Analgesic mechanisms of essential oil components

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Summary

Transient receptor potential (TRP) channels respond to a wide variety of sensory stimuli, including temperature, nociceptive compounds, touch, osmolarity and pheromones. TRPV1 can be activated by chemical ligands such as capsaicin, endocannabinoid, anandamide and protons. It is also activated by physical stimuli such as heat and acts as an integrator of multiple noxious stimuli. TRPA1 is an ion channel targeted by pungent irritants such as those from mustard oil and garlic and is thought to function in diverse sensory processes, including cold nociception and inflammatory pain. Therefore, TRPV1 and TRPA1 ion channels are involved in acute nociception and inflammatory pain and are considered to be promising targets for the development of analgesic agents. Most of the published TRPV1 and TRPA1 antagonists lack optimal properties for clinical development such as selectivity, solubility, oral bioavailability, and/or acceptable pharmacokinetics. Thus, it is important to explore naturally occurring antagonists of these channels, especially those that have an extensive safety profile based on widespread usage.

Essential oils are often used in alternative medicine as analgesic and anti-inflammatory remedies. For example, menthol, the main ingredient of peppermint, is commonly used for pain relief. The analgesic effects of menthol are believed to occur through TRPM8 activation. However, the molecular mechanisms underlying the analgesic effects of these ingredients are largely unknown. In fact, high doses of menthol cause sensory irritation through TRPA1 activation in humans. Camphor, another essential oil component, is now known to exert analgesic effects through inhibition of TRPA1 and activation of TRPM8. However, camphor is not suitable for
use as an analgesic compound because it causes warm or hot sensations, probably through TRPV1 and TRPV3 activation. I concluded that it was important to identify other effective analgesic compounds that activated TRPM8 and inhibited TRPA1 but did not activate TRPV1.

Through the screening of essential oils, I found that eucalyptus oil exhibited a relatively high hTRPM8-activating ability with less activation of hTRPA1. Furthermore, 1,8-cineole, a major component of eucalyptus oil, inhibited hTRPA1 activated by several agonists with a half-maximal inhibitory concentration (IC50) of 3.4 mM for AITC (20 μM)-evoked hTRPA1 currents. In addition, sensory irritation tests in vivo showed that 1,8-cineole conferred an analgesic effect on the sensory irritation produced by menthol and a TRPA1 agonist, octanol. Moreover, 1,4-cineole, another component of eucalyptus oil, activated hTRPA1. Several compounds with similar structures exhibited different effects on hTRPA1. Menthol and 1,4-cineole activated hTRPA1, whereas camphor and 1,8-cineole inhibited hTRPA1. Given these variable effects on hTRPA1, I hypothesized that more detailed analyses would lead to the identification of more effective compounds.

To identify more effective TRPA1 antagonists, I screened camphor analogs among monoterpenes that comprise a group of naturally occurring organic compounds derived from essential oils that have been used for anesthetic, analgesic, anti-inflammatory and anti-pruritic applications. I found that borneol, 2-methylisoborneol and fenchyl alcohol inhibited hTRPA1 activity. The hTRPA1 currents induced by AITC (20 μM) were inhibited by borneol, 2-methylisoborneol or fenchyl alcohol, each in a dose-dependent manner. I obtained IC50 values of 0.20, 0.12 and 0.32 mM for borneol, 2-methylisoborneol and fenchyl alcohol, respectively, values that are much lower than
the IC\textsubscript{50} values of 1,8-cineole (3.43 mM) and camphor (1.26 mM). In addition, sensory irritation tests \textit{in vivo} showed that borneol conferred an analgesic effect on the sensory irritation produced by menthol. Furthermore, I found that the S873, T874, and Y812 residues of hTRPA1 were involved in the inhibitory effects, suggesting that the hydroxyl group in the cyclohexane of the inhibitors may interact with these amino acids.

To understand the analgesic mechanism of menthol, I examined the effects of menthol on hTRPV1. The hTRPV1 currents induced by capsaicin were inhibited by menthol in a dose-dependent manner, with an IC\textsubscript{50} value of 1.17 mM. In addition, an \textit{in vivo} sensory irritation test showed that menthol conferred an analgesic effect on the sensory irritation produced by vanillyl butyl ether (VBE), a TRPV1 agonist. Furthermore, I found that Y511, S512 and T550 of hTRPV1, which are binding sites of capsaicin, were only slightly involved in the inhibitory effects of menthol. These data suggest that menthol interacts with sites different from those of capsaicin. These results show that an analgesic effect of high-dose of menthol is derived, at least in part, from its inhibitory effect on TRPV1.

In this study, I elucidated the molecular mechanisms underlying the analgesic effects of essential oil components. Further analysis of these compounds could lead to better understanding of the structural determinants for the action of these compounds on TRPV1 or TRPA1 and the development of anti-nociceptive agents through TRPA1 or TRPV1 inhibition.
1 Introduction

Monoterpenes, like menthol and camphor, comprise a group of naturally occurring organic compounds derived from essential oils that have been used for anesthetic, analgesic [Xu et al., 2005; Galeotti et al., 2001; Galeotti et al., 2002], anti-inflammatory [Xu et al., 2005; Santos et al., 2001] and antipruritic applications [Umezu et al., 2001; Anand et al., 2003]. However, the molecular mechanisms by which essential oils and these compounds exert their effects are largely unknown.

Transient receptor potential (TRP) channels respond to a wide variety of sensory stimuli, including temperature, nociceptive compounds, touch, osmolarity, and pheromones [Christensen et al., 2007; Minke et al., 2002; Zhang et al., 2008]. TRPV1 is activated by capsaicin as well as heat, protons and some endogenous substances known to be associated with tissue inflammation [Caterina et al., 1997; Tominaga et al., 1998; Tominaga and Caterina, 2004]. Since TRPV1 acts as an integrator of painful stimuli, TRPV1 antagonists can be viewed as promising types of analgesics [Gavva et al., 2008; Gunthorpe et al., 2009; Marcello et al., 2010; Marcello et al., 2013]. A number of potent, small TRPV1 antagonists, including capsazepine, BCTC, CTPC, AMG9810 and SB-452533 have advanced into clinical trials for the evaluation of their analgesic activities [Bevan et al., 1992; Valenzano et al., 2003; Rami et al., 2004; Gavva et al., 2005; Weil et al., 2005]. Although some of these antagonists reduced noxious heat sensation, hyperthermia was a serious side effect that often led to their withdrawal from the clinical trials.

TRPA1 is a receptor that responds to noxious cold temperatures in rodents and
pungent compounds, including allyl isothiocyanate (AITC), a component of mustard oil [Story et al., 2003; Bandell et al., 2004; Jordt et al., 2004; Obata et al., 2005; Kwan et al., 2006]. Although the role of TRPA1 in sensing noxious cold stimuli and somatic mechanosensations in vivo remains unsettled, especially in mammals [Obata et al., 2005; Kwan et al., 2006; Petrus et al., 2007], TRPA1 has been established as a chemical nocisensor for a wide variety of reactive compounds such as flufenamic acid (FFA), 2-aminoethoxydiphenyl borate (2-APB), icilin, menthol, intracellular calcium, and zinc ions [Story et al., 2003; Bautista et al., 2005; Hinman et al., 2006; Doerner et al., 2007; Macpherson et al., 2007; Zurborg et al., 2007; Wang et al., 2008; Andersson et al., 2009; Hu et al., 2009; Hu et al., 2010]. In addition, previous studies identified TRPA1 as a receptor for parabens-mediated irritation of the skin [Fujita et al., 2007] and for pain produced by alkaline pH [Fujita et al., 2008]. On the other hand, menthol has different effects on TRPA1 in humans and mice. Previous studies identified the bimodal action of several compounds such as menthol on mouse TRPA1 (mTRPA1) gating: submicromolar to low micromolar concentrations of menthol caused robust channel activation, whereas higher concentrations led to reversible channel blocking [Karashima et al., 2007; Xiao et al., 2008]. Such bimodal action was not observed with human TRPA1 (hTRPA1) [Xiao et al., 2008]. TRPA1 is reportedly involved in inflammatory processes, including inflammation produced by several airway irritants that cause asthma [Bautista et al., 2006; Caceres et al., 2009; Zhou et al., 2013] and neuropathic pain [Zhou et al., 2013]. Therefore, TRPA1 is an excitatory ion channel targeted by acute nociception and inflammatory pain and is considered to be a promising target for the development of analgesic agents [Nagata et al., 2005; Xu et al., 2005; Klionsky et al., 2007; Eid et al., 2008; McGaraughty et al., 2010; Chen et al., 2011]. There are
several reported TRPA1 antagonists: camphor obtained from the *Cinnamomum camphora* tree [Xu et al., 2005], HC-030031 [Eid et al., 2008], AZ868 [Vallin et al., 2012], A-967079 [Chen et al., 2011], and CMP1, CMP2 and CMP3 that were identified as thioaminal-containing molecules [Chen et al., 2008]. Among these TRPA1 antagonists, naturally occurring analgesic compounds that inhibit hTRPA1 and have a demonstrated safety profile from their lengthy usage would be desirable.

TRPM8 is a thermosensitive receptor that detects cool temperatures and menthol [McKemy et al., 2002; Peier et al., 2002], a natural non-reactive cooling compound that is also involved in anti-nociception to some extent [Dhaka et al., 2007; Proudfoot et al., 2006]. Menthol (the main ingredient of peppermint) is used for pain relief in daily life. It functions through TRPM8 activation [Proudfoot et al., 2006; Galeotti et al., 2002]; however, the molecular mechanisms underlying its analgesic effects are largely unknown. Moreover, high doses of menthol cause sensory irritation [Geen et al., 1992] because it acts as a TRPA1 activator in humans [Karashima et al., 2007]. Camphor, another essential oil component, is now known to exert analgesic effects, most likely through inhibition of TRPA1 [Xu et al., 2005] and activation of TRPM8 [Vogt-Eisele et al., 2007]. However, camphor is not suited for use as an analgesic compound because it causes warm and hot sensations [Green et al., 1990], probably through TRPV1 and TRPV3 activation [Xu et al., 2005]. Therefore, I hypothesized that an effective analgesic compound would activate TRPM8 and inhibit TRPA1, but not activate TRPV1.

Several TRP channels are known to be activated or inhibited by plant-derived substances, such as menthol and camphor, some of which are contained in essential oils. Essential oils have long been used and their side effects are generally considered to be
minimal. Accordingly, essential oils, especially ones acting on TRP channels, could be promising sources for the development of analgesic agents. Therefore, I have screened essential oils for their ability to activate human TRPM8 (hTRPM8) but not hTRPA1, properties distinct from those of menthol. Moreover, I examined the effects of menthol on hTRPV1 to clarify the mechanisms of its analgesic effect.

Through the screening, I found that eucalyptus oil exhibited relatively high hTRPM8-activating ability with comparatively less activation of hTRPA1. Furthermore, 1,8-cineole, a main component of eucalyptus oil, was identified as a novel natural antagonist of hTRPA1. I screened camphor analogs to find more effective TRPA1 antagonists. From the screening, borneol, 2-methylisoborneol, and fenchyl alcohol were found to exhibit higher inhibitory effects than camphor and 1,8-cineole. In addition, the hTRPV1-mediated currents induced by capsaicin were inhibited by menthol.
2 Materials and Methods

2.1 Molecular cloning

Full-length human genes TRPA1, TRPM8, TRPV1, and TRPV2 were obtained from Life Technologies (Carlsbad, CA, USA) and TRPV3 was generously provided by Dr. Hwang (Korea University). cDNAs were cloned into the pcDNA3.1 vector.

2.2 Reagents

Camphor, borneol, fenchyl alcohol, and 2-methylisoborneol were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). (-)-Fenchone, 1,8-cineole, camphorquinone, norcamphor, α,β-thujone, α-pinene oxide, (-)-limonene oxide, (+)-borneol, (-)-borneol and (±)-isobornyl methyl ether were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bornyl acetate, (±)-isoborneol and 3-methylene-2-norbornanone were obtained from Tokyo Kasei Co. Ltd. (Tokyo, Japan). The compounds were used as a mixture of (+) and (-) isomers unless otherwise stated.
2.3 Cell culture

Human embryonic kidney-derived 293T (HEK293T) cells were maintained in DMEM (WAKO Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% FBS (Biowest SAS, Caille, France), 100 units/mL penicillin (Life Technologies Corp., Carlsbad, CA, USA), 100 µg/mL streptomycin (Life Technologies Corp.), and 2 mM L-glutamine (GlutaMAX, Life Technologies Corp.) at 37°C in 5% CO₂. For Ca²⁺-imaging, 1 µg plasmid DNA carrying TRPA1, TRPV1, TRPV2, TRPV3 or TRPM8 in pcDNA3 in OPTI-MEM medium (Life Technologies Corp.) were transfected into HEK293T cells using Lipofectamine Plus Reagent (Life Technologies Corp.). After incubating for 3 to 4 h, the cells were reseeded on coverslips and further incubated at 37°C in 5% CO₂.

2.4 Human subjects

Japanese male subjects in their 20s and 30s were selected as participants to eliminate confounding factors that may have influenced the perception of skin sensitivity, including race, age, gender, and hormonal and psychosocial interactions. To evaluate sensory irritation, I selected skin-sensitive male volunteers. Female volunteers were excluded because of possible hormonal influences. Ethics approval and informed consent was obtained from all participants.
2.5 TRPA1, TRPV1 and TRPM8 mutants

Two TRPA1 mutants (S873V/T874L and Y812A) were constructed by double and single amino acids substitutions using a GeneTailor Site-Directed Mutagenesis System (Invitrogen) [Xiao et al., 2008]. Three TRPV1 mutants (Y511A, S512Y and T550I) were made using a modified QuickChange Site-Directed Mutagenesis method (Agilent Technologies Inc., Santa Clara, CA, USA) [Jordt et al., 2002; Gavva et al., 2004; Shintaku et al., 2012]. Three TRPM8 mutants (Y745H, Y1005F and L1009R) were constructed by single amino acid substitutions using a GeneTailor Site-Directed Mutagenesis System (Invitrogen) [Bandell et al., 2006; Benedikt et al., 2007; Fujita et al., 2013]. In each case, the entire sequence (including the desired substitutions at the site of mutation) was confirmed.

2.6 Transfection

Cells were transfected using one mg of TRPA1, TRPV1, TRPV2 or TRPM8 cDNA with Lipofectamine and OPTI-MEM I Reduced Serum Medium (Invitrogen, Carlsbad, CA) as described previously (Caterina et al., 1997). For patch clamp experiments, cells were co-transfected with these plasmids and green fluorescent protein (GFP) in pcDNA3.1. Green fluorescence from cells expressing GFP was detected with the aid of a Nikon (Tokyo, Japan) microscope equipped with a mercury lamp light source and a GFP filter (emission wavelength, 510 nm).
2.7 Calcium imaging

Ca$^{2+}$-imaging was performed 1 day after transfection. HEK293T cells on coverslips were mounted in an open chamber and superfused with standard bath solution (140 mM NaCl, 5 mM KCl, 2 mM MgCl$_2$, 2 mM CaCl$_2$, 10 mM HEPES, 10 mM glucose, pH 7.4). Cytosolic-free Ca$^{2+}$ concentrations in HEK293T cells were measured by dual-wavelength fura-2 (Molecular Probes, Invitrogen Corp.) microfluorometry with excitation at 340/380 nm and emission at 510 nm. The fura-2 ratio image was calculated and acquired using the IP-Lab imaging processing system (Scanalytics Inc., Fairfax, VA USA).

2.8 Electrophysiology

Whole-cell patch-clamp recordings were performed 1 day after transfection. The standard bath solution was the same as that used in the Ca$^{2+}$-imaging experiments, but extracellular Ca$^{2+}$ was omitted and 5 mM EGTA was added for the recording of AITC-, menthol- and FFA-induced current responses. The pipette solution contained 140 mM KCl, 5 mM EGTA, 10 mM HEPES, pH 7.4 (adjusted with KOH). Data from whole-cell voltage-clamp recordings were sampled at 10 kHz and filtered at 5 kHz for analysis (Axon 200B amplifier with pCLAMP software, Axon Instruments, Sunnyvale, CA, USA). Membrane potential was clamped at -60 mV with 5 sec voltage ramp-pulses from -100 to +100 mV (500 ms). All experiments were performed at room temperature.
2.9 Sensory irritation tests

The study was conducted at a temperature of 21–23°C and a relative humidity of 45-55%. I used the side of the neck, which is innervated by spinal nerves, for the skin irritation analysis because this region is known to be sensitive to various skin irritants. Areas of skin were cleaned with a wet towel and acclimatized for 10 min prior to testing. Blind randomized half-region (left vs. right) trials were performed with two different samples applied to the neck region. A total of 200 µL of 1,8-cineole or 80 µL of menthol was applied. The subjects evaluated pricking, stinging, burning and itching sensations after 1, 3, 5, 7 and 10 min of compound/chemical application in accordance with the criteria summarized in Table 1. The total sensory irritation scores were calculated for the entire period.

2.10 Data analysis

Data in all figures are shown as means ± standard error of the mean (SEM). Statistical significance of the effects of 1,8-cineole on several TRP channels were evaluated using ANOVA followed by two-tailed multiple t-test with Bonferroni correction. The statistical significance of effects of menthol and capsaicin on hTRPV1 and hTRPM8 mutants were evaluated using Student’s t test. Dose-dependent curves were fit with a Hill equation. Sensory irritation tests were evaluated using a Wilcoxon signed-rank test.
3 Results

3-1. Screening essential oils for the ability to activate hTRPM8 but not hTRPA1

3-1-1. Eucalyptus oil possessed hTRPM8-activating ability but little capacity to activate hTRPA1

In order to find promising essential oils that could act as analgesics, I evaluated the ability of individual oils (0.01%) to activate hTRPM8 or hTRPA1. The results were compared with the response to 1 mM menthol. This was accomplished by using a Ca$^{2+}$-imaging method with HEK293T cells expressing hTRPM8 or hTRPA1 proteins. For example, the effect of peppermint oil is presented as the fura-2 ratio (corresponding to cytosolic Ca$^{2+}$ concentrations) compared to that caused by menthol, the main component of peppermint oil. I observed a value of nearly 1.0. Among the essential oils examined, clove oil and eucalyptus oil were found to exhibit some hTRPM8 activation (Figure 1A). Although many of the examined essential oils exhibited hTRPA1 activation (like menthol) sage oil and eucalyptus oil showed less hTRPA1 activation (Figure 1B). When I calculated the ratio of hTRPM8-activating ability versus hTRPA1-activating ability by simply dividing the values in Figure 1A by the values in Figure 1B, the ratio of eucalyptus oil was comparable to that of peppermint oil and much higher than any other oil examined (Figure 1C).

3-1-2. 1,8-cineole activated hTRPM8 but not hTRPA1

Next, I examined the effects of fragrance chemicals, many of which are contained in the essential oils tested above. I compared the same parameters used for the essential oils. As shown in Figure 2A and B, 1,8-cineole, menthone and eugenol showed relatively
high responses compared with other chemicals as measured by the sample/menthol fura-2 ratio in HEK293T cells expressing hTRPM8. This is consistent with the observation that peppermint oil, clove oil and eucalyptus oil, which contain menthone, eugenol and 1,8-cineole, respectively, showed similar high ratios (Figure 1A). Interestingly, 1,8-cineole, but neither menthone nor eugenol, showed low changes in the fura-2 ratio in HEK293T cells expressing hTRPA1 (Figure 2C and D). Although linalool was reported to produce anti-nociception [Peana et al., 2003], the ratio of hTRPM8-activating ability versus hTRPA1-activating ability for linalool was found to be low, suggesting it was a poor candidate as an analgesic. Accordingly, the ratio of 1,8-cineole was found to be very high as shown in Figure 2E. These data indicated that 1,8-cineole contained in eucalyptus oil can activate hTRPM8 without activating hTRPA1.

3-1-3. 1,8-cineole acted on hTRPM8 and hTRPV3, but not on hTRPV1, hTRPV2 or hTRPA1

In order to examine whether 1,8-cineole could activate other TRP channels expressed in sensory neurons, I performed Ca\(^{2+}\)-imaging experiments using HEK293T cells expressing hTRPV1 or hTRPV2 [Caterina et al., 1997; Caterina et al., 1999; Caterina et al., 2000; McKemy et al., 2002; Peier et al., 2002; Behrendt et al., 2004; Bautista et al., 2006; Kwan et al., 2006; Proudfoot et al., 2006; Colburn et al., 2007]. Treatment with 1,8-cineole increased the fura-2 ratio (340 nm/380 nm) in HEK293T cells expressing hTRPM8, but not in cells expressing hTRPA1, hTRPV1 or hTRPV2 (Figure 3A, B and D). These results were consistent with previous findings in rodent cell lines expressing these proteins. Because 1,8-cineole, like menthol, was reported to activate
mouse TRPV3 in a *Xenopus* oocyte expression system [Vogt-Eisele et al., 2007; Sherkheli et al., 2009; Saito et al., 2011], I checked the effect of 1,8-cineole on hTRPV3. Basal fura-2 ratio levels were slightly higher for hTRPV3-expressing HEK293T cells compared to cells expressing hTRPM8, hTRPA1, hTRPV1 or hTRPV2, probably because hTRPV3 can be activated by the warm temperatures to which the cells were exposed during incubation (Figure 3C). 1,8-cineole caused a small but significant fura-2 ratio increase as expected (Figure 3C, D).

3-1-4. 1,4-cineole activated hTRPM8 and hTRPA1 expressed in HEK293T cells

Since eucalyptus oil contains not only 1,8-cineole but also 1,4-cineole, and because these chemicals have similar structures (Figure 4A), the actions of 1,4-cineole on hTRPM8 and hTRPA1 were assessed using a Ca\(^{2+}\)-imaging method. Surprisingly, 1,4-cineole (5 mM) caused increases in the fura-2 ratio not only in cells expressing hTRPM8 (Figure 4B), but also in cells expressing hTRPA1 (Figure 4C). These results might explain the apparent difference in the effects between eucalyptus oil and 1,8-cineole regarding the ratio of hTRPM8-activating versus hTRPA1-activating abilities (Figs. 1C and 2E) in which the ratio for 1,8-cineole was very low while the ratio for eucalyptus oil was comparable to that of peppermint oil.

3-1-5. 1,4-cineole but not 1,8-cineole activated hTRPA1

Next, I performed patch-clamp experiments to confirm the effects of 1,8-cineole and 1,4-cineole on hTRPM8 and hTRPA1 expressed in HEK293T cells. Both 1,8- and 1,4-cineole (5 mM) evoked inward currents with outwardly rectifying current-voltage (I-V) relationships in cells expressing hTRPM8 (Figure 5A, C). On the other hand,
1,8-cineole (5 mM) did not activate hTRPA1 in cells responding to AITC (a TRPA1 agonist) whereas 1,4-cineole evoked an inward current with an outwardly rectifying I-V relationship in cells expressing hTRPA1 (Figure 5B, D). These results showed that chemicals with similar structures exhibited different effects on hTRPA1. Menthol has bimodal action on mTRPA1: lower concentrations of menthol activate mTRPA1 whereas higher concentrations of menthol inhibit it [Xiao et al., 2008]. To confirm that low concentrations of 1,8-cineole activated hTRPA1, I performed patch-clamp experiments. Ten μM and 100 μM 1,8-cineole did not activate hTRPA1 in cells responding to AITC (data not shown).

3-1-6. 1,8-cineole inhibited hTRPA1 currents activated by different agonists

Because 1,8-cineole has analgesic and anti-inflammatory effects in vivo [Santos et al., 2000; Silva et al., 2003], I hypothesized that 1,8-cineole would inhibit TRPA1 [Bautista et al., 2006]. To test this hypothesis, I used AITC (20 μM), which is a TRPA1 agonist that acts through covalent cysteine modification [Hinman et al., 2006; Macpherson et al., 2007]. I observed that induced hTRPA1 currents were inhibited by 1,8-cineole in a dose-dependent manner with an IC_{50} value of 3.4 ± 0.6 mM (Figure 6A, B). The effects of 1,8-cineole on hTRPA1 activated by other TRPA1 agonists were also determined. Several TRPA1 agonists with different activation mechanisms were chosen: (1) menthol, which seems to interact specifically with residues within transmembrane domain 5 to gate TRPA1 [Xiao et al., 2008]; (2) flufenamic acid (FFA), thought to be a cysteine-nonreactive compound [Hu et al., 2010]; and (3) octanol, the mechanism of action of which is largely unknown [Komatsu et al., 2012]. For menthol- or FFA-evoked hTRPA1 currents, I measured the current responses in the
absence of extracellular Ca\(^{2+}\) to minimize desensitization, similar to the experiment examining the effects on AITC-evoked currents. For examination of octanol-evoked hTRPA1 currents, I performed patch-clamp recordings in the presence of extracellular Ca\(^{2+}\) because octanol-evoked responses were too small to analyze in the absence of extracellular Ca\(^{2+}\) (data not shown), thus leading to difficulties in plotting a dose dependency curve. Similar to hTRPA1 currents activated by AITC, hTRPA1 currents activated by menthol (500 \(\mu\)M) or FFA (100 \(\mu\)M) were inhibited by 1,8-cineole in a dose-dependent manner with IC\(_{50}\) values of approximately 0.5 ± 0.1 and 5.3 ± 0.1 mM, respectively (Figure 6C, D, E, F). At 1 mM, octanol-evoked hTRPA1 currents were inhibited reversibly by 1,8-cineole (5 mM) (Figure 6G). These results again suggested that 1,8-cineole was an antagonist of hTRPA1. Interestingly, currents evoked by AITC, menthol or FFA were increased upon washout of 1,8-cineole (Figure 6A, C and E) probably through the reversal of 1,8-cineole-mediated inhibition. I confirmed that 1,8-cineole did not inhibit hTRPV1, hTRPV2 or hTRPV3 responses activated by capsaicin, 2-APB and a cocktail (2-APB + carvacrol), respectively (Figure 6H).

3-1-7. 1,8-cineole inhibited sensory irritation caused by hTRPA1 agonists in vivo

To confirm the inhibitory effect of 1,8-cineole on hTRPA1 in vivo, sensitive human subjects were recruited for sensory irritation tests. Sensory irritation caused by 1,8-cineole itself was comparable to that by vehicle alone (Figure 7A,B), indicating that 1,8-cineole did not cause sensory irritation. Next, I examined the inhibitory effect of 1,8-cineole with concomitant application of the TRPA1 agonist octanol [Komatsu et al., 2012] and 1,8-cineole. Octanol (0.2 wt%) caused sensory irritation for the 7 min, with
a gradual increase thereafter (Figure 7C), as was previously reported [Van Thriel et al., 2003]. The octanol-induced sensory irritation was significantly reduced by concomitant application of 1,8-cineole at the 7-min time point (Figure 7C). Analysis of the total sensory irritation score indicated that 1,8-cineole significantly inhibited sensory irritation caused by octanol (Figure 7D). These data further support the inhibitory action of 1,8-cineole on octanol-induced irritation, which could involve regulation of TRPA1 activity. Menthol is known to activate both TRPM8 and TRPA1, which can simultaneously cause comforting and irritating sensations. In order to examine whether 1,8-cineole can reduce the menthol-induced irritation, I applied menthol with or without 1,8-cineole. Concomitant application of menthol with 1,8-cineole significantly reduced irritation, probably through inhibition of TRPA1 by 1,8-cineole (Figure 7E, F).

3-2. Screening camphor analogs to find more effective TRPA1 antagonists

3-2-1. Screening of naturally occurring compounds affecting hTRPA1

Several compounds with similar structures exhibit different effects on hTRPA1. For example, menthol and 1,4-cineole activate hTRPA1 while camphor and 1,8-cineole inhibit hTRPA1 [Takaishi et al., 2012]. Given these variable effects on hTRPA1, more detailed analyses could lead to a better understanding of the structural basis for the action of these compounds on TRPA1. To identify more effective TRPA1 antagonists, I next screened camphor analogs, many of which are present in essential oils (Table 2). Towards that end, I used a Ca$^{2+}$-imaging method with HEK293T cells expressing hTRPA1. In the experiments, changes in the fura-2 ratio induced by the test compounds and menthol were compared because menthol, which activates hTRPA1,
and the test compounds are members of the monoterpene family. Borneol, 2-methylisoborneol, norcamphor, and fenchyl alcohol showed small changes in the fura-2 ratio similar to 1,8-cineole and camphor (Figure 8). Those data suggests that these compounds did not activate hTRPA1.

3-2-2. Effects of borneol, 2-methylisoborneol, fenchyl alcohol and norcamphor on hTRPA1-mediated current responses

In order to confirm the effects of the above compounds, patch-clamp experiments with HEK293T cells expressing hTRPA1 were performed. As shown in Figure 9, borneol, 2-methylisoborneol, fenchyl alcohol and norcamphor failed to activate hTRPA1, whereas AITC evoked a robust current activation with outward rectification.

3-2-3. Borneol, 2-methylisoborneol, and fenchyl alcohol (but not norcamphor) inhibited hTRPA1 in a Ca\textsuperscript{2+}-imaging method

I next investigated the possibility that the above four compounds inhibited hTRPA1 using a Ca\textsuperscript{2+}-imaging method with HEK293T cells expressing hTRPA1. Increases in the fura-2 ratio caused by menthol were almost completely blocked in the presence of borneol (1 mM), 2-methylisoborneol (1 mM) or fenchyl alcohol (1 mM) similar to that observed with 1,8-cineole and camphor (Figure 10A-C, E). Washing out the three compounds after menthol exposure led to small increases in the fura-2 ratio, which could be due to the loss of inhibition and resumption of hTRPA1 activity from residual menthol in the cell. On the other hand, norcamphor did not inhibit the menthol-induced increase in the fura-2 ratio (Figure 10D, E). It was confirmed that borneol, 2-methylisoborneol, fenchyl alcohol and norcamphor had no effect on
vector-transfected cells, while cells responded normally to ionomycin (5 μM) (Figure 11). These results suggest that borneol, 2-methylisoborneol and fenchyl alcohol, but not norcamphor, inhibited hTRPA1 activity.

3-2-4. Borneol, 2-methylisoborneol and fenchyl alcohol inhibited hTRPA1 current in a dose-dependent manner

To confirm the inhibition of hTRPA1 activity by the three compounds, patch-clamp experiments were performed with HEK293T cells expressing hTRPA1. The current response was measured in the absence of extracellular Ca$^{2+}$ to minimize desensitization. One mM borneol, 2-methylisoborneol and fenchyl alcohol completely inhibited the hTRPA1-mediated current activated by menthol (1 mM) or FFA (100 μM) (Figure 12). Enhancement of the hTRPA1-current was observed upon washing out of the three compounds in response to FFA, but not menthol.

Next, the effective inhibitory concentrations of the three compounds were determined. For this experiment, AITC was chosen as the hTRPA1 agonist because AITC had a higher ability to activate hTRPA1 than did menthol or FFA. One mM borneol, 2-methylisoborneol, and fenchyl alcohol completely inhibited the AITC-induced (20 μM) hTRPA1 current, while 1 mM camphor partially inhibited the AITC-induced response (Figure 13A-D), suggesting that borneol, 2-methylisoborneol and fenchyl alcohol were more capable of inhibiting hTRPA1 than was camphor. Enhancement of the hTRPA1-current was again observed upon washing out of the three compounds.

The dose-dependency of the inhibitory effects of the three compounds on hTRPA1 was then examined using a patch-clamp method. Similar to camphor and 1,8-cineole,
the hTRPA1 current induced by AITC (20 μM) was inhibited by borneol, 2-methylisoborneol and fenchyl alcohol in a dose-dependent manner with IC$_{50}$ values of 0.20 ± 0.06 mM, 0.12 ± 0.03 mM and 0.32 ± 0.06 mM, respectively. Those values were much lower than the IC$_{50}$ of 1,8-cineole (3.43 ± 0.58 mM) and camphor (1.26 ± 0.32 mM) (Figure 13E). These data suggested that borneol, 2-methylisoborneol and fenchyl alcohol have potential to be used as effective analgesic compounds. Commercially available borneol contains both optical isomers. I confirmed that there was no difference in the inhibitory effects on hTRPA1 activity between (+)- and (-)-borneol (Figure 14).

3-2-5. The hydroxyl group of borneol contributed to inhibition of human TRPA1
Xiao et al. showed that menthol acted as an activator of mTRPA1 at low concentrations, and as a blocker at high concentrations [Xiao et al., 2008]. This bimodal effect of menthol on TRPA1 was observed in a mouse clone, but not in a human clone. That author studied a mouse TRPA1 mutant in which serine and threonine residues located in the predicted inner side of TM5 were replaced with valine and leucine (S876V/T877L), respectively. It was found that the mutant was neither activated nor inhibited by menthol [Xiao et al., 2008]. Moreover, the serine and threonine residues were found to be critical for the sensitivity of TRPA1 to menthol in both mammalian TRPA1 channels. It was suggested that T877 of mTRPA1 interacted with menthol through a hydrogen bond [Xiao et al., 2008]. The fact that borneol, 2-methylisoborneol and fenchyl alcohol have similar hydroxyl groups in their structures led us to hypothesize that these compounds interacted with the same serine and/or threonine of TRPA1 as did menthol. To test this hypothesis, I investigated whether the serine and threonine residues were
involved in the inhibitory effect of borneol. The mutant channel hTRPA1-S873V/T874L (corresponding to mTRPA1-S876V/T877L) exhibited significantly lower inhibitory effects at three different concentrations of borneol than did hTRPA1-WT when AITC-activated current was examined (Figure 15A). On the other hand, camphor and 1,8-cineole showed no significant changes in their inhibitory effects with cells expressing mutant hTRPA1 compared with hTRPA1-WT (Figure 15B, C). I asked whether there was involvement of other amino acids in borneol activity. Because a tyrosine residue in TM2 of TRPM8 is known to be involved in interactions with menthol, I screened tyrosine mutants in TM2 and TM3 of hTRPA1 using a Ca\(^{2+}\)-imaging method and found that the effect of borneol was reduced with a Y812A mutant (data not shown). The mutant (hTRPA1-Y812A) showed significantly lower inhibitory effects of borneol on the AITC-activated hTRPA1 current at two different concentrations than did hTRPA1-WT with a patch-clamp method, whereas such differences between WT hTRPA1 and hTRPA1-Y812A were not observed for 1,8-cineole or camphor activity (Figure 15D-F).

3-3. The effects of menthol on human TRPV1

3-3-1. Menthol inhibited sensory irritation caused by an hTRPV1 agonist in vivo

Menthol has been used for its anti-nociceptive effects for more than one thousand years [Van Hellemont et al., 1985]. Preparations containing menthol are used topically to relieve neuralgia in traditional Chinese and European medicine [Wright., 1870]. In addition, menthol demonstrated some anesthetic [Galeotti et al., 2001; McKay et al., 2006; Gaudioso et al., 2012] and κ-opioid-mediated anti-nociceptive properties in mouse hot-plate tests [Galeotti et al., 2002]. At present, the anti-nociceptive
mechanisms of menthol are not fully understood. It has been shown that TRPM8 contributed to mediating the effects of cold analgesia in the rat [Proudfoot et al., 2006] and that it had anti-nociceptive effects in TRPM8-deficient mice [Dhaka et al., 2007]. Since TRPV1 acts as an integrator of painful stimuli, TRPV1 antagonists can be used for promising therapeutics as novel types of analgesics [Gavva et al., 2008; Gunthorpe et al., 2009; Marcello et al., 2010; Marcello et al., 2013]. Therefore, to understand the analgesic mechanism of menthol, I examined the effects of menthol on hTRPV1.

First, to examine the analgesic effects of menthol in vivo, sensitive human subjects were recruited for the sensory irritation test. In this study, I used VBE, which is structurally similar to capsaicin [Mitsudome et al., 2012]. I observed that VBE activated hTRPV1 (Figure 16A), and the activation was inhibited by capsazepine (1 µM) (Figure 16B). VBE did not cause any current activation, but rather decreased basal currents in the vector-transfected cells (Figure 16C). I confirmed that menthol (5 mM) inhibited the hTRPV1 activity evoked by VBE (100 µM) (Figure 16D and E). Thus, VBE and menthol might allow us to examine the inhibitory effect of menthol on sensory irritation by VBE. VBE (0.1 wt %) caused sensory irritation, gradually increasing with time after application (Figure 17A, C). VBE-induced sensory irritation scores were not reduced by concomitant application of 0.1 wt% menthol. However, the scores were significantly reduced by 0.3 wt% menthol (Figure 17A - D). In human subjects, inhibition of VBE-induced irritation by menthol was smaller than TRPV1 inhibitory effects observed in vitro. I conjectured that VBE might activate hTRPA1 as well. Indeed, VBE had a hTRPA1-activating ability, although the effects were observed only at 1 mM whereas VBE activated hTRPV1 at lower concentrations (Figure 18).
3-3-2. Menthol inhibited hTRPV1 activity induced by capsaicin

Next, I examined the inhibitory effects of menthol on hTRPV1 using a Ca\(^{2+}\)-imaging method at room temperature with HEK293T cells expressing hTRPV1. In these cells, the changes in the fura-2 ratios (i.e., cytosolic Ca\(^{2+}\) concentrations, [Ca\(^{2+}\)]\(_i\)) induced by capsaicin (0.1 µM) in the presence of menthol (10 mM) were smaller than those in its absence (Figure 19). Although there was a slight increase in [Ca\(^{2+}\)]\(_i\) upon application of menthol (10 mM) itself, I did not observe any current activation induced by 10 mM menthol in the cells expressing hTRPV1 nor vector-transfected cells using a patch-clamp method (Figure 20A and B), indicating that the slight [Ca\(^{2+}\)]\(_i\) increase could be a non-specific phenomenon. These results suggested that menthol inhibited hTRPV1 activity.

To confirm these possibilities, I performed patch-clamp experiments using HEK293T cells expressing hTRPV1. Capsaicin (0.1 µM) induced TRPV1-mediated current activation with an outwardly rectifying current-voltage (I-V) relationship revealed by 5 sec ramp-pulses from -150 to +100 mV. Addition of menthol (5 mM) reduced the capsaicin-evoked currents at both positive and negative potentials, and these were partly recovered by menthol washout (Figure 21A). The currents evoked by concomitant application of capsaicin with menthol were smaller than those activated by capsaicin alone. These results were consistent with the data in the Ca\(^{2+}\)-imaging experiments (Figure 19).

Next, I examined the dose-dependency of the inhibitory effects. The current responses were measured in the absence of extracellular Ca\(^{2+}\) to minimize desensitization. Menthol inhibited 0.1 µM capsaicin-activated hTRPV1 currents in a
dose-dependent manner, with an IC\textsubscript{50} value of approximately 1.2 ± 0.2 mM (Figure 21B and C). In addition, I examined the effects of different concentrations of capsaicin on hTRPV1 in the absence and presence of 1 mM menthol. I found an apparent shift of the capsaicin dose-dependency towards higher concentrations with menthol (Figure 22). These results indicated that menthol inhibited hTRPV1 activity.

3-3-3. Menthol inhibited the activation of hTRPV1 induced by heat stimulation

Next, I examined the inhibitory effects of menthol on hTRPV1 activities induced by heat stimulation using a Ca\textsuperscript{2+}-imaging method. When I examined the effects of menthol on heat-evoked (> 45°C) TRPV1-mediated [Ca\textsuperscript{2+}]\text{\textsubscript{i}} changes, I compared the effects in different cells to avoid the effects of desensitization. The increases in fura-2 ratios induced by heat stimulation in the absence of menthol (0.54 ± 0.03) were significantly larger than the increases in fura-2 ratios induced by heat stimulation in the presence of menthol (0.35 ± 0.02, p < 0.01) (Figure 23A, B). These data suggested that menthol inhibited hTRPV1 activity induced by heat stimulation. The increases in fura-2 ratios induced by capsaicin after heat stimulation in the presence of menthol were slow, possibly because menthol effects were not completely abolished by washout.

I more thoroughly examined the inhibitory effects of menthol and capsaicin on the thermal responses of hTRPV1. Thus, I performed patch-clamp experiments with HEK293T cells expressing hTRPV1. Menthol (5 mM) inhibited heat-evoked TRPV1-mediated currents in a reversible manner (Figure 24A). The first heat-evoked currents in the presence of menthol (5 mM) were significantly smaller than those in the absence of menthol (36.8 ± 7.1 and 123.3 ± 35.5 pA/pF in the presence and absence of
menthol, respectively, $p < 0.05$) (Figure 24B). Thus, both chemical and thermal responses of hTRPV1 were inhibited by menthol.

I then asked whether the inhibition mediated by menthol affected temperature thresholds. Thus, I assessed the changes in temperature thresholds for hTRPV1 activation through construction of Arrhenius plots for hTRPV1-mediated current responses. The temperature thresholds for hTRPV1 activation were not significantly different between the absence and presence of menthol (41.0 ± 1.3 and 41.2 ± 1.6 °C, respectively) (Figure 24C, D, E). These data indicated that menthol affected hTRPV1 current sizes without changes in the temperature thresholds for activation.

3-3-4. Capsaicin inhibited hTRPM8 activity induced by menthol and cold stimulation

TRPV1 is activated by elevated temperature (> 42°C) whereas cold temperature reportedly decreases capsaicin-induced TRPV1 activity. On the other hand, TRPM8 is activated by low temperatures and menthol, whereas heat stimulation suppresses menthol-evoked TRPM8 currents. These findings suggested that the effects of specific agents on the TRPV1 channel and the TRPM8 channel are intricately related to one another. Therefore, I examined the effect of capsaicin on hTRPM8.

I first examined the effects of menthol on hTRPV1 and the influence of capsaicin on hTRPM8 using a Ca$^{2+}$-imaging method at room temperature with HEK293T cells expressing hTRPM8. The changes in the fura-2 ratios induced by menthol (1 mM) in the presence of capsaicin (1 mM) were smaller than those in its absence in cells expressing hTRPM8 (Figure 25). Fura-2 ratios were slightly reduced by capsaicin
alone in the cells expressing hTRPM8, probably because hTRPM8 was activated even at room temperature. I also confirmed that capsaicin (1 mM) did not cause current activation in the cells expressing hTRPM8 (Figure 26). These results suggested that capsaicin inhibited hTRPM8 activity.

To confirm these possibilities, I performed patch-clamp experiments using HEK293T cells expressing hTRPM8. The hTRPM8 currents induced by menthol (500 µM) were similarly inhibited by capsaicin (100 µM) in a reversible manner and the currents evoked by concomitant application of menthol with capsaicin were smaller than those activated by menthol alone (Figure 27A). These results were consistent with the data in the Ca2+-imaging experiments (Figure 25). Next, I examined the dose-dependency of the inhibitory effects. The current responses were measured in the absence of extracellular Ca2+ to minimize desensitization. The capsaicin-induced inhibition of menthol-activated hTRPM8 currents occurred in a dose-dependent manner, with an IC50 value of approximately 39.9 ± 6.4 µM (Figure 27B, C). These results indicated that capsaicin inhibited hTRPM8 activity.

3-3-5. Capsaicin inhibited the activation of hTRPM8 induced by thermal stimulation

Next, I examined the inhibitory effects of menthol and capsaicin on hTRPV1 and hTRPM8 activities, respectively, by thermal stimulation using a Ca2+-imaging method. In cells expressing hTRPM8, [Ca2+]i increases caused by cold stimulation (< 20°C) in the presence of capsaicin (1 mM) were significantly smaller than [Ca2+]i increases in the absence of capsaicin (0.19 ± 0.01 and 0.44 ± 0.01, respectively, p < 0.01) (Figure 28). I more thoroughly examined the inhibitory effects of capsaicin on the thermal
responses of hTRPM8. Thus, I performed patch-clamp experiments with HEK293T cells expressing hTRPM8. Capsaicin (100 µM) inhibited the cold-evoked TRPM8-mediated currents in a reversible manner (Figure 29A). Furthermore, the first cold-evoked currents in the presence of capsaicin (100 µM) were significantly smaller than those in the absence of capsaicin (3.4 ± 0.9 and 22.2 ± 3.4 pA/pF in the presence and absence of capsaicin, respectively, p < 0.01) (Figure 29B). Thus, both chemical and thermal responses of hTRPM8 were inhibited by capsaicin. I then asked whether the inhibition mediated by capsaicin affected temperature thresholds. However, it was not possible to generate Arrhenius plots for hTRPM8 currents because capsaicin completely inhibited cold-induced hTRPM8-mediated currents (Figure 29A).

3-3-6. The binding site of menthol in TRPV1 was distinguishable from that of capsaicin, whereas the binding site of capsaicin in TRPM8 modestly interacted with that of menthol

Menthol activated TRPM8 and inhibited TRPV1, whereas capsaicin activated TRPV1 and inhibited TRPM8. These results led us to hypothesize that the two compounds interacted with similar sites on each channel in a competitive manner. Therefore, I examined whether menthol interacted with the same binding site as capsaicin on TRPV1 and whether capsaicin interacted with the same binding site as menthol on TRPM8. Towards that end, I examined capsaicin and menthol binding sites that had been mutated. Thus, I investigated whether menthol affected the heat-evoked activities of mutant hTRPV1 and whether capsaicin affected the cold-evoked activities of mutant hTRPM8. Capsaicin was reported to interact with Y511 and S512 in the cytosolic region linking
transmembrane domains 2 and 3 of rat TRPV1 [Jordt et al., 2002] and with T550 in the transmembrane domain 4 of Oryctolagus cuniculus [Gavva et al., 2004], all of which are conserved in hTRPV1 (Figure 30A). Therefore, I examined the effects of menthol on the heat-evoked currents in the three hTRPV1 mutants (Y511A, S512Y and T550I) under naïve conditions. Because the heat-evoked inward currents of Y511A and S512Y mutants at -60 mV were very small (Figure 31B, C, E), I examined the effects of menthol on the currents at both -60 and +100 mV. The effects of menthol on the heat-evoked currents were larger at -60 mV compared with +100 mV in wild-type (WT) and T550I channels (Figure 31A, D, E, F). The very small heat-evoked currents in Y511A and S512Y channels at -60 mV made precise comparisons difficult. Nonetheless, menthol-induced inhibition of the mutant channels at +100 mV were large (Figure 31B, C, E, F). Thus, the levels of inhibition were generally similar between WT and mutants, suggesting that menthol might not share the three amino acids with capsaicin for its inhibitory effects.

In contrast, tyrosine 745 in the middle of transmembrane domain 2 was previously identified as a crucial residue for the menthol-sensitivity of mouse TRPM8 [Bandell et al., 2006], a domain that is conserved in hTRPM8 (Figure 30B). As mentioned above, cold-induced inward currents at -60 mV were very small especially in the presence of capsaicin (Figure 32A - E), preventing us from comparing the effects at negative potentials. However, inhibition of cold-induced TRPM8-mediated currents by capsaicin appeared smaller at +100 mV in the Y745H mutant (Figure 32D, F), and ratios without and with capsaicin were significantly larger in the Y745H mutant ($p < 0.05$) (Figure 32G). Cold-induced TRPM8-mediated currents decreased upon washout of capsaicin (Figure 32A), which probably reflected the inhibition of basal
TRPM8-mediated currents by residual capsaicin remaining in the cells. I examined the involvement of two other amino acids (Y1005 and L1009) that were reported to be involved in menthol action. However, neither of the mutants provided currents sufficient for analysis (data not shown). These findings suggested that Y745 was involved to some extent in the capsaicin-induced hTRPM8 inhibition.
4 Discussion

4-1. Screening essential oils for the ability to activate hTRPM8 but not hTRPA1

TRPA1 is an excitatory ion channel targeted by pungent irritants such as those from mustard oil and garlic. It is thought to function in diverse sensory processes, including cold nociception and inflammatory pain. Therefore, TRPA1 is a promising target for the identification of analgesic drugs. A natural analgesic compound that does not accelerate pain signaling is desirable for pharmaceutical or cosmetic pain relief. TRPA1 antagonists (ruthenium red, HC-030031, AMG5445, A967079 and camphor) possess analgesic properties [Nagata et al., 2005; Xu et al., 2005; Klionsky et al., 2007; Eid et al., 2008; McGaraughty et al., 2010; Chen et al., 2011]. Of these, camphor is the only naturally occurring compound and is often used in cosmetics because it possesses minimal adverse effects. However, camphor is not suited for use as an analgesic compound because it causes warm and hot sensations [Green et al., 1990]. These sensations are mediated through activation of TRPV1 [Xu et al., 2005; Vogt-Eisele et al., 2007]. Moreover, TRPM8 contributes to sensing unpleasant cold stimuli or mediating the effects of cold analgesia [Dhaka et al., 2007; Proudfoot et al., 2006]. Although menthol, the main ingredient of peppermint, is used for pain relief in daily life through TRPM8 activation [Proudfoot et al., 2006], its ability to activate hTRPA1 restricts widespread use of menthol as an analgesic [Galeotti et al., 2002]. Therefore, chemicals that activate TRPM8 and inhibit TRPA1, but do not activate TRPV1, would be ideal analgesic agents.

I found that the activation of hTRPA1 (induced by several agonists with different
activation mechanisms) can be inhibited by 1,8-cineole. Moreover, 1,8-cineole activated hTRPM8 and hTRPV3, but not hTRPA1, hTRPV1 or hTRPV2. It was recently shown that both peripheral and central activation of TRPM8 could produce an analgesic effect that specifically reversed the sensitization of behavioral reflexes elicited by peripheral nerve injury [Galeotti et al., 2002; Proudfoot et al., 2006; Dhaka et al., 2007]. From this point of view, 1,8-cineole appears to be an ideal natural analgesic that activates hTRPM8 and inhibits hTRPA1.

1,8-cineole acts as an agonist of the TRPM8 channel with lower efficacy and potency (3.4 ± 0.4 mM) on TRPM8 than menthol [McKemy et al., 2002; Vogt-Eisele et al., 2007]. 1,8-cineole also activates the TRPV3 channel in mice, but not the western clawed frog TRPV3 [Saito et al., 2011]. Furthermore, 1,8-cineole inhibits the chemical nociception produced by several irritants, and it has anti-inflammatory efficacy in patients with severe asthma [Juergens et al., 2003]. The present study suggests that the known analgesic and anti-inflammatory actions of 1,8-cineole can be attributed to its TRPM8-activating and TRPA1-inhibiting abilities.

1,8-cineole has a fresh smell and elicits a cooling sensation when ingested or applied to the skin and is a common additive in flavorings, food, mouthwashes and cough suppressants. 1,8-cineole is often used in aromatherapy, as a stimulant in skin baths, by the pharmaceutical industry in drug formulations to enhance percutaneous penetration and as a decongestant and antitussive [Williams et al., 1991; Laude et al., 1994; Levison et al., 1994]. Experimental data have shown that 1,8-cineole is an analgesic and anti-inflammatory agent with beneficial effects for patients with severe asthma [Juergens et al., 2003]. 1,8-cineole inhibits the \textit{in vitro} formation of prostaglandins and cytokines by stimulated monocytes. However, the molecular
targets and mechanisms of the analgesic effect of 1,8-cineole remain unclear [Juergens et al., 2004].

In a human study, I examined sensitive volunteers to determine whether 1,8-cineole could inhibit sensory irritation caused by octanol and menthol. In the cosmetic research field, both menthol and octanol are well-known to cause skin irritation, and neither cinnamaldehyde nor allicin is used for human skin studies. 1,8-cineole activated TRPM8 less efficiently than did menthol, and it inhibited menthol-evoked skin irritation. Those results clearly suggested that the inhibitory effects of 1,8-cineole are probably due to inhibition of TRPA1 and not activation of TRPM8.

The inhibitory effects of 1,8-cineole on menthol-induced hTRPA1 activation was a little greater than those for AITC- or FFA-induced hTRPA1 activation (Figure 6B, D, F). Menthol has bimodal action through transmembrane domain 5 of TRPA1 in some species [Xiao et al., 2008]. Therefore, the similarity between the molecular structures of menthol and 1,8-cineole (Figure 4A) suggests that 1,8-cineole could act on the same domain of TRPA1 as menthol, although the structural basis for menthol-evoked hTRPA1 activation is not known. Four compounds with similar structures (Figure 4A) exhibited different effects on hTRPM8 and hTRPA1: i) menthol and 1,4-cineole activated both hTRPM8 and hTRPA1 [Macpherson et al., 2006]; ii) camphor inhibited hTRPA1 [Xu et al., 2005]; iii) 1,8-cineole activated hTRPM8 and inhibited hTRPA1 (Figs. 2-4). The fact that the four compounds exhibited differing effects on hTRPM8 and hTRPA1 suggests that more detailed analyses would lead to a better understanding of the structural basis for the action of these compounds on TRPM8 and TRPA1.
4-2. Screening camphor analogs to identify more effective TRPA1 antagonists

TRPA1 is an excitatory ion channel targeted by pungent irritants such as those from mustard oil and garlic. It is thought to function in diverse sensory processes, including nociception and inflammatory pain. Therefore, TRPA1 is a promising target for the development of analgesic agents. As described above, I found that 1,8-cineole is a naturally occurring hTRPA1 antagonist. However, this natural antagonist exhibited weaker inhibitory effects on TRPA1 activity than other antagonists such as HC-030031, A-967079, and AZ868. Therefore, identification of naturally occurring compounds with greater inhibitory potency of hTRPA1 activation has been sought.

Borneol is a bicyclic monoterpenoid alcohol that has been used in foods as an aromatic spice. It is a valuable medical and chemical material that has been used as a folk medicine in China and India [Almeida et al., 2013]. Additionally, borneol is a fragrant ingredient used in decorative cosmetics, fine fragrances, shampoos, and other toiletries. Previous studies have shown that borneol has a vasorelaxant effect on the rat thoracic aorta [Silva-Filho et al., 2011] and neuroprotective effects [Liu et al., 2011]. Although borneol has been evaluated for anti-nociceptive and anti-inflammatory activities, the molecular targets and mechanisms of its analgesic effect remain unclear. The fact that this monoterpane acts as an agonist of the TRPV3 [Vogt-Eisele et al., 2007] and TRPV1 [Xu et al., 2005] channels and specifically inhibits nAChR-mediated effects in a noncompetitive way [Park et al., 2003] does not explain its anti-nociceptive effects. 2-methylisoborneol has been implicated as the cause of the muddy odor of fish from Cedar Lake, Manitoba. Algae produce 2-methylisoborneol and geosmin that are responsible for the musty odor [Masakazu et al., 1988]. Fenchyl alcohol, which is a component of several essential oils, is a fragrance ingredient used in decorative
cosmetics, fine fragrances, shampoos, and other toiletries. Moreover, this compound has an inhibitory effect on acetylcholinesterase activity [Miyazawa et al., 2005]. Although borneol is the only terpene known to have anti-nociceptive effects, the two other terpenes identified in this study could have similar effects because all three monoterpenes inhibit hTRPA1, and their derivatives might function as analgesics.

Alpizar et al. reported that camphor exhibited a bimodal effect on mTRPA1 [Alpizar et al., 2013]. The authors observed the inhibition of mouse TRPA1-mediated basal current with 1 mM camphor, and the current increased as camphor was washed out, suggesting a bimodal action for camphor on mTRPA1. In addition, 1-5 mM camphor increased the cytosolic Ca\(^{2+}\) concentration upon washing out without any effect during camphor application. I examined the effect of camphor (1 mM) in a Ca\(^{2+}\)-imaging method, but failed to observe changes in the cytosolic Ca\(^{2+}\) concentration (Figure 8). Camphor blocked the menthol-induced increase in cytosolic Ca\(^{2+}\) concentrations (1 mM, Figure 10E). In addition, camphor inhibited the TRPA1 current activated by 20 µM AITC in a dose-dependent manner (Figure 13E). At present, I do not have an explanation for the apparent differences between the two studies, although a species difference might have caused the different outcomes. Transient enhancement of TRPA1 current was observed after washing out of the three test compounds in FFA- or AITC-evoked responses (Figs. 12 and 13), but not in the menthol-evoked response (Figure 12). Several agonists have bimodal actions on mammalian TRPA1, apparently involving non-covalent mechanisms. However, Alpizar et al. reported that cinnamaldehyde (a TRPA1 activator with covalent modification) and camphor, which was thought to be a mammalian TRPA1 antagonist, also exhibit bimodal actions on mTRPA1, indicating that bimodal actions could be a more general phenomenon than
previously thought [Alpizar et al., 2013]. Therefore, the transient enhancement of the hTRPA1 current upon washing out of the antagonists (Figure 12, 13) might result from bimodal actions of the compounds. Interestingly, enhancement was not observed in the menthol-evoked TRPA1 response (Figure 12), possibly because menthol and hTRPA1 inhibitors (borneol, 2-methylisoborneol and fenchyl alcohol) are all monoterpenes that act at similar sites, including ones that were identified in this study, through their cyclohexyl hydroxyl groups.

Analogos of camphor have exhibited variable effects on hTRPA1. Borneol, 2-methylisoborneol and fenchyl alcohol inhibited hTRPA1. In contrast, norcamphor had no effect on hTRPA1, and other related compounds activated hTRPA1 (Figure 8). The mechanisms of action with hTRPA1 remain unclear although these monoterpenes have similar molecular structures. Borneol, which was synthesized by chemical reduction of camphor, inhibited hTRPA1, and the effects were greater than those of camphor. In addition, fenchyl alcohol, which was synthesized by chemical reduction of fenchone, inhibited hTRPA1 activity, while fenchone activated hTRPA1. Common structural differences between camphor and borneol and between fenchone and fenchyl alcohol are hydroxyl and carbonyl groups at the same position of their cyclohexane, which suggests that hydrogen bonding plays a pivotal role in the action of these compounds. This idea is supported by the results from mutation of T874, an amino acid thought to form a hydrogen bond with menthol, resulting in a reduction in the activity of borneol. In our study, S873 and T874 in TM5 and Y812 in TM3 were found to be involved in the inhibitory effects of borneol. Because these two sites are somewhat distant from each other, borneol could fit separately into both sites.
4-3. The effects of menthol on human TRPV1

In the human study, I examined whether menthol could inhibit sensory irritation caused by VBE. In the cosmetic research field, VBE is well-known as a skin irritant, and neither capsaicin nor resiniferatoxin is used for human skin studies. Surprisingly, I found that VBE activated both hTRPV1 and hTRPA1. This property could explain the marked irritation produced by VBE when used in humans. The in vitro study showed that the IC₅₀ value for menthol-induced hTRPV1 inhibition was 1.2 ± 0.2 mM and that 10 mM menthol completely inhibited hTRPV1 currents (Figure 21). However, 0.3 wt% menthol, which exhibited anti-nociceptive effects in the in vivo study (Figure 17), corresponded to 19.2 mM, which is within the attainable concentration range in clinical use [Galeotti et al., 2002; Proudfoot et al., 2006]. Menthol is reported to cause analgesic effects through several different mechanisms, including those that are TRPM8-dependent and -independent [Galeotti et al., 2001; Galeotti et al., 2002; McKay et al., 2006; Proudfoot et al., 2006; Dhaka et al., 2007; Gaudioso et al., 2012]. Menthol also activates hTRPA1 at higher concentrations compared with the concentrations activating hTRPM8 [Klein et al., 2011]. Therefore, in humans, I observed the net result of VBE-hTRPV1 interaction: (1) inhibition of VBE-evoked hTRPV1 activation by menthol, (2) menthol-evoked hTRPA1 activation and (3) VBE-evoked hTRPA1 activation and other mechanisms. This complicated mechanism could partly explain the small inhibition of VBE-induced irritation by menthol in human subjects. Nevertheless, the observation that high concentrations of menthol (0.3%) (which could activate hTRPA1 leading to greater irritation) inhibited VBE-induced irritation could be significant. Thus, the reduction of VBE-induced irritation by 0.3 wt% menthol in vivo might be partly attributed to the inhibition of hTRPV1 activity by
Preparations containing menthol are used topically to relieve neuralgia in traditional Chinese and European medicine [Wright et al., 1870]. In addition, mint oil has been reported to alleviate thermally elicited pain and post-herpetic neuralgia and orally applied menthol can achieve short-term analgesia [Green et al., 2000]. Furthermore, in mice, oral or intracerebroventricular application of menthol decreased nociceptive responses in the hot-plate test and acetic acid writhing test [Liu et al., 2013]. Despite these analgesic actions of menthol in the literature, the mechanism of action has not been fully clarified, although menthol-induced anti-nociception was reduced in TRPM8-deficient mice [Dhaka et al., 2007]. Inhibition of TRPV1 by menthol as shown in the current study could be one of the underlying mechanisms for the analgesic effects of menthol observed in rodents and humans. However, menthol could also achieve its anti-nociceptive effects through activation of TRPM8 and other mechanisms as reported [Galeotti et al., 2002; Proudfoot et al., 2006]. TRPV1 is activated by capsaicin as well as heat, protons and some endogenous substances known to be associated with tissue inflammation [Caterina et al., 1997; Tominaga et al., 1998; Tominaga et al., 2004]. Since TRPV1 acts as an integrator of painful stimuli, TRPV1 antagonists can be viewed as promising and novel types of analgesics [Gavva et al., 2008; Gunthorpe et al., 2009; Marcello et al., 2010; Marcello et al., 2013]. A number of potent, small TRPV1 antagonists such as capsazepine, BCTC, CTPC, AMG9810 and SB-452533 [Bevan et al., 1992; Valenzano et al., 2003; Rami et al., 2004; Gavva et al., 2005; Weil et al., 2005] have advanced into clinical trials for the evaluation of their analgesic activities. Although some of these antagonists reduced noxious heat sensation, hyperthermia as a serious side effect often led to their withdrawal from the clinical trials. Thus, novel
approaches to the development of anti-TRPV1 antagonists are needed, and this study shows that derivatives of menthol could be promising molecules to develop for TRPV1 antagonists.

As discussed above, agonists of TRPV1 and TRPM8 seem to mutually oppose one another. What is the physiological significance of their apparently reciprocal interaction? TRPM8 is not generally co-expressed with TRPV1 in primary afferent neurons [Story et al., 2003; Kobayashi et al., 2005; Abe et al., 2005; Facer et al., 2007], suggesting that the information conducted by TRPM8-expressing neurons and TRPV1-expressing neurons could influence one another. The data presented here suggest the possibility that menthol-induced TRPM8-mediated cold sensation could be enhanced by inhibition of TRPV1 and that capsaicin-induced TRPV1-mediated heat sensation could be enhanced by inhibition of TRPM8. The enhancement could work to strengthen the difference between TRPV1 and TRPM8 activities in some specific concentration ranges. Although the physiological significance of TRPM8 inhibition by capsaicin is not evident, their reciprocal interactions could lead to the enhancement of TRPV1-mediated nociceptive signals that effectively alert us to noxious conditions.
5 Acknowledgement

I would like to express my appreciation to Prof. M. Tominaga for leading me to the physiological sciences and giving me a great opportunity to conduct this study. I also would like to express my appreciation to Dr. F. Fujita (Mandom Corp.), Assistant Professors K. Uchida and Y. Shuzuki (National Institute for Physiological Sciences) for their critical advice and encouragement in the conduct of these experiments, N Fukuta and C Saito (National Institute for Physiological Sciences) for their help in preparation of cultured cells, Dr. R. Kurata, Ms. M. Shimizu, Ms. C. Hatai, Mr. S. Yamamoto, Ms. M. Sawada, Mr. H. Matsui, Mr. T. Shimada, Mr. H. Mekata, Mr. T. Watanabe, Mrs. M. Tsubakihara, Mr. T. Maekawa, Mr. K. Inoue and Dr. F. Okada (Mandom Corp) for their encouragement.
6 References


mouse cold-menthol receptor TRPM8 and vanilloid receptor type-1 VR1 using a fluorometric imaging plate reader (FLIPR) assay. *Br J Pharmacol.* **141**:737-745.


Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR,


Green Chem. 14:610-613


Laude EA, Morice AH, Grattan TJ. (1994) The antitussive effects of menthol, camphor


Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ,


Valenzano KJ, Grant ER, Wu G, Hachicha M, Schmid L, Tafesse L, Sun Q, Rotshteyn Y,


Wright A (1870) Oil of peppermint as a local anesthetic. *Lancet.* **2464**:726.


Figure 1. Abilities of essential oils to activate hTRPM8 or hTRPA1.
(A, B) Comparison of the effects of essential oils (0.01 wt%) on hTRPM8 (n = 34-134) (A) or hTRPA1 (n = 16-67) (B) using a Ca²⁺-imaging method with HEK293T cells expressing hTRPM8 or hTRPA1. Fura-2 ratio (340/380 nm; cytosolic Ca²⁺ concentrations) increases by each oil were normalized to the fura-2 ratio increases by 1 mM menthol. (C) The Ratio of hTRPM8-activating ability versus hTRPA1-activating ability by dividing the values in (A) by the values in (B).
Figure 2. Abilities of fragrance chemicals to activate hTRPM8 or hTRPA1.

(A, C) Effects of 1,8-cineole on fura-2 ratio in HEK293T cells expressing hTRPM8 (n = 32) (A) or hTRPA1 (n = 41) (C).  
(B, D) Comparison of the effects of fragrance chemicals (1 mM) on hTRPM8 (n = 16-80) (B) or hTRPA1 (n = 30-90) (D). Fura-2 ratio increases by each chemical were normalized to the fura-2 ratio increases by 1 mM menthol. (E) The Ratio of hTRPM8-activating ability versus hTRPA1-activating ability by dividing the values in (B) by the values in (D).
Figure 3. Effects of 1,8-cineole on fura-2 ratio in HEK293T cells.

(A-C) Fura-2 ratio changes upon 1,8-cineole (5 mM) application in cells expressing hTRPV1 (n = 65) (A), hTRPV2 (n = 26) (B) or hTRPV3 (n = 79) (C). CAP (capsaicin), LPC (lysophosphatidylcholine), 2-APB (2-aminoethoxydiphenyl borate). Horizontal bars indicate duration of the applied stimuli. (D) 1,8-cineole caused significant fura-2 ratio increases in HEK293T cells expressing hTRPM8 (n = 32) or hTRPV3 (n = 79), but not in cells expressing hTRPV1 (n = 65), hTRPV2 (n = 26) or hTRPA1 (n = 41). Statistical significance was evaluated using ANOVA followed by two-tailed multiple t-test with Bonferroni correction. *: p < 0.05
Figure 4. 1,4-cineole activates and inhibits hTRPA1.
(A) Molecular structures of menthol, camphor, 1,8-cineole and 1,4-cineole. (B) 1,4-cineole increased fura-2 ratio (340/380 nm) in cells expressing hTRPM8 (n = 35). (C) 1,4-cineole increased fura-2 ratio in cells expressing hTRPA1 (n = 17).
Figure 5. Effects of 1,8- or 1,4-cineole on HEK293T cells expressing hTRPM8 or hTRPA1. (A, B) 1,8-cineole (5 mM) activated hTRPM8 (A) with an outwardly rectifying current-voltage relationship, but not hTRPA1 (B). (C, D) 1,4-cineole activated both hTRPM8 and hTRPA1 with an outwardly rectifying current-voltage relationship. The insets (A, C and D) indicate the current-voltage relationship at the point indicated by * in the left trace.
Figure 6. 1,8-cineole inhibits but does not activate hTRPA1-mediated currents in HEK293T cells. 
(A) A representative AITC (20 mM)-evoked hTRPA1 current that was inhibited by 1,8-cineole in a dose-dependent manner in the absence of extracellular Ca\(^{2+}\). (B) Dose-dependent inhibition of AITC (20 mM)-evoked hTRPA1 current by 1,8-cineole. IC\(_{50}\) and Hill’s coefficient values are 3.4 ± 0.6 mM and 1.7 ± 0.4, respectively. Data are shown as the mean ± SEM (n = 5-8). (C) A representative whole-cell menthol (500 mM)-evoked hTRPA1 current that was inhibited by 1,8-cineole (5 mM) in the absence of extracellular Ca\(^{2+}\). (D) Dose-dependent inhibition of menthol (500 mM)-evoked hTRPA1 current by 1,8-cineole. IC\(_{50}\) and Hill’s coefficient values are 0.5 ± 0.1 mM and 1.0 ± 0.2, respectively. Data are shown as the mean ± SEM (n = 5-8). (E) A representative whole-cell FFA (100 mM)-evoked hTRPA1 current that was inhibited by 1,8-cineole (5 mM) in the absence of extracellular Ca\(^{2+}\). (F) Dose-dependent inhibition of FFA (100 mM)-evoked hTRPA1 current by 1,8-cineole. IC\(_{50}\) and Hill’s coefficient values are 5.3 ± 0.1 mM and 2.4 ± 0.8, respectively. Data are shown as the mean ± SEM (n = 6-8). (G) A representative whole-cell octanol (1 mM)-evoked hTRPA1 current that was inhibited by 1,8-cineole (5 mM) in the presence of extracellular Ca\(^{2+}\). (H) 1,8-cineole did not inhibit hTRPV1 (n = 25), TRPV2 (n = 50) or TRPV3 (n = 32) responses by capsaicin, 2-APB or cocktail (2-APB + carvacrol), respectively.
Figure 7. Inhibitory effect of 1,8-cineole in sensory irritation tests in humans.
(A and B) 0.5 (wt%) 1,8-cineole did not cause a difference of sensory irritation scores compared with vehicle (n = 10). (C) Sensory irritation caused by 0.2 (wt%) octanol was significantly inhibited by concomitant application of 0.1 (wt%) 1,8-cineole 7 min after application. (D) Total score of sensory irritation by octanol was significantly inhibited by 1,8-cineole. Statistical significance was evaluated using Wilcoxon signed-rank test. *: p < 0.05. n = 11. (E) Sensory irritation caused by 0.5 (wt%) menthol was significantly inhibited by concomitant application of 0.5 (wt%) 1,8-cineole 5 min after application. (F) Total score of sensory irritation by menthol was significantly inhibited by 1,8-cineole. Statistical significance was evaluated using Wilcoxon signed-rank test. **: p < 0.05. n = 11.
Figure 8. Summary of the inhibitory effects of camphor analogs on human TRPA1 activity. Fura-2 ratios (340 nm/380 nm) by test compounds (1 mM) were normalized to changes in the fura-2 ratio by 1 mM menthol in HEK293T cells expressing human TRPA1 (hTRPA1). Data are presented as the mean ± S.E.M. (n = 27–67).
Figure 9. Effects of borneol, 2-methylisoborneol, and fenchyl alcohol on HEK293T cells expressing hTRPA1.

(A-D) Representative whole-cell current traces in the presence of borneol (1 mM, A), 2-methylisoborneol (1 mM, B), fenchyl alcohol (1 mM, C) or norcamphor (1 mM, D) in HEK293T cells expressing hTRPA1. hTRPA1 activity was confirmed with 20 mM of AITC. Cells were held at -60 mV and ramp-pulses from -100 mV to +100 mV (500 ms) were administered every five seconds.
Figure 10. Effects of borneol, 2-methylisoborneol, fenchyl alcohol and norcamphor on menthol-induced cytosolic Ca\(_{2+}\) increases in HEK293T cells expressing hTRPA1. 

(A-D) Fura-2 ratio changes by menthol (1 mM) in the presence and absence of borneol (1 mM, A), 2-methylisoborneol (1 mM, B), fenchyl alcohol, (1 mM, C) and norcamphor (1 mM, D) application in cells expressing hTRPA1 (n = 14-27). (E) Changes in fura-2 ratios by menthol in the presence of test compounds were normalized to changes in the fura-2 ratio by menthol in the absence of test compounds. Data are presented as the mean ± S.E.M. (n = 47-79).
Figure 11. Effects of borneol, 2-methylisoborneol, fenchyl alcohol and norcamphor with or without menthol on cytosolic Ca\(^{2+}\) concentrations in vector-transfected HEK293T cells. (A-C) No changes in the fura-2 ratio were observed while cells responded normally to ionomycin (5 mM). (n = 48-75)
Figure 12. Effects of borneol, 2-methylisoborneol and fenchyl alcohol on menthol- and FFA-induced hTRPA1 currents in HEK293T cells.

(A-C) Representative menthol (1 mM)-induced hTRPA1 current that was inhibited by borneol (1 mM, A), 2-methylisoborneol (1 mM, B) or fenchyl alcohol (1 mM, C) in the absence of extracellular Ca$^{2+}$.  

(D-F) Representative FFA (100 mM)-induced hTRPA1 current that was inhibited by borneol (1 mM, D), 2-methylisoborneol (1 mM, E) or fenchyl alcohol (1 mM, F) in the absence of extracellular Ca$^{2+}$. 
Figure 13. Effects of borneol, 2-methylisoborneol, fenchyl alcohol, and camphor on AITC-induced hTRPA1 current in HEK293T cells.

(A-D) Representative AITC (20 µM)-induced hTRPA1 currents that were inhibited by borneol (1 mM, A), 2-methylisoborneol (1 mM, B), fenchyl alcohol (1 mM, C) or camphor (1 mM, D) in the absence of extracellular Ca²⁺. (E) Dose-dependent inhibition of AITC (20 µM)-induced hTRPA1 current by 1,8-cineole, camphor, borneol, 2-methylisoborneol, or fenchyl alcohol. IC₅₀ values are 3.43 ± 0.58 mM, 1.26 ± 0.32 mM, 0.20 ± 0.06 mM, 0.12 ± 0.03 mM, and 0.32 ± 0.06 mM for 1,8-cineole, camphor, borneol, 2-methylisoborneol, and fenchyl alcohol, respectively. (n = 5-10)
Figure 14.
Comparison of the inhibitory effects on AITC (20 mM)-induced hTRPA1 current among borneol isomers and (±) isoborneol. (n = 5-6)
Figure 15. Comparison of the inhibitory effects of borneol, camphor and 1,8-cineole on the currents of wild-type hTRPA1 and hTRPA1 mutants expressed in HEK293T cells.

(A-F) Inhibitory effects of borneol at three different concentrations (0.03, 0.3, and 1 mM, A), camphor at three different concentrations (0.03, 0.3, and 3 mM, B), or 1,8-cineole at three different concentrations (1, 5, and 10 mM, C) on TRPA1-mediated current at -60 mV in HEK293T cells expressing wild-type hTRPA1 (hTRPA1-WT) or TRPA1 mutants (hTRPA1-S873V/T874L, A-C; hTRPA1-Y812A, D-F) in the absence of extracellular Ca^{2+}. Current magnitude in the presence of borneol, camphor, or 1,8-cineole was normalized to the current in the absence of test compounds. (n = 5-8) **: p < 0.01.
Figure 16. Effects of VBE on HEK293T cells expressing hTRPV1 or vector alone.

(A, C) Representative traces of the whole-cell currents in the presence of VBE (1 mM) on hTRPV1 (A) and vector-transfected HEK293T cell (C) in the presence of extracellular Ca\(^{2+}\). (B, D) VBE-evoked (100 µM) hTRPV1 currents that were inhibited by capsazepine (1 µM, B) and menthol (5 mM, D) in the presence of extracellular Ca\(^{2+}\). (E) Current-voltage relationships in the absence or presence of menthol at the points indicated by * in the left traces.
Figure 17. Inhibitory effects of menthol on the VBE-induced sensory irritation in humans. (A, C) Changes in sensory irritation scores upon VBE (0.1 wt %) application in the absence and presence of menthol (0.1 wt %, A) (0.3 wt %, C) (n = 10 each). (B, D) Total scores of sensory irritation by VBE during 10 min in the presence and absence of menthol (0.1 wt %, B) (0.3 wt %, D). *p < 0.05. Data are shown as the mean ± SEM.
Figure 18. Effects of VBE on HEK293T cells expressing hTRPA1.
(A) A representative trace of the whole-cell current in the presence of VBE (1 mM) on hTRPA1 in the presence of extracellular Ca^{2+}. (B) Comparison of the current densities activated by 0.1 and 1 mM VBE on hTRPV1 and hTRPA1. n = 5-6.
Figure 19. Effects of menthol and capsaicin on hTRPV1.
Fura-2 ratio changes in response to capsaicin (0.1 μM) application in the presence and absence of menthol (10 mM) in HEK293T cells expressing hTRPV1 (n = 40). Data are shown as the mean ± SD.
Figure 20. Effects of menthol and capsaicin on HEK293T cells expressing hTRPV1, TRPM8 or vector alone.
(A, B) Representative traces of the whole-cell currents in the presence of menthol (10 mM) in vector-transfected HEK293T cell (A), hTRPV1 (B) in the presence of extracellular Ca²⁺.
Figure 21. Inhibition of hTRPV1-mediated currents by menthol in HEK293T cells. 
(A) A representative trace of the whole-cell 0.1 μM capsaicin-evoked hTRPV1 currents that were inhibited by menthol (5 mM) in presence of extracellular Ca$^{2+}$. (B) A representative 0.1 μM capsaicin-evoked hTRPV1 current that was inhibited by menthol in a dose-dependent manner in the absence of extracellular Ca$^{2+}$. (C) Dose-dependent inhibition of 0.1 μM capsaicin-evoked hTRPV1 current by menthol. IC$_{50}$ and Hill’s coefficient values are 1.2 ± 0.2 mM and 1.7 ± 0.3, respectively (n = 5–8). Data are shown as the mean ± SEM.
Figure 22. Inhibitory effects of menthol on hTRPV1 current induced by various concentrations of capsaicin.
Comparison of the current densities activated by 0.01, 0.03 or 0.1 μM capsaicin in the absence and presence of menthol (1 mM). n = 5-8.
Figure 23. Inhibition of heat-evoked hTRPV1 responses by menthol and capsaicin. 
(A, B) Fura-2 ratio changes in response to heat stimulation (> 45 °C) in the absence (A) and presence (B) of menthol (6 mM) in HEK293T cells expressing hTRPV1 (n = 61). Data are shown as the mean ± SD.
Figure 24. Changes in thermal sensitivity of hTRPV1 by menthol.

(A) A representative trace of the hTRPV1-mediated heat-activated currents (upper) with temperature changes (lower). The hTRPV1 response was confirmed by capsaicin (10 µM). (B) Comparison of the first heat-evoked hTRPV1-mediated current densities in the absence and presence of 5 mM menthol in HEK293T cells (n = 10-13). *, p < 0.05. (C, D) Temperature thresholds for heat-evoked hTRPV1 activation determined by Arrhenius plots from the data in the absence (42.3 °C, C) and presence (41.6 °C, D) of 5 mM menthol in HEK293T cells expressing hTRPV1. (E) The average temperature thresholds for hTRPV1 activation after the first heat stimulation in the absence and presence of 5 mM menthol in HEK293T cells (n = 6-7). Data are shown as the mean ± SEM.
Figure 25. Effects of menthol and capsaicin on hTRPM8.
Fura-2 ratio changes in response to menthol (1 mM) application in the presence and absence of capsaicin (1 mM) in HEK293T cells expressing hTRPM8 (n = 94). Data are shown as the mean ± SD.
Figure 26. Effects of menthol and capsaicin on HEK293T cells expressing hTRPV1, TRPM8 or vector alone.
Representative traces of the whole-cell currents in the presence of capsaicin (1 mM) in hTRPM8 in the presence of extracellular Ca$^{2+}$. 
Figure 27. Inhibition of hTRPM8-mediated currents by capsaicin in HEK293T cells.

(A) A representative trace of the whole-cell 500 µM menthol evoked hTRPM8 currents that were inhibited by 100 µM capsaicin in the presence of extracellular Ca²⁺. (B) A representative 0.5 mM menthol-evoked hTRPM8 current that was inhibited by capsaicin in a dose-dependent manner in the absence of extracellular Ca²⁺. (C) Dose-dependent inhibition of 0.5 mM menthol-evoked hTRPM8 current by capsaicin. IC₅₀ and Hill’s coefficient values are 39.9 ± 6.4 µM and 2.5 ± 0.7, respectively (n = 6–8). Data are shown as the mean ± SEM.
Figure 28. Inhibition of cold-evoked hTRPM8 responses by menthol and capsaicin.
Fura-2 ratio changes in response to cold stimulation (< 20 °C) in the presence and absence of capsaicin (1 mM) in HEK293T cells expressing hTRPM8 (n= 117). Data are shown as the mean ± SD.
Figure 29. Changes in thermal sensitivity of hTRPM8 by capsaicin.

(A) A representative trace of the hTRPM8-mediated cold-activated currents (upper) with temperature changes (lower). The hTRPM8 response was confirmed by menthol (1 mM). (B) Comparison of the first cold-evoked hTRPM8-mediated current densities in the absence and presence of 100 µM capsaicin in HEK293T cells (n = 10-12). **, p < 0.01. Data are shown as the mean ± SEM.
Figure 30. Schematic figures representing the topological structures of hTRPV1 (A) and hTRPM8 (B).
Barrels represent the putative transmembrane regions, and the circle indicates the proposed location of the tyrosine residue at position 511, the serine residue at position 512 and the threonine residue at position 550 on hTRPV1 and tyrosine residue at position 745 on hTRPM8.
Figure 31. Comparison of the inhibitory effects of menthol on the currents of wild-type hTRPV1 and hTRPV1 mutants (Y511A, S512Y, T550I) expressed in HEK293T cells.  
(A-D) Representative traces of the first heat-activated currents (upper) in the absence and presence of menthol (5 mM) with temperature changes (lower) in HEK293T cells expressing wild-type (WT, A), Y511A (B), S512Y (C) and T550I (D). Current-voltage relationships (right) in the absence and presence of menthol at the points indicated by asterisk (*) in the left traces. (E, F) Comparison of the current densities activated by the first heat stimulation in the absence or presence of menthol (5 mM) in HEK293T cells at -60 mV (E) and +100 mV (F). **, p < 0.01, *, p < 0.05. n = 6-13. Data are shown as the mean ± SEM.
Figure 32. Comparison of the inhibitory effects of capsaicin on the currents of WT hTRPM8 and hTRPM8 mutants (Y745H) expressed in HEK293T cells.

(A, B) Representative traces of the cold-activated currents (upper) in the absence and presence of capsaicin (100 μM) with temperature changes (lower) in HEK293T cells expressing WT (A) and Y745H (B). (C, D) Whole-cell current-voltage relationships of WT (A) and Y745H (B) expressed in HEK293T cells in the absence and presence of capsaicin at the points indicated by asterisk (*) in the traces (A, B). (E, F) Comparison of the current densities activated by cold in the absence and presence of capsaicin (100 μM) at -60 mV (E) and +100 mV (F). **p < 0.01. n = 8-12. (G) Comparison of the ratios of the cold-activated currents at +100 mV without and with capsaicin from the data shown in (F). **p < 0.01. Data are shown as the mean ± SEM.
Table 1. Sensory irritation scores

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Table 2. Chemical structures of camphor analogs and menthol
Hydroxyl, carbonyl and ether oxygens are indicated in red.

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