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Activation of the cold-receptor TRPM8 by low levels of menthol in tobacco products

Meike Paschke, Anna Tkachenko, Katja Ackermann, Christoph Hutzler, Frank Henkler*, Andreas Luch

German Federal Institute for Risk Assessment (BfR), Department of Chemical and Product Safety, Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany

HIGHLIGHTS

- Menthol in cigarette smoke activates the cold-receptor TRPM8 and mediates a "cooling effect" that is independent from its mint-like aroma.
- We have assessed the minimum menthol contents in cigarettes required for TRPM8 activation.
- A measurable activation of TRPM8 is expected when the content of menthol exceeds 50 μg per cigarette.

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ABSTRACT

Activation of the cold-receptor TRPM8 by menthol or other tobacco additives can suppress natural defense reactions such as coughing that usually would become effective as involuntary resistance against the inhalation of fumes. In Europe menthol is only regulated as flavor, but can be used as additive as long as no characteristic mint-like aroma will become noticeable in the end-product tobacco. The question needs to be addressed of whether such comparatively minor contents would be sufficient to trigger a measurable activation of TRPM8.

In this study, we have analyzed both the contents of menthol and other natural TRPM8 agonists in tobacco products and developed a bioassay to determine the minimum concentrations of selected agonists to activate the TRPM8 receptor in cultured cells.

The data confirm menthol as strongest natural agonist investigated. Based on these experiments and previously published data, we have estimated both the minimum menthol concentrations in cigarette smoke and in tobacco that are expected to trigger measurable physiological effects. According to our assessments, TRPM8 activation is likely to occur when cigarettes contain more than 50 micrograms of menthol. Importantly, menthol contents in cigarettes far below the typical levels that require declaration as "mentholated" would be sufficient to activate sensory receptors.

1. Introduction

Cigarette mentholation was invented in the 1920s, but the procedure did not gain a higher market share until filtered menthol cigarettes were introduced in the 1950s (Reid, 1993). Even then, marketing strategies combined associations of "cooling effects" with low tar contents (Reid, 1993). Nowadays menthol smokers account for about 30% of cigarette smokers in the USA (TPSAC, 2011). In the United States, mentholated cigarettes are preferred by adolescents (12–17 years) and used by a majority of smokers (56.7%) in this age group, followed by young adults (18–24 years) with a proportion of 45.0%. Notably, a considerable decrease in the ratio of menthol smokers (30.5–34.7%) occurs in the age group of 26 years or older (Giovino et al., 2015). This indicates that beginners start with mentholated products and switch later to alternative products. However, other factors that might affect the preference for menthol cigarettes, as for example as gender, ethnic or cultural background or changing perception of fashionable tobacco products are not yet fully understood. In Europe the proportion of menthol smokers is comparatively low and estimated to about 5%, based on cigarette sales figures (EACH, 2013).

* Corresponding author.
E-mail address: Frank.Henkler@bfr.bund.de (F. Henkler).

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In light of the summarized data presented in the TPSAC report, mentholated cigarettes seem to attract adolescents and young adults and are thus believed to contribute to smoke initiation (TPSAC, 2011; Rising and Wasson-Blader, 2011). In addition to its contribution as flavoring compound in the aromatization of cigarette smoke, menthol has also physiological properties that might promote the inhalation of tobacco smoke. Sensory effects of menthol result in an increased smoothness and reduced harshness of the smoke; impact effects describe the perception of strength of a cigarette; cooling effects mask the burning/scratching properties of tobacco smoke (Ahijevych and Garrett, 2004, 2010); finally menthol also acts as anaesthetic (Wayne and Connolly, 2004). Importantly, internal documents of the tobacco industry revealed that these effects had been well known (Yerger and McCandless, 2011), and sensory properties were adopted to attract young smokers (Kreslake et al., 2008a, 2008b). Other additives such as menthol esters can be used as substitute for menthol (Bharate and Bharate, 2012) as well. Known physiological effects of menthol in the respiratory tract are bronchodilation, decreased rates of inhalation and prolonged breath holding (Henningfield et al., 2003). Menthol does also suppress tussive irritations in response to fumes (Millqvist et al., 2013) and symptoms of respiratory diseases, such as chronic cough or thick mucus production (Garten and Falkner, 2004). The physiological effects of menthol are predominantly related to the activation of the transient receptor potential melastatin 8 (TRPM8) receptor, which is also named cold-menthol receptor (McKemy et al., 2002). TRPM8 is a member of the transient receptor potential (TRP) cation channels family. TRPM8 is specifically expressed in subpopulations of neurons instrumental in sensing pain and temperature. Sensation of coldness involves various receptors that distinguish innocuous or pleasant stimuli, such as relief from previous heat or irritation, from high-threshold response levels in relation to frost as potentially harmful condition. The menthol receptor TRPM8 is predominantly involved in the transduction of innocuous "cold" stimuli, possibly paralleled by the suppression of heat responses in peripheral neurons (McKemy, 2013). The overall sensation at moderate temperature levels is pleasant and can be mimicked by chemical agonists, regardless of taste or aromatic properties.

Although physiological properties of menthol that can promote the inhalation of harsh tobacco smoke are well defined, there is ongoing debate on its relevance for smoking related health risks (Heck, 2010). Current studies do not support the conclusion that menthol in general would contribute to addictiveness (Fagan et al., 2010) or increases in lung cancer incidence because of much higher exposure levels against nicotine and prototypic carcinogens present in the smoke (Brooks et al., 2003; Carpenter et al., 1999). However, with regard to addictiveness evidence is somehow contradictory (Sidney et al., 1995). Even though menthol might not lead to additional health risks for experienced smokers (Heck, 2009), physiological properties are adequate and indeed sufficient to promote the inhalation of irritating fumes in unexperienced individuals (Rising and Wasson-Blader, 2011), similar as shown in animals (Willis et al., 2011). In support to this assumption, menthol cigarettes had been classified as starter products (Hersey et al., 2006; Kahnert et al., 2012) that are likely to facilitate product's addictiveness in adolescents (TPSAC, 2011). Menthol might increase addictiveness indirectly by two mechanisms. Firstly, impairment of physiological resistance against inhalation of irritating fumes could enable some individuals to start smoking, who otherwise would rather refrain. Secondly, menthol might alter individual smoking habits, leading to a deeper inhalation and thus uptake of higher amounts of nicotine. Again, this might primarily affect beginners who are not yet adapted to a routine inhalation of smoke. In addition, menthol was proposed to delay the metabolism of nicotine (Benowitz et al., 2004).

According to new European regulation, menthol will be restricted as a potential characterizing flavor. This raises the important question whether physiologically active levels of menthol or of other TRPM8 agonists need to be expected in conventional smoking tobacco products that are—explicitly—not sold as "menthol cigarettes" and that do not release the typical mint-like flavor. Menthol was also included in the EU priority list of tobacco additives and is subject of tighter reporting obligations (EU, 2016), partly because of its "cooling" effects". In this manuscript we have assessed whether menthol levels far below the typical contents of declared menthol cigarettes are sufficient to activate TRPM8 *via* inhalation. Our risk assessment is based on the provided experimental data and on previously published studies. We propose that TRPM8 activation can be expected when menthol levels exceed 50 μg per cigarette. These comparatively low amounts are probably adequate to facilitate inhalation, especially during the initiation phase of unexperienced smokers who are not yet adapted to the irritating properties of tobacco smoke. We also provide first data on menthol contents in cigarettes of the German market. In the products analyzed, the proposed limit for physiological effects was only exceeded in cigarettes declared as mentholated.

2. Materials and methods

2.1. Chemicals

All chemicals, analytical standards and solvents used were of analytical or LC–MS grade. Ethanol (EtOH) (99.9%), poly-L-lysine and sodium chloride (NaCl) were purchased from Merck KGaA (Darmstadt, Germany). ATP (100 mM) was bought from Sigma–Aldrich (Taufkirchen, Germany). Fluo-4 AM (1 mM), HBSS (10x) (Hank's balanced salt solution), lipofectamine 2000 and Opti-MEM were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Standard substances

All analytical standards were used as racemates (Fig. 1). 7-Hydroxycitronellal (95%), carvone (98%), eucalyptol (99%), geraniol (98%), isopulegol (98%), menthol (99%), menthone (99%) and acetophenone-β,β,β-d_3 were purchased from Sigma–Aldrich (Taufkirchen, Germany). Linalool was obtained from Merck KGaA (Darmstadt, Germany) and menthol-d_4 from TRC (Ontario, Canada).

2.3. Samples and their preparation prior to quantitative analysis

Cigarettes of four major manufacturers were obtained from retailers. From each manufacturer a typical American blend product was used as standard brand. Further, additive-free (*i.e.* non-flavored) and declared menthol variations of these standard brands were used in this study. In addition, modified cigarettes, which are sold under the same brand name but contained 0.6 mg nicotine or less, have been analyzed. The latter products are listed here as "light cigarettes", although this designation was not necessarily used by the manufacturer.

Cocoa and liquorice were also obtained from local retailers. Tobacco plants (*Nicotiana tabacum*) were grown from seeds as reference samples. Dried leafs were further processed without curing, as described for cigarette tobacco. An aliquot of each cigarette (100 mg of tobacco) was exactly weighted in 20 mL headspace vials, followed by addition of 5 mL purified water (saturated with NaCl at 60 °C). To this sample, 5 μL of an internal standard mix was added. In the case of mentholated cigarettes a standard mix containing 200 μg/mL of each menthol-d_4 and acetophenone-βββ-d_3, dissolved in EtOH, was used, while for all

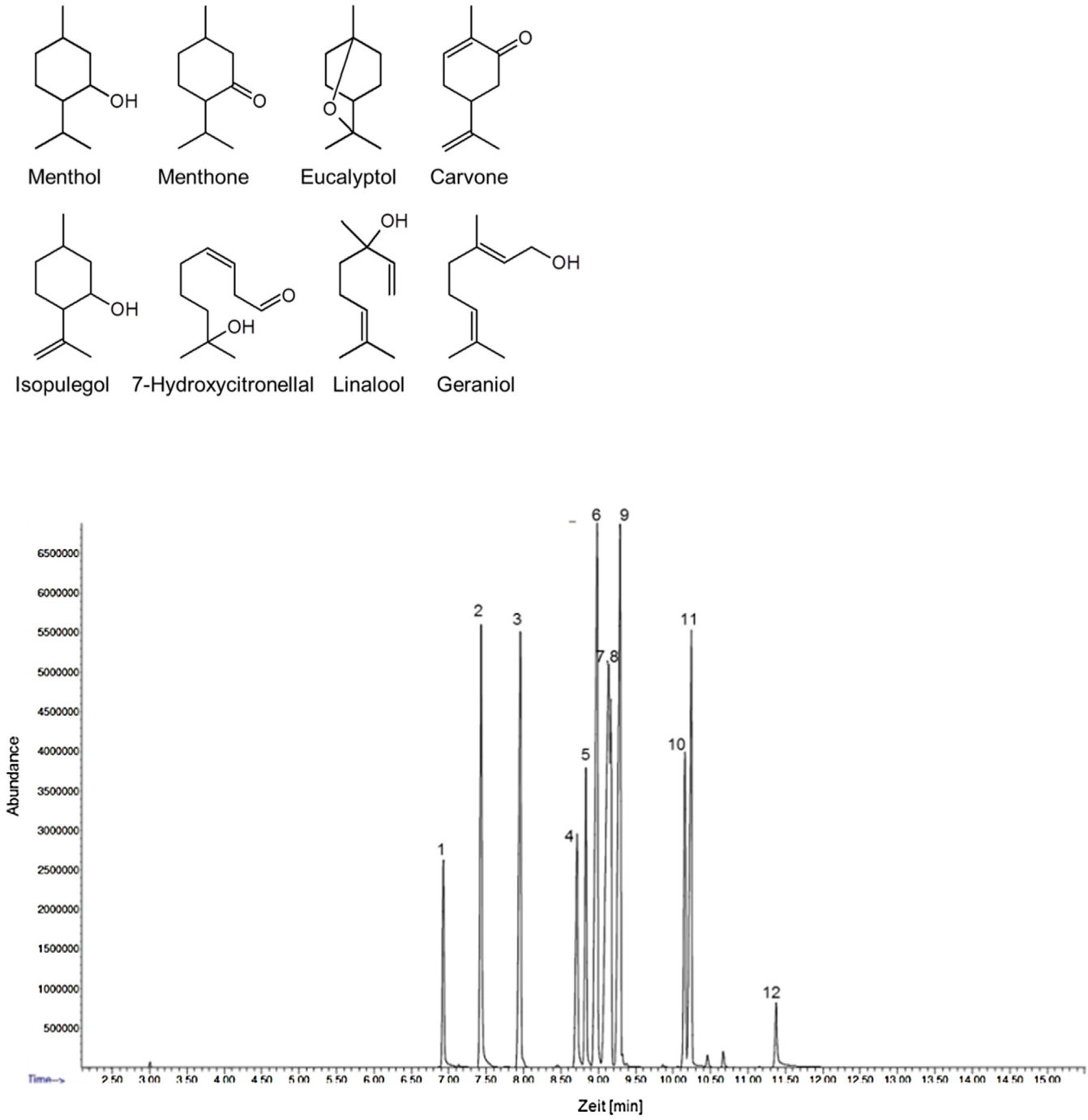


Fig. 1. Chemical structures of the TRPM8 agonists analyzed in this study and the corresponding HS-SPME-GC/MS chromatogram of a mixture of these agonists (20 μg/mL of each) along with internal standards (50 μg/mL of each) in SIM mode (see Materials and methods section for details on the HS-SPME–GC/MS procedure). Compounds are labeled as follows: eucalyptol (1); acetophenone-d_3 (2); linalool (3); isopulegol (4); menthone (5); neomenthol-d_4 (6); menthol-d_4 (7); menthol (8); isomenthol-d_4 (9); carvone (10); geraniol (11); 7-hydroxycitronellal (12).

other samples this standard mix contained only 50 μg/mL of each ingredient. For quantitative analyses the standard addition method was used and six different calibration points were analyzed for each sample. For mentholated cigarettes 5 μL of standard mix in the range of 200–750 μg/mL EtOH was used for each calibration point. For all other samples a standard mix of 5–150 μg/mL EtOH was added to the sample vials. All samples were analyzed in triplicates and 5 μL of EtOH were added to ensure comparability with the calibration samples. The vials were tightly closed with magnetic silicone/PTFE caps before analysis.

2.4. HS-SPME–GC/MS analysis

2.4.1. HS-SPME

For quantification the PDMS/DVB fiber was used as it turned out to be best suited after testing also 4 other fibers with different selectivity (see Supplement, fibers obtained from Supelco (Bellafonte, PA, USA). An MPS2-XL autosampler (Gerstel, Mühlheim, Germany), extended with a SPME fiber holder, was used for automated sampling and injection. The vials were incubated for 5 min at 60 °C. Afterwards, the SPME fiber was exposed to the

headspace for 60 min. Samples were desorbed for 120 s in the cooled injection system (CIS) 4 (Gerstel) at 250 °C, keeping this temperature for 10 min. The CIS was equipped with a bore liner with an inner diameter of 2 mm and operated for mentholated cigarettes with a split ratio of 1:100; all other samples were analyzed in split less mode. Further details on the development of the SPME method are given in the Supplementary Section.

2.4.2. GC/MS

A GC 6890A coupled to the MSD 5975C (Agilent, Waldbronn, Germany) equipped with an HP–5 ms column (30 m × 0.25 mm × 0.25 μm, J&W Scientific, Folsom, CA, USA) was used. Helium gas (purity 99.999%) from Air Liquide (Düsseldorf, Germany) was used as carrier gas at a constant flow of 1 mL/min. The oven temperature of the GC was programmed to start at 50 °C, held for 1 min, and then increased with a rate of 10 °C/min to 180 °C, hold for 1 min, followed by 20 °C/min to reach the final temperature of 320 °C, then held for further 1.5 min. The temperatures of the transfer line, quadrupole and the ion source were 295 °C, 150 °C and 230 °C, respectively. The MS was operated in the combined selective ion monitoring (SIM) scan mode. The mass range was scanned in full scan mode in the mass to charge (m/z) range from 29 to 300 Da. For data acquisition in SIM mode the monitored ions were divided into four groups according to the retention times of the corresponding analytes, with a dwell time of 10 ms for each, using the quantifier and qualifier ions. All analytes under consideration were identified by comparison of their mass spectra and retention times to those of authentic standards (Fig. 1). Data on retention times, quantifiers and qualifiers, limits of detection (LODs) and limits of quantification (LOQs) as well as the description of method validation are given in the Supplementary section (Tables S1–S3) also the method development. Data were evaluated using MSD ChemStation software, Version E.01.00.237 (Agilent, Waldbronn, Germany).

2.5. TRPM8 cloning

The human TRPM8 cDNA was optimized for expression in human cells and synthesized and sequenced by Eurofins Genomics (Ebersberg, Germany). The synthesized TRPM8 fragment was subcloned into the pcDNA3 vector (Invitrogen) using the *Eco*RV/*Not*I sites thereby generating the pcDNA3-TRPM8 plasmid. Sequences of the modified cDNA and the translated protein (http://web.expasy.org) are given in Supplementary Fig. S1. Sequence alignment, using the protein Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins), confirmed 100% conformity of the encoded protein with human TRPM8 (NCBI reference sequence NP_076985.4). CellLight nucleus-CFP BacMam 2.0 was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

2.6. Cell culture media

Dulbecco's phosphate-buffered saline (DPBS), Dulbecco's Modified Eagle's Medium (DMEM) culture medium, containing 10% fetal calf serum (v/v), L-glutamine (2 mM), and penicillin/streptomycin (100 U/mL).

2.7. Calcium imaging

HEK293 cells were seeded at a density of 150,000 cells/dish onto glass bottom dishes coated with poly-L-lysine (dish size of 35 mm, well size of 20 mm; IBL, Gerasdorf, Austria). Cells were grown to confluency in DMEM with 10% FCS at 37 °C and 5% CO_2. Confluent cell cultures were then co-transfected with human TRPM8 and CFP-Nuc as nuclear marker. Expression of TRPM8 was confirmed in control experiments by immunofluorescence, using a rabbit polyclonal antiserum (HPA024117, Sigma) according to the instructions of the manufacturer (Supplementary Fig. S1).

Twenty-four hours after transfection, HEK cells transiently expressing the TRPM8 receptor were treated with the cell permeable calcium indicator Fluo-4 AM. Binding to calcium ions converts this compound into a fluorescent dye (λ_{ex} 488 nm), thus allowing to monitor calcium influx into cells or the release from endoplasmic reticulum or Golgi apparatus. Prior to treatment, cells were first washed three times with HBSS and then 0.5 mL Fluo-4 AM (2 μM in HBSS) was loaded onto the cells. After 30 min of incubation at room temperature the cells were washed again three times with HBSS. Then 1 mL HBSS was added and cells were incubated for further 30 min at room temperature before being analyzed under the fluorescence microscope. A time series of 600 images during 300 s was recorded. For Ca^{2+} imaging, the agonists were dissolved in EtOH and 1 mL was added onto the transfected and Fluo-4 AM-loaded HEK293 cells after a few seconds (Fig. 2). Approximately 150 s later 0.5 mL ATP solution (100 μM in EtOH) was added to the cells as control. ATP non-specifically opens ion channels and thus provides a reproducible fluorescent signal. The fluorescent signals of at least 10 cells were evaluated. Transfection efficiencies of >80% were confirmed in all experiments, using CFP-Nuc as marker (Thermo Fisher Scientific, Waltham, MA, USA).

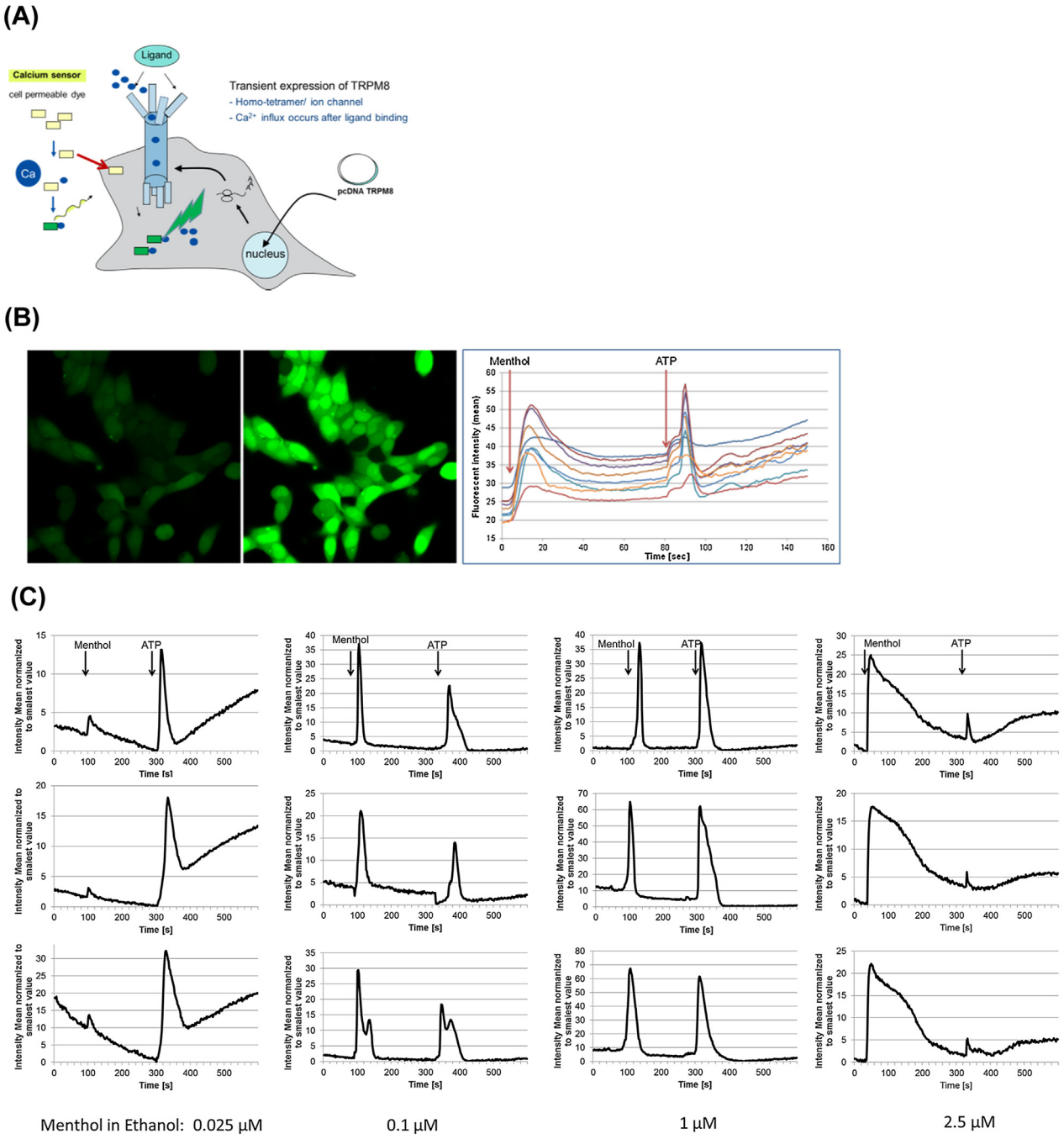
3. Results

3.1. Contents of TRPM8 agonists in cigarettes, tobacco leafs and tobacco additives

Flavoring compounds such as menthol can trigger the activation of the TRPM8 nociceptor for cold pain. Besides menthol, other monoterpenes are described in the literature to activate TRPM8 as well (Behrendt et al., 2004). Behrendt and coworkers demonstrated TRPM8 activation by geraniol, linalool, eucalyptol, isopulegol and 7-hydroxycitronellal in mice. Menthone and carvone are further known agonists (Bharate and Bharate, 2012). In the present study the contents of these selected eight TRPM8 agonists (Fig. 1) were analyzed in four different cigarette brands. Of each brand different types of cigarettes were investigated: American blend cigarettes, additive-free cigarettes, nicotine reduced "light" cigarettes and mentholated cigarettes. The results are compiled in detail in the Supplementary section (Tables S4–S7) and summarized in Table 1.

In mentholated cigarettes we could detect eucalyptol, linalool, isopulegol, menthone, menthol and carvone, but no 7-hydroxycitronellal or geraniol (Table 1). Except for menthol, which was quantified in the milligram range (0.52–4.19 mg/cigarette), all analytes were detected at microgram levels in the cigarette brands investigated. Our data are comparable to the results published by Ai and coworkers in 2016, who analyzed mentholated and non-mentholated cigarettes of the US market (Ai et al., 2016). In mentholated cigarettes they reported an average menthol level of 4.75 mg per cigarette.

In American blend cigarettes only the four analytes linalool, menthone, menthol and carvone could be detected (Table 1). Additive-free cigarettes also contained eucalyptol and geraniol. The detected levels of linalool, menthone, menthol and carvone were about the same in American blend and additive-free cigarettes, a result that indicates that no TRPM8 agonists were voluntarily added to the respective American blend cigarette brands by the manufacturers. However, contents of menthol were found at significantly higher levels in light cigarettes (Table 1). Wayne and Connolly (2004) already described that the amounts of menthol were generally higher in light cigarettes when compared to regular ones. Accordingly, in our study menthol



TRPM8 agonist	concentration [µM]	content per cigarette [mg]*
Menthol	**0.1**	**0.00437**
Linalool	100	4.32
Menthone	580	25.1
Carvone	640	26.9

* Estimate based on a total volume of 280 mL smoke per cigarette (according to DIN ISO 3308)

Fig. 2. Panel (**A**): Cartoon of the approach applied in the present study. Panel (**B**): Ca^{2+} imaging of Fluo-4 AM-loaded HEK293 cells. Cells were imaged before (left) and during onset of Ca^{2+} influx (middle). The method was then used for live imaging of several TRPM8 expressing cells that were treated with menthol (25 µM) and ATP (100 µM) at the time points indicated (arrows). Cells were imaged every 2 s and fluorescence was recorded (see Materials and methods section for details). Panel (**C**): Fluorescence signals of TRPM8 expressing HEK293 cells loaded with Fluo-4 AM and treated with menthol at four different concentrations (0.025, 0.1, 1, and 2.5 µM of menthol), followed by 100 µM ATP at the time points indicated (arrows). Cells were continuously imaged every 2 s and fluorescence was quantified in individual cells using the ZEN software provided by Zeiss. Recorded graphs for 3 representative cells are shown for each menthol concentration applied. Non-transfected cells treated with menthol, and TRPM8 expressing cells

Table 1
Contents of TRPM8 agonists in cigarettes (n = 4), tobacco leaf and selected additives.

Compound	American blend cigarettes ng/cig	Additive-free cigarettes ng/cig	Light cigarettes ng/cig	Menthol cigarettes μg/cig	Tobacco leaf μg/g[a]	Cocoa μg/g[a]	Licorice μg/g[*]
Eucalyptol	n.d.	n.d.–40.5	n.d.	0.15–10.6	n.d.	n.d.	0.66 (9)
Linalool	98.2–143	75.8–229	138–261	0.67–4.05	0.31 (220)	0.31 (4.3)	0.88 (12)
Isopulegol	n.d.	n.d.	n.d.	0.73–6.00	n.d.	0.47 (6.5)	n.d.
Menthone	35.9–37.9	22.6–103	53.3–153	1.38–37.8	n.d.	n.d.	4.21 (59)
Menthol	**58.2–445**	**23.4–256**	**121–3640**	**516–4190**	**0.30 (210)**	**0.20 (3.0)**	**0.65 (9.0)**
Carvone	58.9–88.3	36.2–76.9	43.2–132	0.37–270	0.28 (200)	n.d.	1.41 (20)
Geraniol	n.d.	67.7–125	87.4–206	n.d.	n.d.	n.d.	n.d.
7-Hydroxy-citronellal	n.d.	n.d.	50.4–190	n.d.	n.d.	n.d.	n.d.

n.d.: not detected; cig: cigarette; data for menthol are marked in bold.
[a] For tobacco leaf, cocoa and licorice estimations on the contents per cigarette (expressed as ng/cigarette) are provided in brackets. Estimations are based on 700 mg tobacco per cigarette and proportions of 2% for each cocoa and licorice).

Table 2
Reported activation of cold-receptors by defined menthol concentrations/levels.

Study/publication	Experimental details	Effective menthol concentration/content
Sant' Ambrogio et al. (1991)	Activation of laryngeal cold-receptors in eleven anesthetized dogs after nasal inhalation of L-menthol. Activation of cold-receptors was directly analyzed at internal branches of laryngeal nerves.	140–390 ng/mL = 0.9–2.8 μM
Behrendt et al. (2004)	Transient expression of murine TRPM8 in HEK293 cells. Determination of Ca^{2+} influx using the fluorometric imaging plate reader (FLIPR) assay.	4.1 ± 1.3 μM (EC_{50}, (−)-isomer) 14.4 ± 1.3 μM (EC_{50}, (+)-isomer)
Bödding et al. (2007)	Stable expression of human TRPM8 in HEK293 cells. Determination of Ca^{2+} influx using microfluorimetry.	10.4 μM (EC_{50})
Willis et al. (2011)	Mice were challenged with smoke irritants (acrolein). The irritation response was completely blocked by co-inhalation of menthol.	strong inhibition at 16 ppm (0.65 μM); 2 ppm had no effect

has been quantified in American blend cigarettes and additive-free cigarettes in the range of 58–445 and 23–256 ng/cigarette, respectively. By contrast, in light cigarettes up to 3.6 micrograms menthol were found in the brands investigated. Based on these data, it seems likely that menthol and other TRPM8 agonists have been applied with the intention to enhance the flavor of light cigarettes. Besides linalool, menthone, menthol and carvone also geraniol and 7-hydroxycitronellal were detectable in light cigarettes (Table 1).

We have further analyzed self-grown smoking tobacco to get some idea on endogenous levels of menthol possibly being present in the leaves. In the dried leaves we could detect linalool, menthol and carvone, all of which at about 0.3 μg/g tobacco (Table 1). Based on an average tobacco content of 700 mg/cigarette this would result in 210 ng/cigarette of each of the detected compounds. These levels would be comparable to the concentrations detected in the American blend, additive-free and—except for menthol—also the light cigarettes. Besides endogenous levels in tobacco, natural TRPM8 agonists can also emerge as ingredients of natural additives, including cocoa and licorice. We therefore analyzed the levels of the eight selected TRPM8 agonists in these both kinds of additives as well (Table 1). In cocoa we found linalool, isopulegol and menthol, whereas eucalyptol, linalool, menthone, menthol and carvone were detectable in licorice. Our results show that TRPM8 agonists, such as those monoterpenes analyzed, are present in untreated tobacco leaves, but can also additionally be introduced into cigarettes through additives. However, the overall effect is expected to be rather limited, because these additives do only account for a comparatively low percentage of the total weight.

3.2. Assessment of minimum menthol levels required for activation of TRPM8

Detectable levels of TRPM8 agonists in tobacco products are not necessarily related to receptor activation during smoking. It is therefore important to estimate concentrations and levels of particular agonists that are required for on-target activation of TRPM8. Although TRPM8 activation by various agonists had been analyzed before (Behrendt et al., 2004; Bödding et al., 2007), no minimum levels have yet been determined. In this study, we have transiently expressed TRPM8 in HEK293 cells. Although TRPM8 could not be directly visualized in living cells, successful transfections were confirmed by parallel expression of a fluorescent nuclear marker protein. Cell populations were then labelled with the calcium sensor Fluo-4 AM as described in the method section and monitored by online fluorescence microscopy (Fig. 2A). Binding of calcium ions to the sensor led to the emission of green light when the samples get excited at 488 nm. These signals were recorded every 2 s as an indicator for calcium influx. With this approach, a mixed population was analyzed at the level of individual cells (represented as graphs in Fig. 2B). Application of 25 μM menthol led to a strong, but transient influx of calcium in the transfected cells, indicating activation of TRPM8. Non-specific activation of receptors and subsequent calcium influx was then triggered by treatment with ATP as control (Fig. 2B). Comparable responses were observed in the analyzed cell populations, despite some variations in the basal calcium levels between individual cells.

Next, we analyzed TRPM8 activation in cells by gradually lowering the menthol concentrations from 2.5 μM to 0.025 μM

treated with ethanol (solvent) served as controls. The table beneath summarizes minimum levels for TRPM8 activation as determined for menthol, linalool, menthone and carvone using this cell culture assay, and the estimated agonist contents per cigarette required to trigger TRPM8 activation *in vivo* (according to the parameters laid down in the ISO smoking regime, DIN ISO 3308; cf. Discussion section).

(Fig. 2C). Notably, there was no clear dose response in relation to the initially induced calcium influx. However, at higher concentrations (2.5 μM) a prolonged influx of calcium has been observed, while recuperation of basal levels was delayed. In contrast, only a marginal Ca^{2+} increase was observed after application of 0.025 μM menthol. Our data suggest 0.1 μM menthol as minimum concentration to activate TRPM8 at the molecular level.

4. Discussion

As expected, our estimated minimum menthol concentration required for TRPM8 activation is lower than the previously determined EC_{50} values *in vitro* (Behrendt et al., 2004; Bödding et al., 2007) (Table 2). However, this estimated minimum level is in about the same range as menthol concentrations that had previously been reported to trigger a detectable activation of cold-receptors *in vivo* (Willis et al., 2011; Sant' Ambrogio et al., 1991) (Table 2).

Willis et al. (2011) demonstrated that inhalation of 16 ppm menthol did completely block the irritation response toward acrolein, whereas 2 ppm menthol showed no effect. This corresponds to an active menthol concentration of approximately 0.65 μM, which is about 6-fold higher than our estimated minimum level. We conclude that the threshold level of menthol in tobacco smoke sufficient to trigger a relevant and detectable TRPM8 activation lies somewhere between 0.1 μM and 0.65 μM. Experiments by Sant' Ambrogio et al. (1991) confirmed a similar value, demonstrating that 0.9 μM menthol (140 ng/mL) was sufficient to activate cold-sensing laryngeal receptors/neurons in new born dogs. To assess physiological effects including putative risks of menthol in tobacco products, we postulate a minimum menthol concentration required to trigger relevant *in vivo* effects between 0.3–0.4 μM. In addition, we have also determined the minimum levels of linalool, menthone and carvone for TRPM8 activation (Fig. 2). The numbers obtained confirm that these compounds are less potent than menthol (Behrendt et al., 2004).

Although physiological effects of menthol on cold-receptors are well recognized, the regulation as tobacco additive is still focused on its mint-like flavor. Importantly, activation of TRPM8 needs to be regarded as relevant mechanism to suppress irritating properties of tobacco smoke, and thus as some sort of counteraction of the natural resistance against the inhalation of fumes. In principle, detectable activation of TRPM8 constitutes an additional risk for smokers, despite the likely differential relevance for beginners and experienced smokers. Several investigations and studies provide evidence for a supporting role of menthol during smoking initiation, amongst these—most importantly—the ascertainment of the high prevalence of mentholated products among youths (TPSAC, 2011). There is further evidence indicating that people who start smoking by using mentholated cigarettes are more likely to progress toward regular smoking habits and nicotine dependence (Nonnemaker et al., 2013). This assumption is also supported by higher scores of individuals of this population when nicotine-dependence scales are being applied (Hersey et al., 2006; Fagan et al., 2015). According to previous studies, menthol does not affect long-term health (Brooks et al., 2003; Carpenter et al., 1999), a finding especially relevant in the case of lung cancer disease which mainly affects smokers who had been adapted (and possibly addicted) for a comparatively long time. After adaption, the physiological properties of menthol and other TRPM8 agonists therefore seem less relevant to maintain smoking habits.

In consideration of the data presented, we propose that tobacco products and additives should be assessed in terms of both flavor and putative physiological effects. Based on the parameters of the ISO smoking regime (DIN ISO 3308, see: http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=60404), the proposed minimum menthol concentration of 0.1 μM–0.65 μM would relate to 4.37 μg–28.4 μg per 280 mL (that is, the smoking volume of one cigarette: 8 puffs, 35 mL each). According to the European Tobacco Product Directive, DIN ISO 3308 is used to analyze emissions; however machine smoking regimes can only provide a rough simulation of human smoking behavior. Menthol as flavoring compound is highly volatile; however, little is known on the transition of low-level menthol from dry tobacco into tobacco smoke. For a rough estimate, we considered the analysis of Gordon et al. (2011) who demonstrated a significant transfer of menthol into tobacco smoke, using 0.1% mentholated cigarettes. Notably, menthol was associated with the total particulate matter, while transfer in the gas phase was not determined. This suggests that elevated concentrations might occur in the upper airway by inhalation of particles that carry menthol. Assuming an approximate transfer of 30%, which was also confirmed by earlier studies (Bozinski et al., 1972), minimal menthol contents of about 15–95 μg per cigarette would be expected to trigger a noticeable activation of TRPM8 in the respiratory tract of individuals while smoking. Although data gaps still need to be filled, our calculations allow for some further conclusions. Firstly, even menthol levels far below the milligram scale, the latter well known to be regularly applied in mentholated cigarettes (Ai et al., 2016) (cf. Table 1), presumably will be well sufficient to trigger TRPM8-mediated effects during inhalation. According to our model, TRPM8 activation is likely to occur when menthol contents exceed 50 μg per cigarette. Although the threshold for partial TRPM8 activation might be lower, TRPM8 activation probably can be excluded when menthol contents fall below 4 μg per cigarette. Secondly, endogenous menthol levels are unlikely to trigger TRPM8 activation. Again only limited data are available. Our investigation of self-grown tobacco plants reveals an endogenous content of approximately 0.3 μg menthol per gram tobacco leaf (Table 1), or 0.21 μg per cigarette. Even in a worst case estimate (onset of relevant TRPM8 activation at 4.37 μg per cigarette), endogenous contents thus would still be 20-fold below physiologically relevant levels. Nevertheless, endogenous menthol contents of industrially processed tobacco should be monitored by product surveillance, keeping in mind that newly bred or genetically modified tobacco might end up with much higher endogenous menthol levels as our self-grown plants. Thirdly, other natural TRPM8 agonists, especially linalool, menthone and carvone require 1000–6500-fold higher levels to trigger similar effects on the cold-receptor as menthol (Fig. 2). Nevertheless it seems feasible to achieve TRPM8 activation if comparatively high levels were to be used as additives. On the other side, some synthetic compounds, especially the TRPM8 super-agonist icilin [*i.e.*, 1-(2-hydroxyphenyl)-4-(3-nitrophenyl)-3,6-dihydropyrimidin-2-one], may mediate similar or even stronger effects compared to menthol (Behrendt et al., 2004). There is as yet no hint that such highly potent TRPM8 agonists already advanced into practical application though.

5. Conclusions

Although we did not find menthol levels sufficient to activate TRPM8 in the non-mentholated products analyzed here (highest levels of about 3.6 μg/cigarette were found in light cigarettes, brand A; see Table S7), others did (Ai et al., 2016; Richter et al., 2016). Hence some concern exists that products might be adjusted to avoid a mint-like characterizing flavor on the one hand, but remain to be capable of triggering a cooling effect within the upper respiratory tract on the other. Conversely, all of the mentholated cigarette brands investigated (see Table S4), revealed menthol levels well beyond the level of 50 μg per cigarette and are thus likely capable of inducing TRPM8 activation *in vivo*. In principle,

agonists of the TRPM8 receptor are suited to contribute to the enhancement of tobacco smoke inhalation *via* defined and detectable physiological effects in the respiratory tract. This premise remains valid, although the relevance of TRPM8 activation to enhance inhalation seems less apparent for experienced smokers after adaption. Still, the tobacco industry might be tempted to continue efforts to utilize synthetic compounds as alternative cooling agents, being much more potent than the natural menthol-like flavorings that were in the focus so far (Bharate and Bharate, 2012). European regulators should consider further restrictions for menthol and synthetic TRPM8 agonists, based on its innate physiological properties at the TRPM8 receptor.

Conflict of interest

None

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2017.02.020.

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