Supplementary Online Content


eAppendix. Supplemental Methods
eReferences.

This supplementary material has been provided by the authors to give readers additional information about their work.
eAppendix. Supplemental Methods
Sampling of “non-menthol” cigarettes in California and Massachusetts

Methods

Newly marketed “non-menthol” cigarette products in California and Massachusetts were identified through complementary strategies using information sources from marketing studies, manufacturer and governmental resources. Initially, a marketing report provided by SRITA, the Stanford Research Into The Impact of Tobacco Advertising group directed by Dr. Robert Jackler, was consulted that reviewed the California tobacco marketplace and advertisements immediately after December 2022 flavor ban:
https://stanfordmedicine.app.box.com/s/sbqrl5lmhrjciws0q9f4dx9wtk8h2z6

This report first identified the RJ Reynolds (Newport, Camel) and ITG Brands (Kool) products we tested, receiving strong advertising and sales support by these two major tobacco companies. Altria, the remaining of the “big three” tobacco companies (Marlboro brand) decided against marketing “non-menthol” products, according to a statement by an executive cited by the New York Times: https://www.nytimes.com/2023/01/11/health/cigarettes-flavor-ban-california.html

Sampling for RJ Reynolds products was done for brands and brand styles labelled “non-menthol” that appeared in December 2022 in the product list on the company’s website:
https://rjrt.com/commercial-integrity/ingredients/brand-compounds/

Additionally, we consulted the California Tobacco Directory of approved manufacturers and brands maintained by the California Attorney General’s Office in late 2022, early 2023 and June 2023, and the equivalent directory in Massachusetts in June 2023:
https://oag.ca.gov/tobacco/directory
https://www.mass.gov/service-details/tobacco-product-manufacturers-directory

California-marketed products were purchased in three convenience stores chosen for their location in high consumer traffic areas in the major population centers in both Southern (Ontario, Irvine) and Northern California (San Francisco). Similarly, two stores in Massachusetts were chosen from two major suburbs of Boston (Canton, Sharon). When visiting the convenience stores, we requested all “non-menthol” brands listed in the Stanford report and the state directories. We found that the convenience stores in California had the same subset of state directory-listed products available. Newport branded “non-menthol” cigarettes (three varieties) were available in all stores visited in California and Massachusetts, while the Camel- and Kool-branded “non-menthol” brands (4 and 2 varieties, respectively) were only available in California at the times we purchased (January and June 2023). Six Menthol (1 for Newport, 3 for Camel and 2 for Kool, 6 varieties total) and 2 unflavored (one each for Newport and Camel) reference cigarettes were purchased in convenience stores in New Haven, CT (Yale), and Durham, NC (Duke)

Chemical Analysis

Materials

Chemicals used for extraction and as reference materials were methanol (LC-MS grade, Fisher Scientific, Waltham, MA); menthol (99%), vanillin (99%), ethylvanillin (~98%; all Sigma-Aldrich, St. Louis, MO); WS-3 (~98%, CAS No. 39711-79-0), WS-23 (>98%; 51115-67-4, both TCI America, Portland, OR); WS-5 (~98%; 68489-14-5; Penta Manufacturing, Livingston, NJ), WS-12 (99%; 68489-09-8; Xi’an Taima), Frescolat ML (>97%; 17162-29-7; Sigma-Aldrich), Frescolat MGA (63187-91-7), and Frescolat XCool (1122460-01-8; both Symrise, Teterboro, NJ).

Methods

For initial analysis, a cigarette of each variety was separated into the filter material, wrapping paper, and the tobacco rod; Crushable capsules, if present, were removed from the filters and set aside. The cigarette materials were weighed and extracted separately with 10mL methanol in 20mL glass vials at 150rpm for 2h using an orbital shaker

© 2023 American Medical Association. All rights reserved.
Samples were filtered (0.22 µm, Millex-GP, Sigma-Aldrich), and 1 µL of sample was injected into a GC/MS for extract characterization (Perkin-Elmer Clarus 580-SQ8S with an Elite-5MS column [length 60 m, id 0.25 mm, 0.25 µm film]), and a GC/FID for quantification of selected compounds (Shimadzu GC-2010 Plus with an Agilent J&W DB-5 column [length 60 m, id 0.25 mm, 0.25 µm film]) using previously established methods.1

Because no cooling agent was detected in the filters, we subsequently extracted only the tobacco rod and wrapping paper in 5 mL methanol. Extractions were carried out in triplicate or quintuplicate (n=3 or 5) as indicated in Table 1 of the manuscript.

Crushable capsules were placed inside a 2 mL autosampler vial, filled with 1mL methanol + 1 drop of dichloromethane (Fisher Scientific), crushed using a needle, mixed thoroughly, followed by filtration and analysis as above.

Recovery was 97.8% (menthol) and 85.1% (WS-3; both n=5). Precision was 1.3%RSD (menthol) and 0.7%RSD (WS-3; both n=5). Limit of detection (LOD) was 3 µg/mL (menthol) and 5 µg/mL (WS-3), and limit of quantification (LOQ) was 9 µg/mL (menthol) and 15 µg/mL (WS-3).

Testing for sensory cooling activity by calcium microfluorimetry

Extract preparation for calcium microfluorimetry:

Extracts were prepared by stirring tobacco rod material overnight in 10 mL methanol. Contents were transferred to a 50 mL tube attached with a Falcon 100 µm nylon cell strainer (Falcon, Corning Inc. Corning, NY) to strain the tobacco leaf material, followed by centrifugation (@4000 rpm for 5 minutes). Supernatant was collected, aliquoted and dried down of methanol using vacuum concentration (Eppendorf Vacufage, Eppendorf, CT). Dried down contents were reconstituted in calcium assay buffer (Hank’s Balanced Salt Solution with 10 mM HEPES). Further dilutions of these reconstituted extracts (diluted 1X-200X in assay buffer) were prepared to test for receptor activity. 1X dilution is defined as the extract of one tobacco rod contents in 50 mL assay buffer, and 200X is 200-fold dilution thereof.

Calcium microfluorimetry

Tobacco rod extracts (diluted 2X-200X in buffer) were tested for cold/menthol receptor, TRPM8, activity by intracellular calcium microfluorimetry in HEK-293t cells (RRID:CVCL1926) expressing human TRPM8 isoform and as previously described.1–3 To control for any variability in receptor expression levels and loading of Ca2+ indicator across experiments, Ca2+- influx responses from these extracts were normalized to the Ca2+-response elicited by a maximally activating concentration of agonist L-menthol (1 mM; TRPM8). Further, specificity of TRPM8-mediated activity was validated using TRPM8 specific inhibitor, N-(3-aminopropyl)-2-[(3-methylphenyl)methyl]oxy]-N-(2-thienylmethyl)benzamide hydrochloride salt (AMTB).4 For these validation experiments, 25 µM AMTB was added to the microplate wells containing TRPM8-expressing HEK-293t cells, 20-30 minutes before superfusing with tobacco rod extracts. Dose-response curves for receptor activity and associated calcium influx changes were plotted using non-linear regression analysis with a 4-parameter logistic equation (Graphpad Prism 9.0, San Diego, CA). The efficacies of receptor activation by “non-menthol” cigarettes and their corresponding menthol reference products were compared at maximal receptor activation at the lowest dilution (2x) tested. Experiments were repeated 2-5 times (n = 2-5) and each conducted in triplicates (N = 3) with independent tobacco rod extractions.

Extracts tested for menthol receptor activity and shown in this study include,

1) California-Marketed Non-Menthol Kool Green,
2) Kool Green-True Menthol (banned in California and Massachusetts)
3) California- and Massachusetts-marketed Newport Non-Menthol Green
4) California-and Massachusetts-marketed Newport EXP Non-Menthol Mix
5) US-marketed Newport-Box-(Green)(Originally called ‘Full-Flavor’ menthol cigarettes, banned in California and Massachusetts)
6) US-marketed Newport Non-Menthol-Box-(Red) an unflavored Newport brand introduced in 2010 to appeal to smokers of regular cigarettes, marketed throughout the US)
7) California-Marketed Camel Crisp Non-Menthol Green
8) California- Marketed Camel Crush Oasis

© 2023 American Medical Association. All rights reserved.
9) US-marketed Camel unflavored

cReferences.


