀ Values (µg/ml)			
	DMSO-extracted smokeless tobacco (as-is) basis			
	Rep 1	Rep 2	Rep 3	Avg
Camel Snus Frost	*	2290	*	2290
2S3	2323	*	*	2323
Camel Fresh Strips	*	*	*	NA
Camel Mellow Sticks	*	*	*	NA
Copenhagen Long Cut	*	*	*	NA
Ariva Wintergreen	*	2074	2285	2179
Camel Fresh Orbs	*	*	*	NA

* Minimum relative absorbance was greater than 50% for all four assay plates, therefore, IC₅₀ value was not estimated.

NA – not applicable

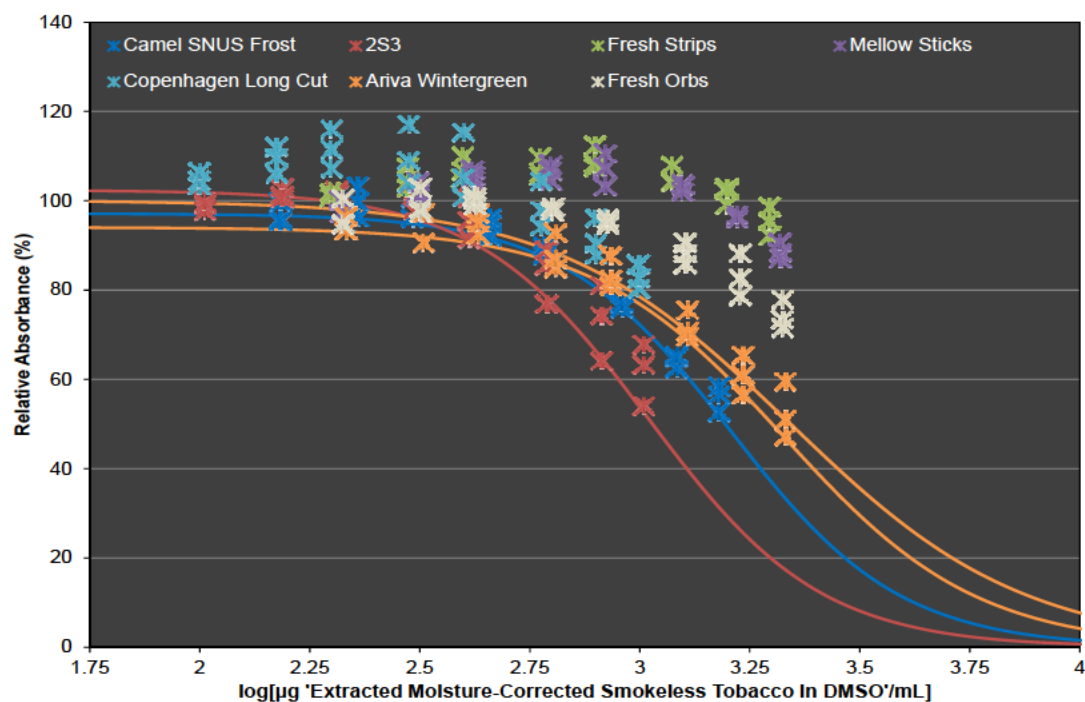


B. Results on DMSO-extracted moisture-corrected smokeless tobacco (dry weight) basis

Sample	IC ₅₀ Values (µg/ml)			
	DMSO-extracted moisture-corrected smokeless tobacco (dry weight) basis			
	Rep 1	Rep 2	Rep 3	Avg
Camel Snus Frost	*	1563	*	1563
2S3	1068	*	*	1068
Camel Fresh Strips	*	*	*	NA
Camel Mellow Sticks	*	*	*	NA
Copenhagen Long Cut	*	*	*	NA
Ariva Wintergreen	*	1998	2201	2099
Camel Fresh Orbs	*	*	*	NA

* Minimum relative absorbance was greater than 50% for all four assay plates, therefore, IC₅₀ value was not estimated.

NA – not applicable

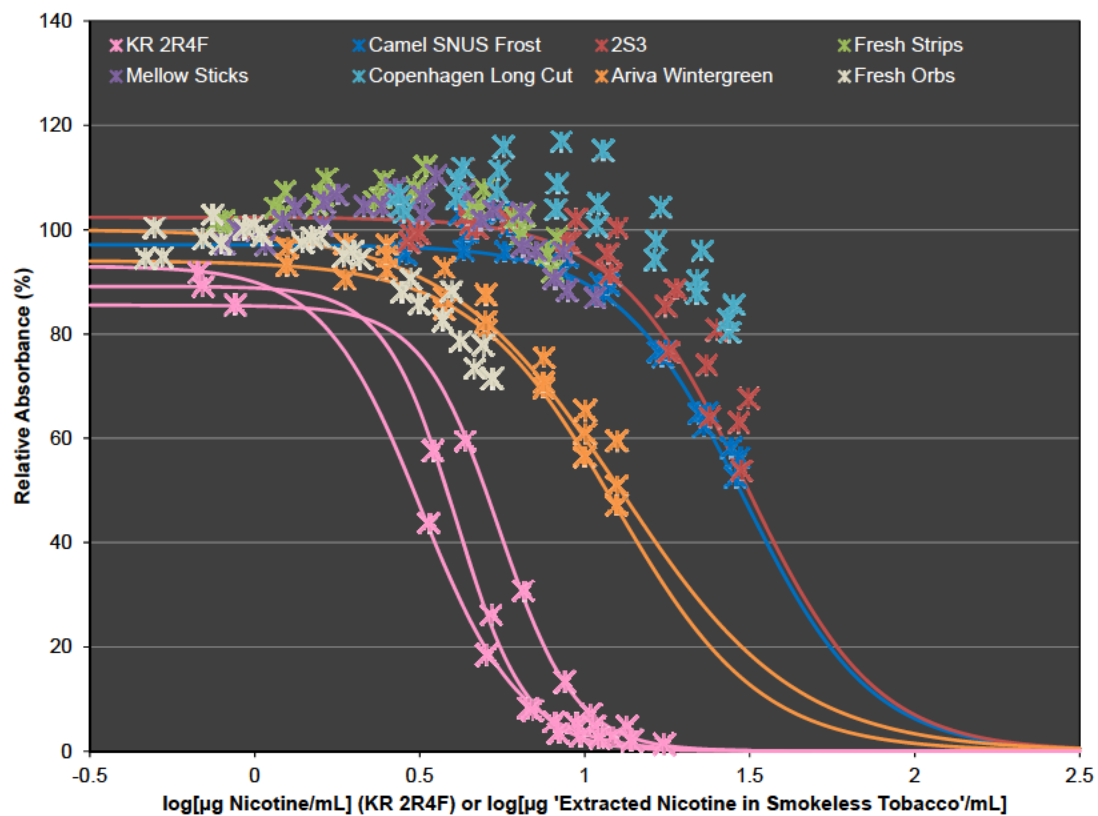
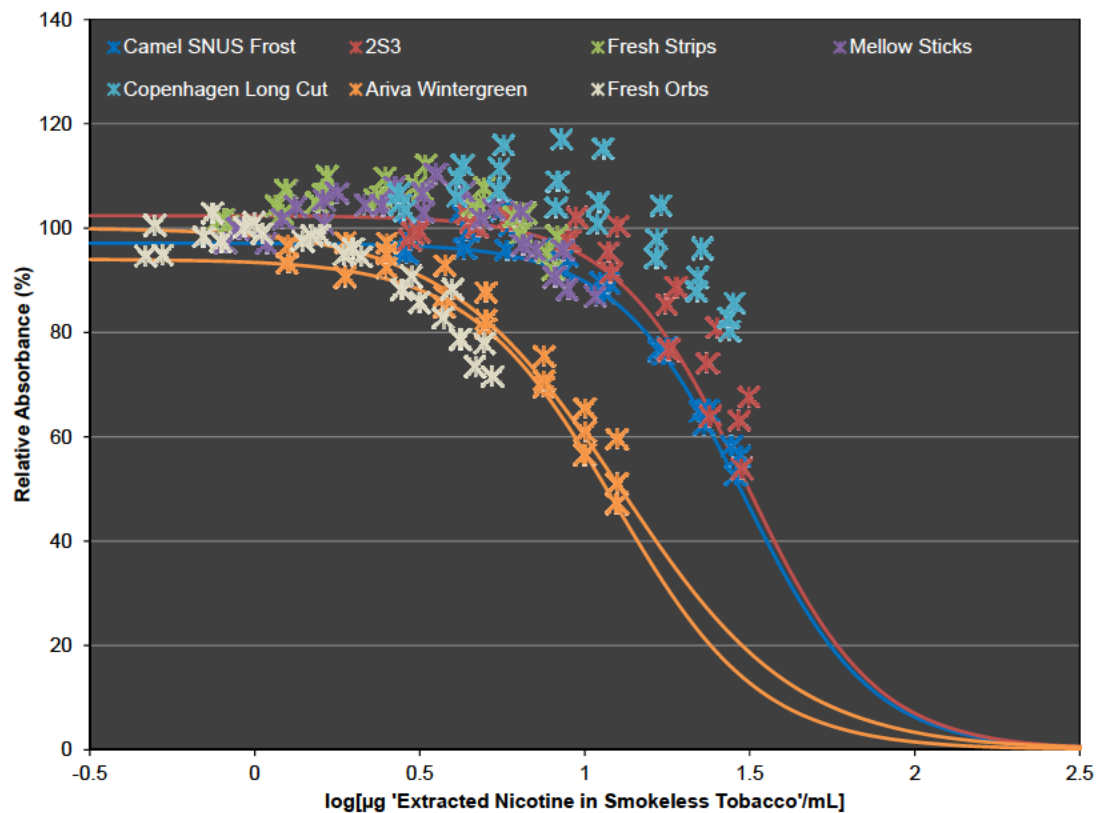


C. Results on DMSO-extracted nicotine basis for smokeless samples and 2R4F

Sample	IC ₅₀ Values (µg/ml)			
	DMSO-extracted nicotine in smokeless tobacco basis			
	Rep 1	Rep 2	Rep 3	Avg
Camel Snus Frost	*	29.6	*	29.6
2S3	31.4	*	*	31.4
Camel Fresh Strips	*	*	*	NA
Camel Mellow Sticks	*	*	*	NA
Copenhagen Long Cut	*	*	*	NA
Ariva Wintergreen	*	11.6	12.8	12.2
Camel Fresh Orbs	*	*	*	NA
KR 2R4F	5.01	3.03	3.83	3.96

* Minimum relative absorbance was greater than 50% for all four assay plates, therefore, IC₅₀ value was not estimated.

NA – not applicable



D. ANOVA-based comparisons on smokeless tobacco samples:

ANOVA-based comparisons were performed on the three smokeless tobacco samples (Camel Snus Frost, 2S3, Ariva Wintergreen) for which at least one IC_{50} estimate could be calculated. There were no statistically significant differences between the three samples on any comparison basis. Although Ariva Wintergreen was more cytotoxic than Camel Snus Frost and 2S3 on moisture-corrected and nicotine bases, the difference was not statistically significant.

E. ANOVA-based comparisons between smokeless tobacco samples and 2R4F:

ANOVA-based comparisons were performed between each of the three smokeless tobacco samples (Camel Snus Frost, 2S3, Ariva Wintergreen) for which at least one IC_{50} estimate could be calculated and 2R4F on a DMSO-extracted nicotine basis. 2R4F was statistically significantly more cytotoxic than Camel Snus Frost, 2S3 and Ariva Wintergreen.

SUMMARY AND CONCLUSIONS

In many cases, weak or non-cytotoxic responses from the smokeless samples resulted in minimum relative absorbance of greater than 50%. In assays where 50% reduction relative to control was not achieved, IC_{50} values were not calculated since in these instances it is not possible to determine an IC_{50} value with any degree of confidence. For all replicates of Camel Fresh Strips, Camel Mellow Sticks, Copenhagen Long Cut, and Camel Fresh Orbs, plus one replicate of Ariva Wintergreen and two replicates for each of Camel Snus Frost and 2S3, a 50% reduction in relative absorbance was not achieved at any concentration and an IC_{50} value was not calculated. Therefore, statistical comparisons were conducted on only the three smokeless samples (Camel Snus Frost, 2S3, and Ariva Wintergreen) for which at least one replicate IC_{50} was calculated.

Camel Snus Frost, 2S3 and Ariva Wintergreen were compared on “DMSO-extracted smokeless tobacco” basis, “DMSO-extracted moisture-corrected smokeless tobacco” basis and “DMSO-extracted nicotine” basis. There were no statistically significant differences observed between these three smokeless samples in any comparison. Although Ariva Wintergreen was more cytotoxic than Camel Snus Frost and 2S3 on moisture-corrected and nicotine bases, the difference was not statistically significant.

Camel Snus Frost, 2S3 and Ariva Wintergreen were compared to 2R4F on “DMSO-extracted nicotine” basis. 2R4F was significantly more cytotoxic than these three smokeless samples on an extracted nicotine basis.

IC_{50} values could not be calculated for Camel Mellow Sticks, Camel Fresh Strips, Camel Fresh Orbs and Copenhagen Long Cut due to lack of appreciable cytotoxicity. Therefore, these samples were not compared to 2R4F.

In summary, smokeless samples elicited weak or non-cytotoxic responses, with no statistically significant differences between the three smokeless samples where IC_{50} values could be calculated for at least one replicate; TPM from Kentucky Reference 2R4F was statistically significantly more cytotoxic than these three smokeless samples.