OXFORD UNIVERSITY PRESS | Toxicological Sciences

Smoke Chemistry, In vitro Cytotoxicity, and Genotoxicity Demonstrates Enhanced Toxicity of Cigarillos Compared to Cigarettes

Journal:	*Toxicological Sciences*
Manuscript ID	TOXSCI-20-0304.R1
Manuscript Type:	Research Article
Date Submitted by the Author:	02-Sep-2020
Complete List of Authors:	Crosby, Lynn; US FDA, CTP; US FDA, CFSAN Yucesoy, Berran; US FDA, CTP Leggett, Carmine; US FDA, CTP; American Association for Cancer Research, Science and Health Policy Tu, Zheng; US FDA, CTP Belinsky, S.A.; LRRI, Lung Cancer McDonald, Jacob; Lovelace Biomedical Leng, Shuguang; Lovelace Respiratory Research Institute Wu, Guodong; Lovelace Respiratory Research Institute Irshad, Hammad; Lovelace Respiratory Research Institute Valerio, Jr, Luis; US FDA, Rosenfeldt, Hans; Food and Drug Administration, Center for Tobacco Products, Office of Science
Category - Please select one category that is most applicable to your manuscript.:	Genetic and Epigenetic Toxicology
Category 2 - Optional: select a second category that is also applicable to your manuscript.:	Mixtures Toxicology
Key Words:	polycyclic aromatic hydrocarbons, carbonyls, tobacco-specific nitrosamines, Ames assay, Thymidine kinase assay, Micronucleus Assay, total particulate matter, volatile organic compounds, neutral red assay, nicotine, cotinine, propylene glycol, vegetable glycerin, particle size distribution

Smoke Chemistry, In vitro Cytotoxicity, and Genotoxicity Demonstrates Enhanced Toxicity of Cigarillos Compared to Cigarettes

Lynn Crosby[1*], Berran Yucesoy[1], Carmine Leggett[1], Zheng Tu[1], Steven A. Belinsky[2], Jake McDonald[2], Shuguang Leng[2], Guodong Wu[2], Hammad Irshad[2], Luis G. Valerio, Jr.[1], and Hans Rosenfeldt[1]

[1]Center for Tobacco Products, Office of Science, U.S. Food and Drug Administration, Silver Spring, MD 20993; [2]Lovelace Respiratory Research Institute, Albuquerque, NM, 87108

*Corresponding author: Center for Tobacco Products, Office of Science, U.S. Food and Drug Administration, Silver Spring, MD 20903, USA. email lynn.crosby@fda.hhs.gov

Keywords: Cigarillos, cigarettes, cytotoxicity, genotoxicity, carbonyls, tobacco-specific nitrosamines, polycyclic aromatic hydrocarbons

Disclaimers: The authors have no conflicts of interest to declare. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Food and Drug Administration.

Funding: This work was supported by the U.S. Food and Drug Administration Center for Tobacco Products.

ABSTRACT

There has been limited toxicity testing of cigarillos, including comparison to cigarettes. The present study compared the smoke chemistry and the cytotoxic and genotoxic potential of ten conventional cigarettes and ten cigarillos based on the greatest market share. Whole smoke and total particulate matter (TPM) were generated using the Canadian Intense (CI) and International Organization for Standardization (ISO) puffing protocols. Tobacco specific nitrosamines (TSNAs), carbonyls, and polycyclic aromatic hydrocarbons (PAHs) were measured using GC-MS. TPM smoke extracts were used for the in vitro assays. Cytotoxicity was assessed in HBEC4 cells using the neutral red uptake assay. Genotoxic potential was assessed using the micronucleus (MN; A549 cells), Ames, and thymidine kinase (TK) assays. TPM from all cigarillos tested was more cytotoxic than cigarettes. MN formation was significantly greater for cigarillos compared to cigarettes at the highest dose of TPM, with or without rat liver S9 fraction. In the Ames test +S9, both tobacco products exhibited significant dose-dependent increases in mutation frequency (MF), indicating metabolic activation is required for genotoxicity. In the TK assay +S9, cigarillos showed a significantly enhanced MF although both tobacco products were positive. The levels of all measured PAHs, TSNAs, and carbonyls (except acrolein) were significantly greater in cigarillos than

cigarettes. The CI puffing protocol demonstrated increased smoke constituent levels compared to ISO. Even though the gas vapor phase was not tested, the results of this study showed that under the tested conditions the investigated cigarillos showed greater toxicity than comparator cigarettes. This study found that there is significantly greater toxicity in the tested US marketed cigarillos than cigarettes for tobacco constituent levels, cytotoxicity, and genotoxicity. These findings are important for understanding the human health toxicity from the use of cigarillos relative to cigarettes and for building upon knowledge regarding harm from cigarillos to inform risk mitigation strategies.

LIST OF ABBREVIATIONS

A549	human lung adenocarcinoma continuously-cultured cell line
BA	benzo[a]anthracene
BaP	benzo[a]pyrene
BF	benzo[b]fluoranthene
BF	benzo[b]fluoranthene
BkF	benzo[k]fluoranthene
BLOQ	below the limit of quantitation
CH	chrysene
CI	Canadian Intense
DMSO	dimethyl sulfoxide
GC-FID	Gas Chromatography – Flame Ionization Detector

GC-MS	gas chromatography-mass spectrometry
GFF	glass fiber filter
GVP	gas vapor phase
HBEC4	human bronchial epithelial continuously-cultured cell line
HPHC	harmful and potentially-harmful constituents
ISO	International Organization for Standardization
LC/GC-MS	liquid/gas chromatography-mass spectrometry
LC-MS	liquid chromatography/mass spectrometry
LC-MS/MS	liquid chromatography tandem triple-quad mass spectrometry
MF	mutant frequency
MN	micronucleus assay
MSS	mainstream smoke
NHANES	National Health and Nutrition Examination Survey
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	N-nitrosonornicotine
NRU	neutral red uptake assay
NS	not significant
OR	odds ratio
PAH	polycyclic aromatic hydrocarbons
PG	propylene glycol
QC	quality control
SDS	sodium dodecyl sulfate

SVOC	semivolatile organic compounds
TK	thymidine kinase assay
TPM	total particulate matter
TSNA	tobacco-specific nitrosamines
VG	vegetable glycerin
VOC	volatile organic compounds

Introduction

Little cigars and cigarillos are a public health concern and are increasingly popular in the U.S. Because cigarillos usage data are bundled with all cigar products (CDC 2014; Trapl et al., 2017) and cigarillo users are more likely to be dual or multiple tobacco product users (Soneji, 2016), actual cigarillo usage data are difficult to ascertain. However, the Population Assessment of Tobacco and Health (PATH) questionnaire (PATH Wave 4, years 2016-2017) reported current cigarillo usage among youth (12-17 years old) of 0.9%, young adults (18-24 years) of 2.6%, and adults (aged 25+ years) of 1.3% (Rostron et al., 2020). Increased youth uptake may be partly due to the availability of appealing flavored products such as candy, fruit, and menthol (www.truthinitiative.org). Since characterizing flavors except menthol have been banned in US cigarettes, (US Family Smoking Control and Prevention Act, 2009) but are currently still allowed in cigars and cigarillos, flavoring cigarillos could make them more attractive to youth than cigarettes. Rostron et al. (2019) estimated that prohibiting characterizing flavors in the U.S. would result in approximately 800 fewer cigar smoking-attributable deaths in the U.S. each year and 112,000 fewer cigar smokers in each cohort of 18-year-olds.

Reports indicate a perception that cigars/cigarillos are less hazardous than cigarettes despite evidence demonstrating that cigarillo smoke is at least as toxic as cigarette smoke (Sterling et al., 2016; Amrock et al., 2016; Nyman et al., 2018) and is linked to multiple health consequences such as COPD and cancer (oral, esophageal, laryngeal,

lung, renal, ureter) (Jensen 1988, US DHHS 2012). NHANES data from 1999-2012 reported that cigar smokers, on average, have higher levels of cotinine, NNAL, cadmium, and lead than non-tobacco users (Chen et al., 2014). A recent study showed that 'little cigars' have higher yields of NNN, NNK and BaP per mass of TPM compared to the Kentucky 3R4F reference cigarette (Hamad et al., 2017).

Koszowski et al. (2017) studied smoking behavior and smoke constituents from cigarillos and little cigars in dual users but did not find differences between the mainstream smoke of cigars and cigarettes. A recent study (Jablonski et al., 2019) compared carbonyl delivery of 12 brands of cigars/cigarillos (global study mean) smoked according to CRM-64 and found they yielded more acetaldehyde (+3.3-fold), acrolein (+1.25-fold), and crotonaldehyde (+3.4-fold) than cigarettes smoked under ISO 3308, and cigars/cigarillos (global study mean) smoked under CRM-64 yielded more acetaldehyde (+1.5-fold) than cigarettes under ISO 20778, highlighting the importance of smoking method and puff topography. Pickworth et al. (2018) compared 3 VOC and 7 SVOC components of cigarillos and little cigars, finding cigarillo MSS contained significantly more toxicants in o2 out of 3 SVOC (1.1 to 3.6-fold higher) and 5 out of 7 VOC (2 to 17-fold higher) compared with little cigars when adjusted for nicotine content. Majewski et al. (2018) examined the elemental content of tobacco products including cigarillos, cigars and cigarettes and detected 18 elements in leaves or stalks. The topography of dual usage of cigarettes, little cigars, cigarillos and large cigars (Pickworth et al., 2017) was studied by plasma nicotine and exhaled CO concentrations. Cigar smoking was associated with increased mortality (HR = 1.52, 95% CI = 1.12 to

2.08, Inoue-Choi et al., 2019). Cigar smoking was strongly-associated with increased risk of oral, esophageal, pancreatic, laryngeal, and lung cancers and coronary heart disease and aortic aneurysm (Chang et al., 2015).

Using CHO (Chinese Hamster Ovary) cells with in vitro toxicity testing, Rickert et al. (2011) found cytotoxic, mutagenic, and genotoxic potential of the mainstream smoke from Canadian market cigarillos to be either equally or more toxic than mainstream smoke of 3R4F Kentucky reference cigarettes. Recently, Ghosh et al. (2017) studied the effects of whole smoke from little cigars on airway epithelial cells as compared to Kentucky reference cigarettes. They reported greater cytotoxicity, increased pro-inflammatory cytokine secretion, gene expression and proteomic alterations from little cigar smoke exposure compared with cigarette smoke exposure. In addition, higher quantities of tobacco constituents were found in little cigar smoke.

To expand upon the comparative nonclinical toxicity of smoke constituents from little cigars and cigarettes, the present study compared the in vitro cytotoxicity to human HBEC4 cells, genotoxicity to human lung A549 cells and mouse lymphoma L5178Y TK+/- cells, and mutagenicity of total TPM to *Salmonella typhimurium* strains TA98 and TA100 ±S9, generated from 10 each conventional cigarettes and cigarillos marketed in the U.S. In addition, carbonyls, tobacco-specific nitrosamines (TSNA), and polycyclic aromatic hydrocarbons (PAH) were measured and compared between product types. The objective of this study was to determine the relationships between biological endpoints and types and amounts of HPHC's. To the best of our knowledge, this is the

first study comparing smoke constituents and in vitro toxicological endpoints between US marketed cigarillos and cigarettes, according to brand using both ISO and CI methods.

Methods

Figure 1 gives the schematic illustration of the workflow of in vitro testing for these products.

Selection of test articles

Ten commercially available cigarettes and ten cigarillos were selected based on market share using CDC Data, from *The Maxwell Report: Year End and Fourth Quarter, 2013 and 2014* (Table 1). These data indicate that Marlboro (40.8%), Newport (12.4%), Camel (7.9%) and Pall Mall Box (7.8%) constitute the top market share of the U.S. cigarette market. Swisher Little (43.7%), Swisher Sweets (10.6%), Black and Mild (9.4%), White Owl (5.6%), Dutch Masters (5.0%) and Winchester (2.3%) were the top-selling cigarillos in the US. Winchester Little Cigars in January-February 2016.

TPM Collection and Smoke Analysis

TPM collection and smoke analysis were conducted at Lovelace (Albuquerque, NM) as follow: cigarettes and cigarillos were conditioned in a humidor (temperature 22±3°C) for at least 48 hr prior to use. Smoke was generated using a cigarette smoke machine (Mark II, AMESA Technologies, Switzerland) under ISO and CI smoking regimens. Each cigarette or cigarillo of each brand was puffed by machine 8 or 9 times, or until the butt length (unburned fraction) was the greater of 23 mm, the length of the filter + 8 mm,

or the length of the overwrap + 3 mm. Most of the cigarillos in this study did not have filters. In those cases, 23 mm was used as butt length. Smoke was passed through previously tared GFF and the filters were re-weighed to determine the mass of TPM collected. For the in vitro studies, 7 (for CI) and 13 (for ISO) cigarettes and 6 (for CI) and 10 (for ISO) cigarillos were used to generate a minimum of 100 mg TPM from each type of cigarette/cigarillo. TPM was extracted into DMSO to a final concentration of 40 mg/ml. Filtered aliquots were stored in glass vials at -20°C for use in chemical and in vitro assays.

Nicotine, cotinine, propylene glycol, glycerin, and particle size were measured in the TPM. The levels of TSNAs, PAHs, and carbonyls were measured from the GVP. Data are presented in Tables 2-5 as means and standard deviations (SD) which were summarized from all 10 cigarettes and cigarillos products (either as combination of both ISO and CI regimens or as single regimen). Test of product type difference (cigarillo versus cigarette) was implemented in ANOVA (analysis of variance), while products' puffing effect was adjusted in the Type III Sum of squares.

Nicotine in TPM

LC-MS was used to quantify nicotine levels in TPM DMSO extracts. One hundred microliters (µL) of filter extract was diluted with 1900 µL of water/methanol (80/20, v/v) in two series with a final 400-fold dilution factor (20-fold dilution for each step, sample/diluent, 100/1900, v/v, 2 steps in series). One hundred µL of 400-fold diluted filter extract was further mixed with 900 µL of the internal standard working solution

(nicotine-d4, at 40 ng/ml in water with 0.1% formic acid) for the LC-MS assay. A standard curve was used for each run at the concentration range from 5.0 ng/ml to 1000 ng/ml for nicotine. To ensure the accurate quantitation of unknown samples, QC samples were used throughout the run. Relative standard deviation was kept at ≤ 15%.

Nicotine and Cotinine in Smoke

GVP samples from cigarillos and cigarettes were collected on 25 mm GFF during exposure in a smoke chamber. Smoke samples were generated in triplicate for each puffing protocol and product, and expressed as mean µg/puff ± SD. Post exposure, 5 µg nicotine-D4 and 5 µg cotinine-D3 was spiked onto the filters. After drying, filters were extracted with 1 ml of dichloromethane. GLP-compliant GC-MS was used to measure nicotine and cotinine levels. QC samples were used throughout the run. Repeatability for the relative standard deviation was ≤ 15%. Limits of detection for nicotine and cotinine were 10 and 0.25 µg/ml, respectively. Cotinine was determined in the GVP only.

Tobacco Specific Nitrosamines (TSNA)

LC-MS/MS was used to determine TSNA levels (NNN, NNK, and NNAL). Smoke was collected in triplicate on GFF for each puffing protocol and product. TSNAs were extracted from filters with aqueous ammonium acetate following the addition of the internal standard (NNAL-d5) and were expressed as mean ng/puff ± SD. The results were quantitated in a linear range of 1 to 1000 ng/mL and were qualified using QC standards throughout the run. Concentrations were back calculated from dilutions to

determine the amount of each TSNA deposited onto GFF. The limit of detection for all analytes was 1 ng/ml.

Polycyclic Aromatic Hydrocarbons (PAH)

A GC-MS method was used to determine PAH levels deposited on GFF. BF, BkF, BA, BaP, and CH were selected for analysis based on their abundance in tobacco products and availability of a good standard. Chrysene-d12 was used as an internal standard for all five analytes. The CI or ISO smoking regimens were used to generate the smoke from each product from three puffs collected on a GFF. PAHs were extracted from the filters by adding aqueous ammonium acetate to each filter following the addition of the internal standard (NNAL-d5). The results were quantitated over a linear range of 2.5 to 50 ng/mL and qualified with QC standards and spikes to meet pre-defined acceptance criteria for the five analytes. Concentrations were then back-calculated to determine the amount of each PAH and expressed as ng/puff. The limit of detection for each analyte was 2.5 ng/ml.

Carbonyls

A LC-MS/MS method was used to determine the amount of acetaldehyde, acetone, acrolein, crotonaldehyde, diacetyl, and formaldehyde in the smoke generated from the cigarette and cigarillo products. Acrolein-DNPH_d3 and Crotonaldehyde-DNPH_d3 were used as internal standards for all five analytes. A DNPH Cartridge Column was used to collect smoke from cigarettes and cigarillos. A single puff from each tobacco product was collected in a syringe and 5 mL of the sample was then pushed through the DNPH cartridges using a syringe pump at a flow rate of 10 mL/min. 2 mL of acetonitrile

was added to each cartridge column and the extract collected. 200 µL of extract was diluted with 800 µL of water/methanol (90/10, v/v) in a 2 mL sample vial, then analyzed via LC-MS/MS. The results were quantitated over a linear range of quantitation of 1 to 1000 ng/mL for all five analytes, and qualified using quality control samples. The calibration and qualification met pre-defined QC acceptance criteria. The limit of detection was 20 ng/mL for each analyte and the amounts of carbonyls were expressed as µg/puff for mean ± SD from 3 independent measurements. Analytes below the limit of quantification were set to zero for statistical analyses.

Propylene Glycol (PG) and Vegetable Glycerin (VG)

A GC-FID method was used to determine the amount of PG and VG in cigarettes and cigarillos. A six-point standard calibration curve from 50 µg/mL to 1000 µg/mL with an internal standard of 1,4-Butanediol was used to quantify target compounds for the samples. Samples were collected onto 25 mm GFF. The exposed filters were extracted with a 90:10 mixture of methanol and water containing an internal standard of 1,4-Butanediol. Three independent samples were analyzed for each product and reported as mean ± SD. Analytes below the limit of quantification were set to zero for statistical analyses.

Particle Size Distribution

Particle size distribution was measured using a TSI Aerodynamic Particle Sizer (APS, Model 3321, TSI, Inc., Shoreview, MN) with a Smoke Diluter (3302A, 100:1 dilution ratio, TSI, Inc., Shoreview, MN) and a TSI Fast Mobility Particle Sizer (FMPS, Model

3091, TSI, Inc., Shoreview, MN). Each test article was collected in a syringe using both CI and ISO methods. The collected product was then delivered into a 0.28 m^3 chamber connected to the APS or FMPS for sampling. The count median aerodynamic diameter (CMAD) and mass median aerodynamic diameter (MMAD) with geometric standard deviations (GSDs) were determined for both instruments.

Cytotoxicity Assay

The NRU assay was used to measure cytotoxicity in a lung cell line (HBEC4, Repetto et al., 2008). Cells were grown to confluence of ~80% in a 96-well plate. Negative (1% DMSO) and positive (1% SDS) controls along with increasing exposure concentrations of TPM generated from the cigarettes and cigarillos were added to the wells (N = 3 replicates). The test plate was incubated with the test articles for 24 hours at 37°C. Studies were conducted without S9 microsomes. The extent of cytotoxicity was determined by dividing the absorbance readings of the different test article doses by the absorbance seen with the vehicle control. Dose-finding studies were conducted using TPM obtained from the mainstream smoke of the Kentucky 3R4F reference cigarette using the ISO and CI smoking regimens. The dose response studies with the Kentucky 3R4F cigarette identified a final concentration range for TPM of 0.0039 to 0.125 mg/ml. The six doses of TPM selected for study were 0.0039, 0.0078, 0.0156, 0.0313, 0.0625, and 0.125 mg/ml. The IC_{50} for each test article was calculated using probit analysis.

Genotoxicity Assays

Ames Assay

The Ames Modified ISO kit was used according to the manufacturer's instructions (https://www.ebpi-kits.com). Dose finding studies were conducted using TPM from the 3R4F research cigarette ±S9 microsomes, smoked by the ISO and CI regimens. Ten doses over a 3–4 log range with 5 mg/ml as the top concentration were tested for signs of cytotoxicity (reduction in turbidity at 600 nm of the TA98 and TA100 bacteria strains). Four doses spanning half-log intervals from 0.01 to 0.4 mg/mL were selected, with survival ranging from equivalent to negative control, to 10-15%. our doses of test article were selected for testing (0.01, 0.04, 0.13, or 0.4 mg/ml) +/-S9. *Salmonella typhimurium* strains TA100, which detects base pair substitutions, and TA98, which detects frame-shift mutations, were used in the assay, as they have shown mutagenic activity in response to different tobacco smoke constituents (e.g., BaP, CH, BA; Apostoli et al., 1993; Yuan et al., 2007). In the absence of S9, positive controls were sodium azide and 2-nitrofluorene for TA100 and TA98, respectively. In the presence of S9, the positive control for either bacterial strain was 2-aminoanthracene.

MN Assay

A flow cytometry-based MN assay was carried out (Bryce et al., 2007). Lung A549 cells were used for this assay as they are more adherent and resistant to multiple washing steps compared to HBEC4 cells. The four doses studied with the test articles, as determined from the cytotoxicity assay, were 0.0156, 0.0313, 0.0625, and 0.125 mg/ml TPM. This was based on the highest dose causing 40-50% cell death and the lowest dose having no effect on viability in the NRU assay using A549 cells. The half-log dose intervals were used as the generation of micronuclei is most informative in cells that

have completed one round of mitosis during or after treatment with the test substance. Micronuclei formation was quantified using the Litron *in vitro* micro-flow kit (Litron Laboratories, Rochester, NY) in conjunction with flow cytometry. Cells were incubated in quadruplicate with negative (1% DMSO) and positive controls (BaP +S9 as the clastogenicity control, vinblastine without S9 as the aneugenicity control). Micronucleus scoring was performed as described in the Litron protocol (http://litronlabs.com/in_vitro_micronucleus.html) using a Becton Dickinson FACSCaliber flow cytometer and FACS DIVA8 software.

Thymidine Kinase Assay

L5178Y TK+/- cells were treated with four doses of test article (0.015, 0.031, 0.062, and 0.125 mg/m) +/-S9 at 37 ± 0.5 °C for 4 hours. The selection of doses followed the same strategy used for the Ames test. Negative control and positive control (4-nitroquinolineoxide without S9 and BaP with S9) were included in each batch of experiments. The MF was calculated and adjusted based on the survival percentage. The toxicity of the test agent was indicated by a decrease in colony forming efficiency in plates without selection medium. Mutagenicity is evidenced by the increase in mutation frequency based on the number of mutants. For individual test articles, an increased mutation frequency with biological relevance was defined as total MF equal or greater than 126 mutants per 10^6 plated cells (OECD Method 490).

Statistical Analysis

NRU Assay: An ANOVA test was used for the comparison of IC_{50} between products with adjustment for puffing protocol and the paired t-test was used for the comparison between puffing protocols.

Micronucleus Assay: The difference between any of the treatment groups versus control was tested using Poisson regression. The number of cells with micronuclei or apoptotic cells per 10,000 interrogated cells was used as the readout for the assay. Group-wise comparisons were conducted for the comparison between cigarettes and cigarillos and between puffing protocols using Poisson regression, followed by effect size analysis. A generalized estimating equation (GEE) was used to assess whether product type and smoking regimen had any effect on induction of micronuclei and apoptosis. The effects of product type and puffing protocol on micronuclei or apoptosis were measured under different doses with different nicotine products. Doses and nicotine products were included in the model and adjusted as covariates.

Ames Assay: Group-wise comparisons were conducted for the comparison between cigarettes and cigarillos and between puffing protocols using logistic regression. Logistic regression was also used to evaluate product differences as a function of dose with adjustment for puffing protocol.

Thymidine Kinase Assay: The repeated measurement model was used to assess whether product type and puffing had any effect on induction of total mutant frequency and cloning efficiency in the viability assay.

Results

Nicotine and Cotinine

The magnitude and range of nicotine levels was greater for the GVP fraction than for TPM for both cigarettes and cigarillos (mean values of both ISO and CI regimens shown, Table 2. Nicotine levels in cigarettes exceeded those seen in cigarillos by 51% (2.59 [sd = 0.24] vs. 1.71 [sd = 0.46]). Nicotine content in the GVP fraction was greater in the CI puffing protocol. Cotinine levels varied from 0.37 – 1.18 µg/puff for cigarettes and 0.55 – 4.26 µg/puff for cigarillos (mean values shown in Table 2, results are from GVP only). In contrast to nicotine, cotinine levels were 3.4-fold greater in cigarillos compared to cigarettes ($p < 0.05$).

TSNAs

The amounts of NNN and NNK (ng/puff) in cigarillos were significantly higher than in cigarettes ($p = 0.05$, Table 3). For cigarettes, the range of NNN and NNK was 4.2 – 28.6 and 3.3 – 17.6 ng/puff, respectively (mean values of combined ISO and CI regimens or single regimen shown in Table 3). For cigarillos, the range of NNN and NNK was greater and varied more dramatically across products (NNN, 9.8 – 120.4; NNK, 6.6 – 57.0, mean values shown in Table 3). NNAL was BLOQ for all but one cigarette product (which was set to zero for statistical purposes) and was detected at low levels in most cigarillos. The CI regimen resulted in significantly increased TSNA levels compared to the ISO regimen for both product types (NNN and NNK; $p<0.05$, respectively, Table 3 and Fig. 3c).

PAHs

All PAHs except BF were significantly greater in cigarillos than cigarettes, with mean fold differences of 3 – 7 (p <0.05 except BF, Table 4). CH and BA were the most abundant PAHs, irrespective of product or puffing protocol, followed by BP and BF/BkF (Table 4). There was less variation in the magnitude of PAH measured across cigarettes and cigarillos than seen for TSNAs, and PAH levels were much lower as a group than TSNAs. In general, the CI protocol yielded higher levels of PAHs than the ISO regimen (Fid. 4a) although statistical differences were not always seen due to low absolute levels (e.g., BF, Table 4).

Carbonyls

Acetaldehyde and acetone were the most abundant carbonyls detected in all products, followed by diacetyl (Table 5). Acetaldehyde also showed the most variation in concentration across both product types, or from 2.8–3.4-fold. The variation in diacetyl was also high in the cigarillo products ~15-fold for ISO and 20-fold for CI. All carbonyls except acrolein were significantly more abundant in the cigarillos compared to cigarettes (Table 5). The CI regimen led to increased carbonyls in cigarettes and cigarillos compare to the ISO regimen (p<0.05 for both cigarettes and cigarillos, Fig. 3b).

PG and VG

Cigarettes contained low to undetectable levels of propylene glycol, while glycerin was present at levels of 37.8 – 108 µg/puff (mean values shown, Table 6). Most cigarillos contained quantifiable levels of propylene glycol, while detection of glycerin was more sporadic. Group comparison showed on average 3-fold greater amounts of propylene

glycol in cigarillos than cigarettes, while glycerin was more abundant in cigarettes (Table 6). The more intensive CI puffing protocol compared to ISO yielded higher levels of these constituents from both tobacco products (Table 6).

Particle Size

There was no statistically significant difference across products with respect to the size of large particles quantified through APS (Table 6). In contrast, both the CMAD and MMAD small particle quantitation were slightly, but significantly higher for cigarillo products compared to cigarettes (Table 6). There was little to no effect seen for the puffing protocol, although some individual values were statistically significant (Table 6).

Cytotoxicity (NRU assay)

The IC_{50} calculated from TPM generated from the cigarettes ranged from 3.6 to 28.8 µg/ml, while the range seen for cigarillos was 2.4 to 35.5 µg/mL (Table 7). Seven of the ten most potent products for inducing cytotoxicity were cigarillos including Black & Mild Cigarillos Natural Apple, Black & Mild Tip Natural Cigarillos, Dutch Masters Palma Natural Corona, Swisher Sweets Natural Sweet Cigarillos, Swisher Sweets Sweet Original Cigarillos, and Swisher Sweets Tropical Fusion Cigarillos (data not shown). In addition, the group comparison revealed that TPM from cigarillos was significantly more potent than cigarettes in inducing cell death, with mean of 12.3 ± 9.3 versus 21.0 ± 10.5 µg/ml. There was no significant difference in cytotoxicity as a function of puffing protocol (Table 7, see Supplementary Material 1 for the full set of results).

MN Assay

The geometric means ± geometric standard deviations for DMSO controls with and without S9 were 136.5 ± 1.4 and 135.3 ± 1.5, respectively. Significant induction of micronuclei was observed for BaP with S9 (205 ± 1.4, p=0.05) and vinblastine without S9 (194.5 ± 1.8, p =0.05). A significant induction for apoptosis was also observed for BaP with S9 (504.1 ± 2.6 versus 284.5 ± 2.8 [control], p=0.05), but not for vinblastine without S9 (94.1 ± 2.0 versus 93.0 ± 1.8 [control], NS). Test articles showing at least one dose with significant induction of micronuclei are given in Table 8 (see Supplementary Material 2 for the full set of results). This significance was also evident as shown by increased effect size (e.g., Marlboro Red). A significantly greater induction of micronuclei in cigarillos versus cigarettes was identified for the highest TPM dose (0.125 mg/ml) with (FR=1.9, 95%CI=1-3.5, p=0.05) and without S9 (FR=2.5, 95%CI=1.4-4.3, p<0.05). Significant induction of apoptosis in cigarillos versus cigarettes was identified for the second highest TPM dose (0.0625 mg/ml) with S9 (FR=3.1, 95%CI=1.6-6.3, p<0.05). However, group-wise comparisons did not show any significant difference between cigarettes and cigarillos with respect to micronuclei formation or induction of apoptosis (Table 8). There was also no effect of smoking protocol on these endpoints with exception being apoptosis in the absence of S9 (Table 8). An exploratory trend test was also conducted to evaluate the dynamics between dose and increase in micronuclei and apoptosis. For every 1 mg/ml increase in dose of TPM, the frequency ratio (FR) of micronuclei was shown to increase 2.1 (95% CI=0.7-6.0, p=0.16) with S9 or 2.2 (95% CI=1.2-4.1, p=0.01) without S9. The FRs for apoptosis

increased dramatically to 33.1 (95% CI=1.0-1089.5, $p<0.05$) with S9 and to 1704.9 (95% CI=5.8-486195.1, $p<0.01$) without S9 under the same conditions.

Ames Assay

Although a few significant findings were seen with any test article or strains in the absence of S9, there was no clear dose response when statistical significance was achieved. In contrast, TA98 in the presence of S9 showed high mutation positivity in response to cigarettes and cigarillos that often was saturated by the 0.13 mg/ml dose. At the lowest dose (0.01 mg/ml), two cigarettes and eight cigarillos were positive for mutation induction. The TA100 (+S9) showed significantly increased mutation frequency for cigarillos compared to cigarettes at the 0.04 and 0.13 mg/ml doses. Overall comparison between product groups, after adjusting for dose and puffing protocol, showed a significant difference for only the TA100 (+S9) group ($p<0.05$, Table 8; see Supplementary Material 3 for the full set of results). Puffing protocol with adjustment for dose showed very modest differences for cigarettes and cigarillos with only cigarillos TA100 –S9 reaching statistical significance ($p<0.05$) (Table 7 and 8).

TK Assay

Average mutation frequencies for DMSO controls with and without S9 were 97.8 ± 45.2 and 89.6 ± 25.1 per 10^6 cells (p=NS), respectively (Table 8; see Supplementary Material 2 for the full set of results). All test articles except “Black & Mild Tip Natural Cigarillo” had at least one dose showing an induced total MF. These samples showed either dose response or positivity at only the highest dose (Table 9; see Supplementary Material 2).

The average percentage of small colonies for the highest dose (0.125 mg/ml) was 84% (range of 59-100%) and 76% (range of 51-97%) for positive test articles with and without S9, respectively. Group-wise comparisons for dose response in the presence of S9 showed significantly increased MF for cigarillos compared to cigarettes for three of the four doses (0.0156, 0.0625, and 0.125 mg/ml, means shown in Table 8). In the absence of S9, mutation frequency did not differ significantly between cigarillos and cigarettes, indicating the need for metabolic activation of the mutagens. Group comparisons adjusting for dose also showed significantly greater mutation frequency for cigarillos compared to cigarettes with S9 (Table 8). There was no effect of puffing protocol by group (Table 8).

Genotoxicity Comparison Across Assays

Dose response was assessed for Ames (TA98 and TA100), micronuclei, and TK assays with S9 to estimate slopes, standard errors, and *p* values for all test articles with TPM generated by the two puffing protocols. Correlation analysis based on ranking suggested a significant correlation of the results between TK and micronuclei (Spearman correlation coefficient=0.37, $p<0.05$), while these two assays showed no correlation with Ames assay (all *p*-values NS). The lack of correlation among the three assays may be because TK and micronuclei assays use mammalian cells, while Ames assay uses bacteria. Table 9 shows the rank by assay with #1 being the most mutagenic and #40 being the least mutagenic. We observed that TA98 was most informative for mutagenicity, compared with TA100, for which many products did not demonstrate a dose response. There were also a number of products which did not

demonstrate a dose response in the MN assay, and several for the TK assay, as shown in Table 9.

Correlation Between Nicotine, TSNA, PAH and Carbonyl Levels and In Vitro Cytotoxic and Genotoxic Endpoints

Additional analyses were conducted to assess the contribution of nicotine, TSNAs, total PAHs, and total carbonyls to IC_{50} determined through NRU and all genotoxicity endpoints. Pearson correlation analysis identified either minimal or no correlations (< 0.5, > -0.5) among the four categories of chemicals across the twenty test articles and two puffing protocols (Figure 5). Nicotine has a significant inverse correlation with PAHs (Pearson correlation coefficient=-0.35, p=0.02), while carbonyls have a positive correlation with PAHs and nitrosamines (Pearson correlation coefficients ≥ 0.45, p<0.01). A GEE model used to assess the association of these four categories of chemicals and the outcomes with four treatments found no consistent pattern of genotoxicity of chemical categories under different assays (Figure 5).

Discussion

The findings of the present study showed that, under the test conditions (both ISO and CI), smoke from ten commercially available cigarillos demonstrated increased toxicity in vitro compared to ten commercially available cigarettes in the US. Compared to cigarettes, tested cigarillos contained greater levels of three major classes of harmful and potentially harmful constituents (HPHCs), but a lower level of nicotine. Notably,

mean levels of carcinogenic HPHCs NNN and NNK were 2.0 – 2.4-fold higher in the smoke of cigarillos compared with cigarettes. A mean 3 to 7-fold increase in PAHs was also seen in the smoke of cigarillos compared to cigarettes. In addition, mean values of all measured carbonyl compounds except acrolein were higher (~1.5 fold) in the smoke of cigarillos compared to cigarettes. These findings are in line with previous findings reporting higher levels of chemical compounds in tar extracts from little cigars (not cigarillos) relative to Kentucky reference cigarettes (Ghosh et al., 2017).

There was, on average, a 3-fold greater amount of propylene glycol in the more toxic cigarillos than cigarettes, while glycerin was more abundant in cigarettes, and there were higher levels of PG and VG from both tobacco products under the CI puffing protocol. A previous study found that PG produced in vitro DNA-damage leading to chromosome mutations in the presence and absence of the S9 Mix (Aye, 2010). However, it is not clear whether PG-induced chromosomal mutations occur in vivo and whether this effect occurs with exposure to PG via the inhalation pathway.

Particle size is important in predicting the deposition of the inhalation of particles by smokers, with particles below 3 to 5 μm being in the respirable range for humans (Brown, 2013) and particles in the range of 0.1 – 2.0 μm contributing heavily to the mass of TPM (Li, 2014). There was no statistically significant difference across products with respect to the size of large particles however small particle quantitation was slightly but significantly higher for cigarillo products compared to cigarettes, while puffing protocol had no effect.

Significantly, the TPM generated from the cigarillos was more toxic across products compared to cigarettes for inducing in vitro cytotoxicity (human respiratory HBEC4 cells), genotoxicity (*S. typhimurium* TA98/TA100), and mutagenicity (human lung A549, mouse lymphoma L5178Y TK+/- cells) under the test conditions. Seven of the ten most potent cytotoxic products as measured by the NRU assay were cigarillos. Increased HPHC levels generated by cigarillos may drive this toxicity. The increased levels of the TSNA's NNN and NNK could result from greater burley tobacco content (Hofmann and Hoffmann, 1997; Shi et al., 2013). The increased PAH content could result from lower porosity of the cigarillo wrapping material compared to cigarette paper. The inclusion of S9 for genotoxicity assays showed the most significant and dose-dependent effects for both cigarettes and cigarillos, consistent with the fact that many mutagens are pro-mutagens requiring metabolic activation.

Comparing genotoxic responses induced by cigarettes and cigarillos, Ames and TK assays showed differences between these products. Group comparison showed significant mutation induction for the TA100 treatment group (+S9, $p=0.003$) and a greater TK mutation frequency (+S9, $p=0.04$) for cigarillos compared to cigarettes. The genotoxicity ranking of samples (Table 9) showed that among all forty samples tested, 39, 10, 35, and 16 samples had a dose-dependent response for TA98, TA100, TK, and MN assays, respectively. Correlation analysis based on ranking suggested a significant correlation between TK and MN results ($P=0.018$). However, the results of these two assays were not correlated with the results from Ames assay (p-values ≥ 0.08). This

may be consistent with the fact that bacteria and mammals have different chromatin structure, antioxidant capacity, and DNA repair mechanism. The contribution of nicotine, total TSNAs, PAHs, or carbonyls to in vitro cytotoxicity and genotoxicity endpoints was not consistently associated by chemical category. For example, for treatments with S9, nitrosamines and carbonyls were significantly associated with mutation frequency in the Ames assay, while PAHs were significantly associated with mutation frequency in the TK assay. The differences between the results of these two assays may be due in part to the specificity of test strains in detecting mutations and sensitivity of TK cells to bulky adducts such as those formed by PAH's. HPHC yields under the ISO and CI regimens were not equivalent or linearly correlated. The CI smoking regimen showed higher amounts of TSNA, PAH, and carbonyls than the ISO regimen which is expected given the difference in puffing regimens. However, there was no consistent contribution of these increases to the measured cytotoxicity or genotoxicity endpoints. This might be due to the omission of the GVP from in vitro testing, and therefore needs to be interpreted with caution.

Kirkland et al. (2005, 2006) evaluated the ability of three in vitro genotoxicity tests (Ames, MLA, MN) to discriminate between rodent carcinogens and non-carcinogens in terms of sensitivity, specificity, and relative predictivity. 93% of the rodent carcinogens evaluated in at least one assay gave positive results in at least one of the three tests, and combinations of two and three test systems had greater sensitivity than individual tests, with resultant sensitivity of about 90%. However, 75-95% of non-carcinogens gave falsely positive results in at least one out of the three tests, indicating low

specificity. Using the relative predictivity (RP), the ratio of real:false results, the authors reported that 3 positive test results indicates a chemical is greater than 3 times more likely to be a rodent carcinogen than a non-carcinogen, and 0 positive result in all tests shows that the chemical is greater than two times more likely to be a rodent non-carcinogen than a carcinogen, a useful adaptation. Five of the 40 comparisons made in the present study yielded only 1 of 3 assay results positive (Ames), while the other 35 comparisons yielded 2 or more different assay results positive and 16 of the product comparisons had positive results in all 3 assays, indicating likely positivity by the above criteria.

There are no in vivo studies of cigarillo toxicity and only a sparse amount of in vitro toxicity testing. Rickert et al. (2011) compared the cytotoxic, mutagenic, and genotoxic properties of mainstream smoke from Canadian market cigarillos to mainstream smoke of Kentucky reference research cigarettes. They found that mainstream cigarillo smoke was equally or more toxic than mainstream cigarette smoke, however, no cigarillos were identified by brand. Although this study also used Ames, NRU, and MN assays, the study by Rickert el al. (2011) did not characterize the smoke chemistry and used only the CI regimen for testing. Therefore, no direct comparison of smoking regimen-dependent toxicity can be made. However, our data is in line with their conclusions and reports additional differences in toxicity between ISO and CI regimens for the two types of products, based on chemical analyses. In addition, Blank et al. (2011) studied the cardiovascular response, toxicant exposure, and puffing topography for Black & Mild cigarillos and reported significant amounts of carbon monoxide exposure in users of

these products compared with non-users. More recently, Ghosh et al. (2017a) showed that little cigars pose more harm than cigarettes under the conditions of their study as they exhibited greater cytotoxicity and pro-inflammatory cytokine secretion, decreased cilia function, changes in airway gene expression, and alterations in airway genomic and proteomic profiles compared to Kentucky reference research cigarettes. This group also analyzed the chemical profile of little cigars and cigarettes and identified 49 unique compounds and higher levels of tobacco constituents in little cigars compared to Kentucky reference research cigarettes, which could help explain drivers of toxicity. For both flavored and non-flavored Swisher Sweets little cigars, the authors found that acute smoke exposure significantly increased cell death compared with controls, while 4 days whole smoke exposure increased necrosis and apoptosis. The observed toxicity of little cigars was attributed to the increased chemical load. Overall, our results confirm previous findings that the smoke of cigar products exhibits greater toxicity compared to that of cigarettes under testing conditions.

Finally, the perception that cigarillos are less harmful than cigarettes might be related to smoking behavior patterns such as smoking them less frequently and not smoking a whole product at once (Baker et al., 2001) and lack of effective communication to the public on health risks of these products. Although cigarillo-specific information is not available, occasional cigar smokers are reported to smoke more frequently or inhale more deeply potentially leading to increased toxicity to the user and may account for greater toxicant exposure compared with cigarette smoking (Monograph 9: Cigars: Health Effects and Trends, NCI, 1998).

The strengths of the present study are, primarily, that it provides the first comparative toxicity assessment of selected marketed, branded cigarillos and cigarettes in the US using a basic battery of in vitro tests. Secondly, this study used two different standardized smoke machine protocols, non-intense ISO and intense HCI smoking regimens, which encompasses a wider spectrum of potential HPHC exposures for most smokers and allows for more complete evaluation of toxicity. Lastly, we found greater HPHC generation from the smoke of cigarillos compared to cigarettes, confirming previous findings on cigar products and further extending them by evaluating the correlation between chemical profile and in vitro toxicity in top marketed cigarillos and cigarettes. A limitation of this study, however, is the inclusion of only TPM in testing. As the GVP fraction contains several known carcinogens (e.g., formaldehyde) and acutely-toxic substances (e.g. acrolein), testing both the TPM and GVP phases of tobacco smoke would more completely represent overall smoke toxicity. Therefore, the results of comparative in vitro genotoxicity need to be interpreted with caution since we evaluated only one phase of smoke, and therefore, may not reflect the overall genotoxic potential of these products under physiological conditions. Moreover, while, interpretation of standard in vitro genotoxicity studies is usually binary (positive or negative) based on specific criteria, relative comparisons such as the one made herein are useful but must be interpreted carefully. In this case, there were clear differences between the classes of products tested that were important to report. Given the increasing reliance of in vitro methods, more work that addresses the relative

comparison of in vitro genotoxicity results for tobacco mixtures, constituents, and other test articles is a fruitful avenue for future research.

In summary, this is the first study comprehensively examining the smoke chemistry and comparative in vitro toxicity of a selection of commercially available U.S. cigarillos and combustible cigarettes. Our findings show that there is a significant difference between the tested US marketed cigarillos and cigarettes for tobacco constituent levels, cytotoxicity, and genotoxicity, and importantly that, on a 'puff for puff' basis, cigarillos demonstrated a higher mutation frequency and greater level of cytotoxicity than cigarettes under testing conditions. These findings are important, not only for improved understanding of the toxicity from the use of cigarillos for defined endpoints relative to cigarettes, but also for building upon knowledge regarding harm from cigarillos to inform risk mitigation strategies.

References

Amrock SM, Lee L, Weitzman M. (2016). Perceptions of e-Cigarettes and Noncigarette Tobacco Products Among US Youth. Pediatrics 138, e20154306.

Apostoli P, Crippa M. Fracasso ME, Cottica D, Alessio L. (1993). Increases in polycyclic aromatic hydrocarbon content and mutagenicity in a cutting fluid as a consequence of its use. Int Arch Occup Environ Health 64, 473-477.

Aye M, Di Giorgio C, De Mo M, Botta A, Perrin J, Courbiere B. (2010). Assessment of the genotoxicity of three cryoprotectants used for human oocyte vitrification: dimethyl sulfoxide, ethylene glycol and propylene glycol. Food Chem Toxicol, 48, 1905-1912.

Baker F, Dye JT, Denniston MM, Ainsworth SR. Risk perception and cigar smoking behavior. Am J Health Behav. 2001;25(2):106.

Blank, M.D., Nasim, A., Hart, A. Jr, Eissenberg, T. (2011). Acute Effects of Cigarillo Smoking. Nicotine Tob Res 13, 874–879.

Bryce SM, Bemis JC, Avlasevich SL & Dertinger SD. (2007). In vitro micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity. Mutat Res 630, 78-91.

Brown, J. (2013). Thoracic and respirable particle definitions for human health risk assessment. Part Fibre Toxicol 10,12.

CDC: US Centers for Disease Control and Prevention, Youth Risk Behavior Surveillance – United States, 2013. Morbidity and Mortality Weekly Report, June 13, 2014, Vol. 63, No. 4

Chang CM, Corey CG, Rostron BL, Apelberg BJ. (2015). Systematic review of cigar smoking and all cause and smoking related mortality. BMC Public Health, 24;15:390.

Chen J, Kettermann A, Rostron BL, Day HR. (2014). Biomarkers of exposure among U.S. cigar smokers: an analysis of 1999-2012 National Health and Nutrition Examination Survey (NHANES) data. Cancer Epidemiol Biomarkers Prev 23, 2906-2915.

Ghosh A, Abdelwahab SH, Reeber SL, Reidel B, Marklew AJ, Garrison AJ, Lee S, Dang H, Herring AH, Glish GL, Kesimer M, Tarran R. (2017a). Little Cigars are More Toxic than Cigarettes and Uniquely Change the Airway Gene and Protein Expression. Sci Rep 27, 7:46239.

Ghosh A, Nethery RC, Herring AH, Tarran R. (2017). Flavored little cigar smoke induces cytotoxicity and apoptosis in airway epithelia. Cell Death Discov, 3:17019.

Hamad SH, Johnson NM, Tefft ME, Brinkman MC, Gordon SM, Clark PI and Buehler SS. (2017). Little Cigars vs 3R4F Cigarette: Physical Properties and HPHC Yields. Tob Regul Sci 3, 459-478.

Hoffmann D and Hoffmann I. (1997). The changing cigarette 1950-1995. J Toxicol Environ Health 50, 307-364.

Inoue-Choi M, Shiels MS, McNeel TS, Graubard BI4, Hatsukami D, Freedman ND. (2019). Contemporary Associations of Exclusive Cigarette, Cigar, Pipe, and Smokeless

Tobacco Use With Overall and Cause-Specific Mortality in the United States, JNCI Cancer Spectr 3, pkz036.

Jablonski JJ, Hunter Maines, AG, Cheetham I, Gillman G. (2019). Comparative levels of carbonyl delivery between mass-market cigars and cigarettes. Regul Toxicol Pharmacol 108, 104453.

Jensen OM, Knudsen JB, McLaughlin JK, Sørensen BL. (1988). The Copenhagen case-control study of renal pelvis and ureter cancer: role of smoking and occupational exposures. Int J Cancer 41,557-561.

Kirkland D, Aardema M, Henderson L and Muller L. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens I. Sensitivity, specificity and relative predictivity. Mutat Res. 2005 Jul 4; 584(1-2):1-256. Doi: 10.1016/j.mrgentox.2005.02.004

Kirkland, D, Aardema M, Muller L and Makoto H. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens II. Further analysis of mammalian cell results, relative predictivity and tumour profiles. Mutat Res. 2006 Sep 19;608(1):29-42. Doi: 10.1016/j.mrgentox.2006.04.017.

Koszowski B, Rosenberry ZR, Yi D, Stewart S, Pickworth WB. (2017). Smoking Behavior and Smoke Constituents from Cigarillos and Little Cigars. Tob Regul Sci 3(Suppl 1), S31-S40.

Li X, Kong H, Zhang X, Peng B, Nie C, Shen G and Liu H. (2014). Characterization of particle size distribution of mainstream cigarette smoke generated by smoking machine with an electrical low pressure impactor. J Env Sci 26, 827-833.

Majewski U, Piotrowska M, Sychowska I, Banas D, Kubala-Kukus A, Wudarczyk-Mocko J, Strabrawa I, Gozdz S. (2018). Mutielemental Analysis of Tobacco Plant and Tobacco Products by TXRF. J Anal Toxicol 42, 409-416.

Maxwell JC. The Maxwell Report: Year End & Fourth Quarter 2013 and 2014 Cigarette Industry. Richmond, VA: John C. Maxwell, Jr., 2014.

Monograph 9: Cigars: Health Effects and Trends, NCI, 1998, https://cancercontrol.cancer.gov/brp/tcrb/monographs/9/index.html

Nyman AL, Sterling KL, Majeed BA, Jones DM and Eriksen MP. (2018). Flavors and Risk: Perceptions of Flavors in Little Cigars and Cigarillos Among U.S. Adults, 2015. Nicotine Tob Res 20, 1055-1061.

OECD Test No. 490: In Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene, OECD Guidelines for the Testing of Chemicals, Section 4, Organisation for Economic Cooperation and Development

Pickworth WB, Rosenberry ZR, Yi D, Pitts EN, Lord-Adem W, Koszowski B. (2018). Cigarillo and Little Cigar Mainstream Smoke Constituents from Replicated Human Smoking. Chem Res Toxicol 31, 251-258.

Pickworth WB, Rosenberry ZR, O'Grady KE, Koszowski B. (2017). Dual Use of Cigarettes, Little Cigars, Cigarillos, and Large Cigars: Smoking Topography and Toxicant Exposure. Tob Regul Sci 3(Suppl 1), S72-S83.

Repetto G, del Peso A, & Zurita JL. (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc 3, 1125-1131.

Rickert WS, Trivedi AH, Momin RA, Wagstaff WG, Lauterbach JH. (2011). Mutagenic, cytotoxic, and genotoxic properties of tobacco smoke produced by cigarillos available on the Canadian market. Regul Toxicol Pharmacol 61,199-209.

Rostron BL, Cheng Y, Gardner LD and Ambrose BK. (2020). Prevalence and Reasons for Use of Flavored Cigars and ENDS among US Youth and Adults: Estimates from Wave 4 of the PATH Study, 2016-2017. Am J Health Behav 44,76-81.

Rostron BL, Corey CG, Holder-Hayes E, Ambrose BK. (2019). Estimating the Potential Public Health Impact of Prohibiting Characterizing Flavors in Cigars throughout the US. Int J Environ Res Public Health 16, 3234.

Shi H, Wang R, Bush LP, Zhou J, Yang H, Fannin N, Bai R, (2013). Changes in TSNA contents during tobacco storage and the effect of temperature and nitrate level on TSNA formation. J Agric Food Chem 61, 11588–11594.

Soneji S, Sargent J, Tanski S. (2016). Multiple tobacco product use among US adolescents and young adults. Tobacco Control 25,174-180.

Sterling KL, Fryer CS and Fagan P. (2016). The Most Natural Tobacco Used: A Qualitative Investigation of Young Adult Smokers' Risk Perceptions of Flavored Little Cigars and Cigarillos. Nicotine Tob Res 18, 827–833.

Trapl ES, O'Rourke-Suchoff D, Yoder LD., Cofie LE, Frank JL, and Fryer CS. (2017). Youth Acquisition and Situational Use of Cigars, Cigarillos, and Little Cigars: A Cross-Sectional Study. Am J Prev Med 52, e9-e16.

US DHHS, NIH, NCI, Division of Cancer Control & Population Sciences. Behavioral Research Program. Monograph 9: Cigars: Health Effects and Trends. May 21, 2012. Accessed May 17, 2019

Yuan T, Fournier AR, Proudlock R, Marshall WD. (2007). Continuous catalytic hydrogenation of polyaromatic hydrocarbon compounds in hydrogen-supercritical carbon dioxide. Environ Sci Technol 41,1983-1988.

Table 1. Cigarettes and Cigarillos Selected as test articles for the Study

Cigarettes*	**Cigarillos***
Camel	Black & Mild Cigarillos Natural Apple
Camel Menthol	Black & Mild Tip Natural Cigarillos
Marlboro Blue Fresh (Menthol)	Black & Mild Classic/Natural Cigarillos
Marlboro Gold (Lights)	Dutch Masters Palma Natural Corona
Marlboro Red	Swisher Sweets Grape Natural Cigarillos
Newport Box (Full Flavor)	Swisher Sweets Natural Sweet Cigarillos
Newport Menthol Blue	Swisher Sweets Sweet Original Cigarillos
Newport Red (Non-Menthol)	Swisher Sweets Tropical Fusion Cigarillos
Pall Mall Menthol	White Owl Cigarillos
Pall Mall Red	Winchester Original Blend Little Cigar

*Commercial brand names at the time of purchase.

Table 2. Nicotine and Cotinine: Cigarettes & Cigarillos Group Comparisons

Product Type	Mean nicotine in TPM mg/mL (SD)	Nicotine comparison (P-value)	Mean nicotine in GVP µg/puff (SD)	Nicotine comparison (P-value)	Mean cotinine µg/puff (SD)	Cotinine Comparison (P-value)
Cigarettes[a]	2.59 (0.24)	<0.0001*	119.45* (44.37)	0.006*	0.67* (0.25)	<0.0001*
Cigarillos[a]	1.71 (0.46)		82.29* (40.42)		2.27* (0.85)	
Cigarettes with CI	2.50 (0.22)	0.13	142.40* (45.19)	0.003	0.81* (0.23)	0.002
Cigarettes with ISO	2.67 (0.24)		96.50* (30.73)		0.53* (0.53)	
Cigarillos with CI	1.58 (0.47)	0.02	88.18 (44.10)	0.29	2.53 (0.96)	0.086
Cigarillos with ISO	1.84 (0.43)		76.40 (37.78)		2.01 (0.69)	

[a]ANOVA with adjustment for puffing protocol
Puffing comparisons are based on paired T-test
[#]as determined from GVP only
Sample size (cigarettes): N= 7 (CI), N=13 (ISO), (cigarillos): N= 6 (CI), N = 10 (ISO)

Table 3. TSNAs: Cigarettes & Cigarillos Group Comparisons

Product Type	Mean NNN ng/puff (SD)	NNN Comparison (P-value)	Mean NNK ng/puff (SD)	NNK Comparison (P-value)	Mean NNAL ng/puff (SD)	NNAL Comparison (P-value)
Cigarettes	14.44*(7.88)	0.002*	8.35* (4.31)	0.007*	nc	nc
Cigarillos	34.50* (27.56)		16.32* (12.09)		1.45 (0.74)	
Cigarettes with CI	18.95*(8.23)	<0.001	10.45* (4.52)	<0.001	nc	nc
Cigarettes with ISO	9.92* (4.26)		6.25* (3.00)		nc	
Cigarillos with CI	44.35* (32.54)	0.003	20.17* (14.16)	0.002	1.67*(0.89)	0.016
Cigarillos with ISO	24.64* (18.12)		12.46* (8.66)		1.18* (0.39)	

*ANOVA with adjustment for puffing protocol
nc = not calculated
Sample size (cigarettes): N= 7 (CI), N=13 (ISO), (cigarillos): N= 6 (CI), N = 10 (ISO)
Comparison of ISO and CI smoking regimens based on paired T-test

Table 4. PAHs: Cigarettes & Cigarillos Group Comparisons

Product Type	Mean BA ng/puff(SD)	P-value	Mean BF ng/puff(SD)	P-value	Mean BkF ng/puff(SD)	P-value	Mean BP ng/puff (SD)	P-value	Mean CH ng/puff (SD)	P-value
Cigarettes	1.01* (0.27)	<0.0001*	0.58 (0.23)	0.51*	0.31* (0.09)	<0.0001	0.55* (0.15)	<0.0001*	1.51 *(0.34)	<0.0001*
Cigarillos	3.34 *(1.26)		0.65 (0.49)		2.15* (1.00)		2.43* (1.45)		4.45* (1.69)	
Cigarettes w/ CI	1.21* (0.19)	<0.0001	0.70* (0.25)	0.008	0.37* (0.07)	0.0002	0.65* (0.11)	0.001	1.76* (0.20)	0.0002
Cigarettes w/ ISO	0.82* (0. 81)		0.46* (0.15)		0.24* (0.05)		0.46* (0.12)		1.26* (0.26)	
Cigarillos w/ CI	4.07* (0.82)	0.0004	0.86 (0.61)	0.11	4.46 (1.06)	0.15	3.01* (1.41)	0.001	5.23* (1.52)	0.0003
Cigarillos w/ ISO	2.60* (1.22)		0.45 (0.24)		1.88 (0.82)		1.85* (1.30)		3.67* (1.52)	

*ANOVA with adjustment for puffing protocol
ISO vs. CI comparisons are based on paired T-test
Sample size (cigarettes): N= 7 (CI), N=13 (ISO), (cigarillos): N= 6 (CI), N = 10 (ISO)

Table 5. Carbonyls: Cigarettes & Cigarillos Group Comparisons

Product Type	Mean Acetaldehyde µg/puff (SD)	P-value	Mean Acetone µg/puff (SD)	P-value	Mean Acrolein µg/puff (SD)	P-value	Mean Croton-aldehyde µg/puff (SD)	P-value	Mean Diacetyl µg/puff (SD)	P-value	Mean Form-aldehyde µg/puff (SD)	P-value
Cigarettes	54.3 (19.1)	<0.0001*	25.1 (7.84)	<0.001*	0.23 (0.15)	0.33*	0.36 (0.11)	<0.0001*	1.56 (0.72)	0.001*	0.52 (0.20)	0.0004*
Cigarillos	81.1 (27.2)		57.0(16.3)		0.19 (0.09)		0.49 (0.16)		4.26 (3.40)		0.33 (0.17)	
Cigarettes with CI	68.8 (13.1)	<0.0001	31.4 (4.9)	<0.0001	0.32 (0.12)	0.001	0.43 (0.10)	<0.0001	1.99 (0.45)	0.009	0.65 (0.19)	<0.0001
Cigarettes with ISO	39.8 (11.1)		18.9(4.3)		0.13 (0.01)		0.29 (0.06)		1.42 (0.70)		0.40 (0.14)	
Cigarillos with CI	102.1 (21.2)	<0.0001	68.9(13.2)	<0.0001	0.24 (0.09)	0.001	0.61 (0.15)	<0.0001	5.0 (4.0)	0.03	0.42 (0.16)	<0.0001
Cigarillos with ISO	60.0 (11.1)		45.1(8.5)		0.15(0.06)		0.38 (0.06)		3.52 (2.72)		0.24 (0.12)	

*ANOVA with adjustment for puffing protocol
Sample size (cigarettes): N= 7 (CI), N=13 (ISO), (cigarillos): N= 6 (CI), N = 10 (ISO)

Table 6. PG, VG, and Particle Size

Product Type	Mean Propylene Glycol µg/puff (SD)	P-value	Mean Glycerin µg/puff (SD)	P-value	Particle size APS (SD)		P-value CMAD	P-value MMAD	Particle size FMPS (SD)		P-value CMAD	
					CMAD	MMAD			CMAD	MMAD		
Cigarettes	27.5* (34.8)	0.0002*	69.4* (21.4)	0.03*	0.896 (0.12)	1.22 (0.23)	0.57*	0.74*	215.8* (13.8)	338.5* (22.6)	0.0003*	0.0006*
Cigarillos	98.6* (76.1)		42.7* (48.7)		0.877 (0.10)	1.20 (0.18)			238.6* (21.4)	371.7* (32.2)		
Cigarettes with CI	48.3* (33.9)	0.003	82.4* (19.8)	<0.0001	0.878 (0.15)	1.20 (0.229)	0.4	0.72	212.9 (17.4)	218.6 (9.0)	0.45	0.19
Cigarettes with ISO	6.7* (21.1)		56.5* (14.1)		0.913 (0.08)	1.23 (0.16)			330.9 (30.0)	346 (7.2)		
Cigarillos with CI	126.8* (72.0)	0.001	51.6 (55.1)	0.06	0.823* (0.06)	1.11* (0.12)	0.01	0.02	235.5 (27.4)	241.6 (13.8)	0.48	0.66
Cigarillos with ISO	70.4* (72.7)		33.7 (42.4)		0.93* (0.1)	1.28* (0.20)			375.7 (34.3)	367.7 (31.3)		

*ANOVA with adjustment for puffing protocol
Puffing comparisons are based on paired T-test
Sample size (cigarettes): N= 7 (CI), N=13 (ISO), (cigarillos): N= 6 (CI), N = 10 (ISO

Table 7. Neutral Red Assay: Cigarettes & Cigarillos Group Comparisons

Product Type	Mean IC_{50} µg/ml (SD)	Comparison (P-value)
Cigarettes	21.02* (10.53)	<0.01*
Cigarillos	12.28 (9.30)	
Cigarettes with CI	19.59 (7.72)	0.59
Cigarettes with ISO	22.45 (13.03)	
Cigarillos with CI	17.79 (11.22)	0.29
Cigarillos with ISO	9.76 (6.52)	

*ANOVA with adjustment for puffing protocol
Puffing comparisons are based on paired t-test
Sample size (cigarettes): N= 7 (CI), N=13 (ISO), (cigarillos): N= 6 (CI), N = 10 (ISO)

Table 8. Group Comparison for Genotoxicity Assay Results

Ames Assay[a] **p value**				
Comparison	**Strain**			
	TA98 +S9	**TA98 -S9**	**TA100 +S9**	**TA100 -S9**
Cigarettes vs. Cigarillos	0.119[a]	0.545[a]	0.003[a]	0.650[a]
Cigarettes – CI vs. ISO	0.660[b]	0.507[b]	0.502[b]	0.963[b]
Cigarillos – CI vs. ISO	0.811[b]	0.275[b]	0.268	0.010[b*]
MN Assay **Frequency ratio (95% CI)**				
	MN+S9	**MN -S9**	**Apoptosis +S9**	**Apoptosis -S9**
Cigarettes vs. Cigarillos	1.42 (0.88 -2.30)[a]	1.65* (1.02 – 2.68)[a]	1.28 (0.77 – 2.14)[a]	1.14 (0.57 – 2.26)[a]
Cigarettes – CI vs. ISO	1.40 (0.44 -4.43)[b]	1.27 (0.51 – 3.13)[b]	1.07 (0.49 – 2.35)[a]	2.49* (1.14 – 5.42)[b]
Cigarillos – CI vs. ISO[b]	0.93 (0.43-2.00)	0.92 (0.58 – 1.47)	1.27 (0.70 – 2.30)	0.51* (0.32 – 0.80)
TK Assay[#] **MF Difference (95% CI)**				
	+S9		**-S9**	

Cigarettes vs. Cigarillos	36.40[a] (1.19 – 71.62*)	-17.35[a] (-37.53 – 2.82)
Cigarettes – CI vs. ISO	-0.80[b] (-36.89 – 35.29)	-20.19[b] (-70.84 – 30.46)
Cigarillos – CI vs. ISO	38.41[b] (-28.43 – 105.26)	-2.30[b] (-31.27 – 26.68)

[a]Adjusted for dose and puffing protocol, cigarette is the reference;
[b]Adjusted for dose, ISO is the reference
Comparisons are based on ANOVA with repeated measurements
*p<0.05
Sample size (cigarettes): N= 7 (CI), N=13 (ISO), (cigarillos): N= 6 (CI), N = 10 (ISO)

Table 9. Genotoxic Ranking of Products by Assay*

RANK 1 = highest, 40 = lowest	Ames Assay TA98	Ames Assay TA100	TK	MN
1	Camel Menthol, CI	Marlboro Red, ISO	Black & Mild Cigarillos Natural Apple, CI	Camel, ISO
2	Marlboro Gold Lights, CI	Swisher Sweets Sweet Original Cigarillos, CI	Swisher Sweets Grape Natural Cigarillos, CI	Black & Mild Cigarillos Natural Apple, CI
3	Black & Mild Tip Natural Cigarillos, ISO	White Owl Cigarillos, CI	Marlboro Gold Lights, CI	Swisher Sweets Grape Natural Cigarillos, ISO
4	Marlboro Red, CI	Black & Mild Tip Natural Cigarillos, CI	Pall Mall Menthol, CI	Black and Mild Classic/Natural Cigarillos, ISO
5	Pall Mall Red, CI	Marlboro Blue Fresh Menthol, CI	Swisher Sweets Sweet Original Cigarillos, ISO	Swisher Sweets Sweet Original Cigarillos, ISO
6	White Owl Cigarillos, CI	Swisher Sweets Tropical Fusion Cigarillos, ISO	Newport Red Non-menthol, ISO	Marlboro Blue Fresh Menthol, ISO
7	Newport Menthol Blue, ISO	Winchester Original Blend Little Cigars, ISO	Swisher Sweets Sweet Original Cigarillos, CI	Swisher Sweets Grape Natural Cigarillos, CI
8	Newport Box Full Flavor, CI	Swisher Sweets Grape Natural Cigarillos, CI	Marlboro Blue Fresh Menthol, ISO	Swisher Sweets Tropical Fusion Cigarillos, CI
9	Dutch Masters Palma Natural Corona, CI	Black & Mild Tip Natural Cigarillos, ISO	Swisher Sweets Grape Natural Cigarillos, ISO	Marlboro Gold Lights, CI
10	Swisher Sweets Original Cigarillos, CI	Winchester Original Blend Little Cigars, CI	Swisher Sweets Tropical Fusion Cigarillos, CI	Newport Menthol Blue, CI
11	Winchester Original Blend Little Cigars, CI	#	Camel, CI	Swisher Sweets Sweet Original Cigarillos, CI
12	Marlboro Blue Fresh Menthol, ISO	#	White Owl Cigarillos, ISO	White Owl Cigarillos, CI
13	Camel Menthol, ISO	#	Pall Mall Red, ISO	Dutch Masters Palma Natural Corona, ISO

RANK 1 = highest, 40 = lowest	Ames Assay TA98	Ames Assay TA100	TK	MN
14	Marlboro Red, ISO	#	Newport Menthol Blue, ISO	Swisher Sweets Tropical Fusion Cigarillos, ISO
15	Newport Red Non-Menthol, CI	#	Swisher Sweets Natural Sweet Cigarillos, ISO	Marlboro Gold Lights, ISO
16	Swisher Sweets Tropical Fusion Cigarillos, ISO	#	White Owl Cigarillos, CI	Newport Red Non-Menthol, CI
17	Pall Mall Red, ISO	#	Camel Menthol, CI	#
18	Winchester Original Blend Little Cigars, ISO	#	Pall Mall Red, CI	#
19	Marlboro Gold Light, ISO	#	Swisher Sweets Tropical Fusion Cigarillos, ISO	#
20	Swisher Sweets Natural Sweet Cigarillos, ISO	#	Winchester Original Blend Little Cigars, CI	#
21	Camel, CI	#	Black and Mild Classic/Natural Cigarillos, ISO	#
22	Newport Red Non-Menthol, ISO	#	Swisher Sweets Natural Sweet Cigarillos, CI	#
23	Swisher Sweets Natural Sweet Cigarillos, CI	#	Black & Mild Tip Natural Cigarillos, CI	#
24	Newport Menthol Blue, CI	#	Black & Mild Cigarillos Natural Apple, ISO	#
25	Black & Mild Cigarillos Natural Apple, ISO	#	Dutch Masters Palma Natural Corona, ISO	#
26	Black & Mild Tip Natural Cigarillos, CI	#	Black & Mild Tip Natural Cigarillos, ISO	#
27	Black & Mild Classic Natural Cigarillos, CI	#	Camel, ISO	#

RANK 1 = highest, 40 = lowest	Ames Assay TA98	Ames Assay TA100	TK	MN
28	Swisher Sweets Grape Natural Cigarillos, CI	#	Marlboro Red, CI	#
29	Marlboro Blue Fresh Menthol, CI	#	Black and Mild Classic/Natural Cigarillos, CI	#
30	Swisher Sweets Sweet Original Cigarillos, ISO	#	Winchester Original Blend Little Cigars, ISO	#
31	Black & Mild Classic Natural Cigarillos, ISO	#	Newport Box Full Flavor, CI	#
32	Swisher Sweets Grape Natural Cigarillos, ISO	#	Marlboro Red, ISO	#
33	Swisher Sweets Tropical Fusion Cigarillos, CI	#	Camel Menthol, ISO	#
34	Newport Box Full Flavor, CI	#	Newport Red Non-Menthol, CI	#
35	Dutch Masters Palma Natural Corona, ISO	#	Marlboro Blue Fresh Menthol, CI	#
36	Pall Mall Menthol, ISO	#	#	#
37	Camel, ISO	#	#	#
38	White Owl Cigarillos, ISO	#	#	#
39	Pall Mall Menthol, CI	#	#	#
40	Black & Mild Cigarillos Natural Apple, CI	#	#	#

*Slope estimates were statistically significant at $p<0.05$
the products for these rank numbers did not show a dose response in the indicated test
Sample size (cigarettes): N= 7 (CI), N=13 (ISO), (cigarillos): N= 6 (CI), N = 10 (ISO)

Figure Legends

Figure 1. Overall work flow for in vitro toxicity testing of a test set of commercially available cigarillos and cigarettes.

Fig. 2a-c. CI produces more a) benzo[a]pyrene, b) acetaldehyde, and c) NNN per puff than ISO in 20 major brands of cigarettes and cigarillos (blue = CI, red = ISO). Error bars = SEM. N = 7 (for CI) and 13 (for ISO) cigarettes and 6 (for CI) and 10 (for ISO) cigarillos. 100 mg TPM was generated from each type of cigarette/cigarillo and extracted into DMSO to [40 mg/ml].

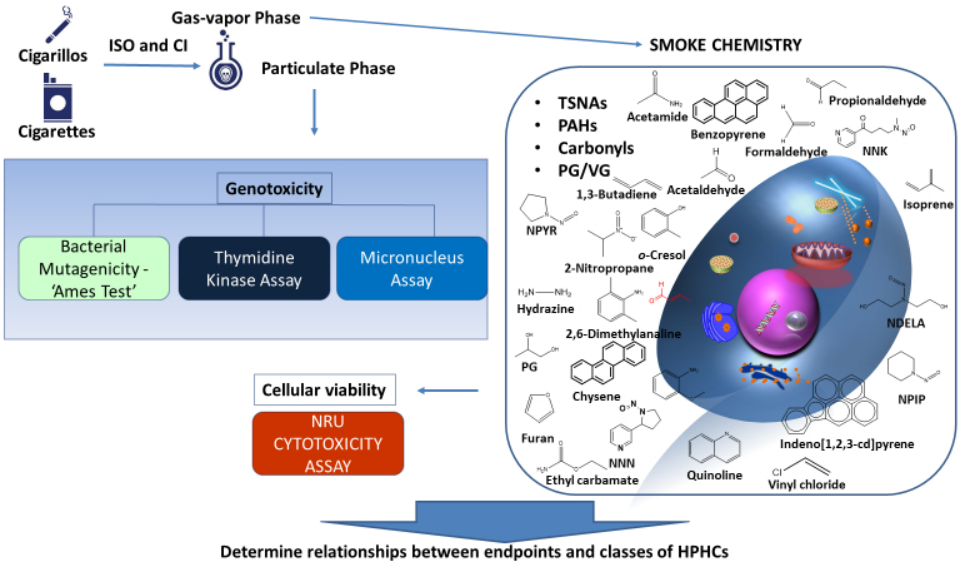

Figure 1. Overall work flow for in vitro toxicity testing of a test set of commercially available cigarillos and cigarettes.

169x95mm (120 x 120 DPI)

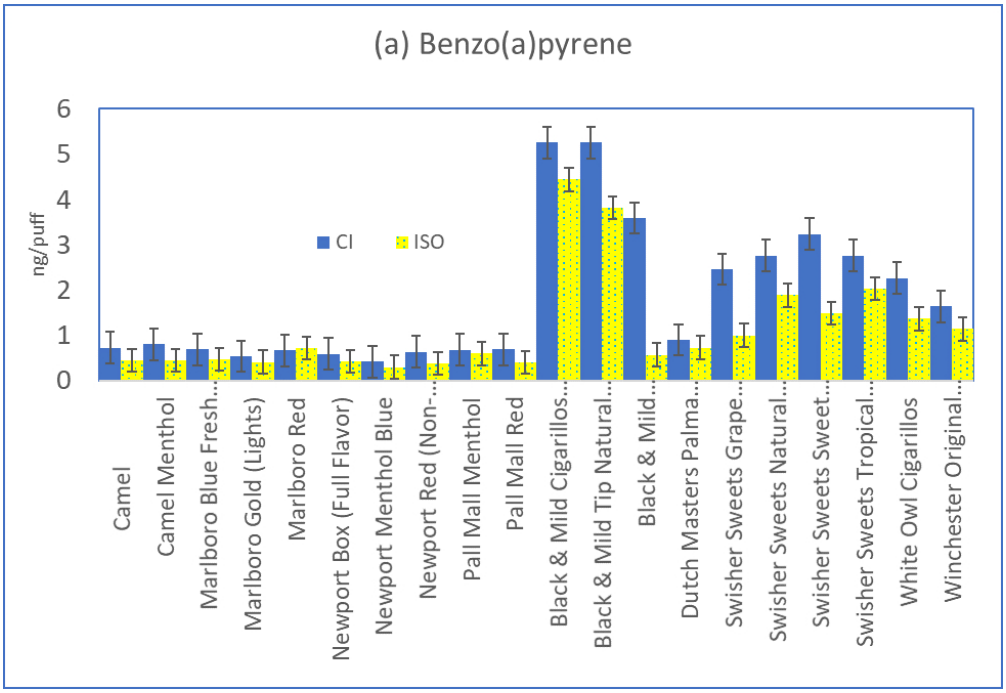

Fig. 2a-c. CI produces more a) benzo[a]pyrene, b) acetaldehyde, and c) NNN per puff than ISO in 20 major brands of cigarettes and cigarillos (blue = CI, red = ISO). Error bars = SEM. N = 7 (for CI) and 13 (for ISO) cigarettes and 6 (for CI) and 10 (for ISO) cigarillos. 100 mg TPM was generated from each type of cigarette/cigarillo and extracted into DMSO to [40 mg/ml].

165x113mm (150 x 150 DPI)

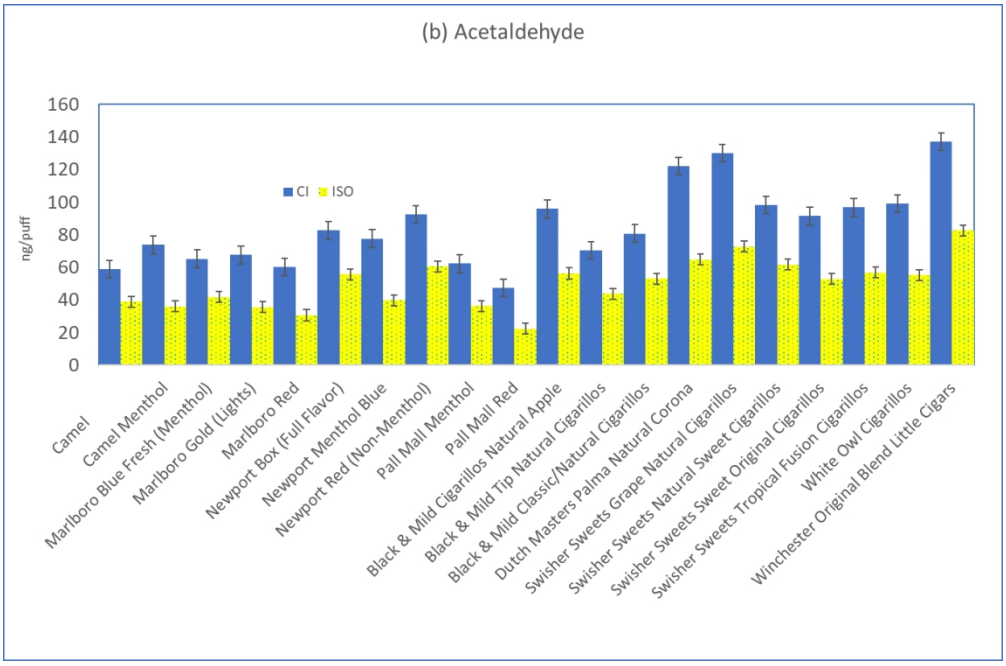

Fig. 2a-c. CI produces more a) benzo[a]pyrene, b) acetaldehyde, and c) NNN per puff than ISO in 20 major brands of cigarettes and cigarillos (blue = CI, red = ISO). Error bars = SEM. N = 7 (for CI) and 13 (for ISO) cigarettes and 6 (for CI) and 10 (for ISO) cigarillos. 100 mg TPM was generated from each type of cigarette/cigarillo and extracted into DMSO to [40 mg/ml].

234x154mm (150 x 150 DPI)

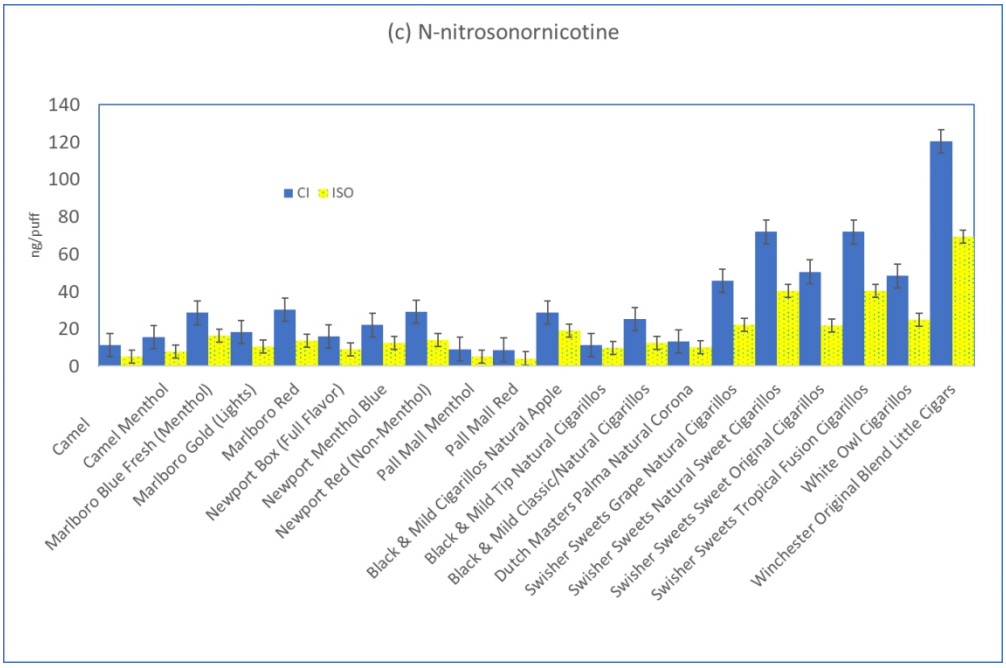

Fig. 2a-c. CI produces more a) benzo[a]pyrene, b) acetaldehyde, and c) NNN per puff than ISO in 20 major brands of cigarettes and cigarillos (blue = CI, red = ISO). Error bars = SEM. N = 7 (for CI) and 13 (for ISO) cigarettes and 6 (for CI) and 10 (for ISO) cigarillos. 100 mg TPM was generated from each type of cigarette/cigarillo and extracted into DMSO to [40 mg/ml].

234x154mm (150 x 150 DPI)