British Journal of Pharmacology (2007) 151, 530–540
 0007–1188/07 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Monoterpenoid agonists of TRPV3

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Background and purpose: Transient receptor potential (TRP) V3 is a thermosensitive ion channel expressed predominantly in the skin and neural tissues. It is activated by warmth and the monoterpene camphor and has been hypothesized to be involved in skin sensitization. A selection of monoterpenoid compounds was tested for TRPV3 activation to establish a structure-function relationship. The related channel TRPM8 is activated by cool temperatures and a number of chemicals, among them the monoterpene (-)-menthol. The overlap of the receptor pharmacology between the two channels was investigated.
Experimental approach: Transfected HEK293 cells were superfused with the test substances. Evoked currents were measured in whole cell patch clamp measurements. Dose-response curves for the most potent agonists were obtained in *Xenopus laevis* oocytes.
Key results: Six monoterpenes significantly more potent than camphor were identified: 6-tert-butyl-m-cresol, carvacrol, dihydrocarveol, thymol, carveol and (+)-borneol. Their EC_{50} is up to 16 times lower than that of camphor. All of these compounds carry a ring-located hydroxyl group and neither activates TRPM8 to a major extent.
Conclusions and implications: Terpenoids have long been recognized as medically and pharmacologically active compounds, although their molecular targets have only partially been identified. TRPV3 activation may be responsible for several of the described effects of terpenoids. We show here that TRPV3 is activated by a number of monoterpenes and that a secondary hydroxyl-group is a structural requirement.
British Journal of Pharmacology (2007) **151,** 530–540; doi:10.1038/sj.bjp.0707245; published online 10 April 2007

Keywords: TRPV3; TRPM8; terpenes; skin sensitisation; GABA(A); screening; HEK293; *Xenopus laevis*; receptor pharmacology

Abbreviations: 2-APB, 2-aminoethoxy diphenyl borate; HEK293, human embryonic kidney 293; TRP, transient receptor potential; YFP, yellow fluorescent protein

Introduction

Monoterpenes, like camphor, borneol or menthol comprise a group of naturally occurring organic compounds derived from two isoprene units. Most of them are fragrant and form major constituents of many plant-derived essential oils. While most commonly used as antimicrobial agents, they also have a wide range of applications in pharmaceutical, medical and cosmetic fields. These uses range from anesthetic and analgesic (Galeotti *et al*., 2001, 2002; Xu *et al*., 2005) to anti-inflammatory (Santos and Rao, 2001) and antipruritic applications (Umezu *et al*., 2001; Anand, 2003).

Although the mechanisms of action are not completely understood for many of the described effects, different monoterpenes have been shown to activate, inactivate or modulate ion channels. Thus, borneol, thymol, α-thujone and menthol modulate γ-aminobutyric acid $(GABA)_A$ channels (Priestley *et al*., 2003; Hall *et al*., 2004; Granger *et al*., 2005); camphor and borneol are non-competitive inhibitors of nicotinic acetylcholine receptors (Park *et al*., 2003), and thymol affects calcium and potassium channels (Magyar *et al*., 2002; Szentandrassy *et al*., 2004). A number of monoterpenes have also been described as agonists or antagonists of different members of the transient receptor potential (TRP) channel family (Mckemy *et al*., 2002; Peier *et al*., 2002a, b; Behrendt *et al*., 2004; Moqrich *et al*., 2005; Xu *et al*., 2005, 2006; Macpherson *et al*., 2006).

The TRP ion channel family contains several thermosensitive members, named thermoTRPs. ThermoTRPs are believed to serve as temperature sensors on the molecular level. While some members of the TRPV family activate on heating (TRPV1 and TRPV2 in the noxious range, at 42 and 52°C, respectively, TRPV3 at 39°C, and TRPV4 at 27–42°C), TRPM8 serves to detect innocuous cooling below 25°C (Smith *et al*., 2002; Xu *et al*., 2002; Patapoutian *et al*., 2003). TRPA1 has been described as being activated in the noxious cold temperature range below 17°C (Story *et al*., 2003), although

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Received 15 December 2006; revised 13 February 2007; accepted 20 February 2007; published online 10 April 2007

the temperature sensitivity of this channel is controversial (Bautista *et al*., 2006).

In addition to temperature, thermoTRPs can be activated by other stimuli, such as osmolarity, pH or chemical agonists. Therefore, compounds such as (−)-menthol or capsaicin also cause changes in temperature perception through activation or inhibition of particular TRP channels. The ability of camphor, a naturally occurring monoterpene produced by the Camphor Laurel (*Cinnamomum camphora*), to modulate sensations of warmth in humans has been attributed to its ability to activate TRPV3 (Moqrich *et al*., 2005). TRPV3 is expressed in keratinocytes, the dorsal root ganglia, brain and spinal cord ([Peier *et al*., 2002a, b; Xu *et al*., 2002). It has been implicated in hyperalgesia in inflamed tissues (Hu *et al*., 2006; Xu *et al*., 2006) and possibly skin sensitization (Xu *et al*., 2006).

TRPV3 is a sensitizing receptor (Peier *et al*., 2002b; Xu *et al*., 2002), such that upon prolonged or repeated stimulation, currents evoked by chemical or thermal stimuli increase in amplitude. The synthetic TRPV3 agonist, 2-aminoethoxy diphenyl borate (2-APB), which is structurally unrelated to camphor, has been shown to activate significant currents upon first application at concentrations of 100–300 μM. In contrast, camphor as a relatively weak agonist, activates sizeable currents only at concentrations of 10 mM or higher on first exposure. However, after preceding stimulation with higher doses, pronounced currents can already be observed with 2 mM camphor. Also, camphor's dose dependence is shifted to lower activating concentrations after prestimulating exposures to 2-APB before camphor treatment (Xu *et al*., 2005).

In addition to camphor, carvacrol, thymol and menthol have also been shown to activate TRPV3 (Macpherson *et al*., 2006; Xu *et al*., 2006). Interestingly, (−)-menthol is also a well-known agonist for the cool-sensitive channel TRPM8 (Mckemy *et al*., 2002; Peier *et al*., 2002a).

As still only few ligands are known for TRPV3, we aimed to investigate systematically the pharmacological profile of this receptor by testing compounds covering several important classes of terpenoids and thus establish a structure–function relationship. The most potent of the identified agonists were also tested on TRPM8 to determine whether similar structural requirements were relevant for activation of this channel and to assess the overlap of the receptor pharmacology.

Methods

Cell culture of HEK293 cells

HEK293 cells were maintained under standard conditions in a minimum essential medium supplemented with 10% foetal bovine serum, 100 U ml^{-1} penicillin and streptomycin, and 2 mM L-glutamine.

Cloning and expression of TRPV3 and TRPM8

Expression vectors for murine TRPV3 and TRPM8 were gifts from M Schaefer (Charité Universitätsmedizin, Berlin, Germany) and D Julius (University of California, San Francisco, CA, USA), respectively. TRPV3 was expressed as a fusion protein with a C-terminal YFP (Hellwig *et al*., 2005). Semiconfluent cells were transfected in 35-mm dishes (Becton Dickinson, Heidelberg, Germany) by using the CaP-precipitation technique as described (Zufall *et al*., 1993). Measurements were done 48–72 h after transfection.

Synthesis and injection of TRPM8 and TRPV3 cRNA

The generation of cRNA was performed by standard methods. To use plasmids containing cloned murine TRPM8 or TRPV3 cDNA as a template for *in vitro* transcription, plasmids were linearized downstream of the end of the cDNA. Capped RNAs were synthesized in the presence of capping analogue $m^7G(5')ppp(5')G$ using the AmpliCap-T7 MessageMaker Kit (Epicentre, Oldendorf, Germany). RNA was ethanol-precipitated and redissolved in water to give a final concentration of 1 μg μl^{-1}. Ovarian lobes were obtained from mature female *Xenopus laevis* anaesthetized by immersion in 0.15% 3-aminobenzoic acid ethyl ester. Ovarian tissue was removed and placed in Barth's solution (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 2.4 mM $NaHCO_3$, 5 mM Tris-HCl, pH 7.4; 100 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin). After treatment of the ovarian tissue with collagenase (Type I, 4 mg ml^{-1} in Ca^{2+}-free Barth's solution) for 2 h at room temperature, the oocytes were incubated overnight in fresh Barth's solution (15°C). After 24 h, mature healthy oocytes (stages V–VI) were selected for cytoplasmic injection of cRNA (about 50 nl per oocyte; approximate cRNA concentration 1 μg μl^{-1}) with a sharp pipette using a pressure injector (npi PDES 04 T; Tamm, Germany). Afterwards, injected oocytes were placed again in fresh Barth's solution and incubated at 16°C. Oocytes were tested for functional expression of TRP channels after 3–5 days.

Electrophysiology in HEK293 cells

Recordings were performed using the whole-cell mode of the patch-clamp technique. Cells were maintained in an extracellular recording solution containing (in mM) 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid (HEPES), 10 glucose, pH 7.4. Patch electrodes were pulled from borosilicate glass (1.2 mm OD × 1.17 mm ID; Harvard apparatus, Edenbridge, Kent, UK) and fire polished to 4–6 MΩ tip resistance using a horizontal pipette puller (Zeitz Instruments, Munich, Germany). The pipette solution contained (in mM) 140 KCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 5 ethylene glycol tetraacetic acid, 10 HEPES, pH 7.4 for recordings. Patch-clamp recordings were carried out at 32°C for TRPV3, as the sensitivity of this channel to chemical stimulation is reduced at temperatures below 30°C (Hu *et al*., 2004). In contrast, the sensitivity of TRPM8 to chemical stimulation is reduced by warm temperatures (Peier *et al*., 2002a); therefore, measurements on TRPM8 were performed at room temperature (22–25°C). In each case, a HEKA EPC7 amplifier was used. Membrane potential was held at −40 mV. Data were acquired using Pulse software. Compounds were applied in the assay buffer and could transiently (5 s for recordings on TRPM8 or 10 s for recordings on TRPV3) superfuse the cells; washes between applications lasted 30 s.

Before each recording, TRPV3 expressing cells were sensitized with 10–15 pulses of 300 μM 2-APB, as this concentration reliably activates the channel. Afterwards, camphor and other monoterpenes were applied alternately.

Electrophysiological recordings in oocytes

Two-electrode voltage-clamp recording was used to obtain current responses to applied substances. Agonists and antagonists were diluted to the concentrations indicated with *Xenopus*-Ringer solutions, containing calcium (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 200 μM flufenamic acid, 10 mM HEPES, pH 7.4) or calcium-free (115 mM NaCl, 2.5 mM KCl, 10 mM HEPES, pH 7.4). Agonists were applied by means of a multibarrel single tip superfusion device or by manual application. Application time was usually 10 s. Electrodes were pulled from borosilicate glass using a Kopf vertical pipette puller. Electrodes were backfilled with 3 M KCl. Membrane potential was controlled and current signals were recorded with a two-electrode voltage-clamp amplifier (TURBO TEC-03, npi, Tamm, Germany) and the PCLAMP software (Axon Instruments, Wokingham, Berkshire, UK).

Data analysis

Comparison of currents evoked by different monoterpenes was performed by applying camphor before and after each tested compound. Evoked currents were then normalized to the average of the preceding and the subsequent camphor amplitude. Values were compared by multiple *t*-test; $P<0.05$ was taken as significant.

For the dose–response data, statistical analysis and curve fitting were carried out by Hill equation using SigmaPlot V8.0 (Systat Software, San Jose, CA, USA). EC_{50} repesents mean $\pm$ s.d. of three to five individual fits of independent measurements.

Reagents

All cell culture reagents were obtained from Invitrogen (Karlsruhe, Germany). Chemicals for intracellular and extracellular solutions in patch clamp measurements were obtained from Sigma Aldrich (Munich, Germany), as well as the following compounds: 1-isopropyl-4-methyl-bicyclo[3.1.0]hexan-4-ol, 1,8-cineole, 2-aminoethoxydiphenyl borate, (1*S*)-(−)-α-pinene, *endo*-(1*R*)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-ol (referred to in the text as (+)-borneol), camphor, (1*S*)-(+)-camphorquinone, carvacrol, (L)-(−)-carveol, (*S*)-(−)-carvone, *p*-cymene, dihydrocarveol, (2*R*,5*R*)-(+)-dihydrocarvone, geraniol, isoborneol, isobornyl acetate, (1*R*,3*R*,4S)-(−)-*p*-menth-8-en-3-ol (referred to in the text as (−)-isopulegol), (*R*)-(+)-limonene, linalool, (1*R*,4*S*)-(−)-*p*-menthan-3-one (referred to in the text as (−)-menthone), terpineol, thymol, (1*S*,3*R*,5*S*)-(−)-*trans*-pinocarveol. Obtained from Symrise (Holzminden, Germany) were 6-tert-butyl-*m*-cresol, (4*S*,8*S*)-(−)-á-bisabolol, α-pinene oxide, (1*S*,4*R*)-(−)-1-isopropyl-4-methylbicyclo[3.1.0]hexan-3-one (referred to in the text as (−)-α-thujone), carvacrol methylether, (1*S*)-(+)-1,3,3-trimethylbicyclo[2.2.1]-heptan-2-one (referred to in the text as (+)-fenchone), (1*R*,2*R*,3*R*,5*S*)-(−)-3-pinanol (referred to in the text as (−)-isopinocampheol), kreosol, (1*R*,2*S*,5*R*)-(−)-2-isopropyl-5-methylcyclohexanol (referred to in the text as (−)-menthol), mugetanol, *p*-xylenol. Where the stereochemistry is not indicated, racemates were used. Propofol was obtained from Tocris (Bristol, UK), icilin was a gift from ET Wei (Berkeley, CA, USA).

Results

Screening of monoterpenes for TRPV3 activation

For the identification and characterization of new TRPV3 agonists, the activity of 33 monoterpenes and related compounds on TRPV3 was compared to that of camphor. Screening was performed by whole-cell patch-clamp measurements of HEK293 cells expressing murine TRPV3. The tested compounds were grouped with respect to structural similarity: Twelve bicyclic monoterpenes were tested, including the known agonist camphor and its biosynthetic precursor (+)-borneol. The monocyclic monoterpenes as the largest group were divided into eight aromatic and nine non-aromatic compounds. In addition, three related cyclic compounds not classified as monoterpenes, among them the sesquiterpene (−)-α-bisabolol as well as two acyclic monoterpenes were tested.

All compounds were applied at a concentration of 2 mM as this was the highest concentration at which all compounds were soluble without an excessive amount of solvent (>0.1%) in the final solution. As camphor only activates significant currents at a concentration of 2 mM after sensitization, TRPV3-expressing HEK293 cells were first sensitized with the structurally unrelated agonist 2-APB (see Methods section). Owing to the sensitizing properties of TRPV3, several consecutive applications of the same substance result in increasing currents rather than stable amplitudes. To compare different compounds, camphor was given as a reference before and after the application of the respective test substance, typical traces are shown in Figure 1. Reference camphor applications on average induced currents of 390 $\pm$ 50 pA (mean $\pm$ s.e.m; $n=79$). Current amplitudes of all compounds relative to camphor are summarized in Table 1. As a control, all tested compounds were also applied to at least three untransfected HEK293 cells; none elicited a response.

In all, six compounds were identified that activated significantly larger currents than camphor ($P<0.05$). While the bicyclic compound (+)-borneol showed 150% of the camphor activation, even more potent agonists were encountered both among the aromatic and the non-aromatic monocyclic monoterpenes: 6-tert-butyl-m-cresol (290%), carvacrol (265%) and thymol (245%) among the aromatic and dihydrocarveol (255%) and (−)-carveol (150%) among the non-aromatic compounds.

To test whether the identified agonists sensitized the receptor as did camphor, cells were challenged with two consecutive pulses of the same agonist and the increase in amplitude of the second versus the first pulse was evaluated. Sensitization was similar for all compounds: While camphor currents typically increased to 122 $\pm$ 11% of the preceding response, borneol currents increased to 118 $\pm$ 15%,

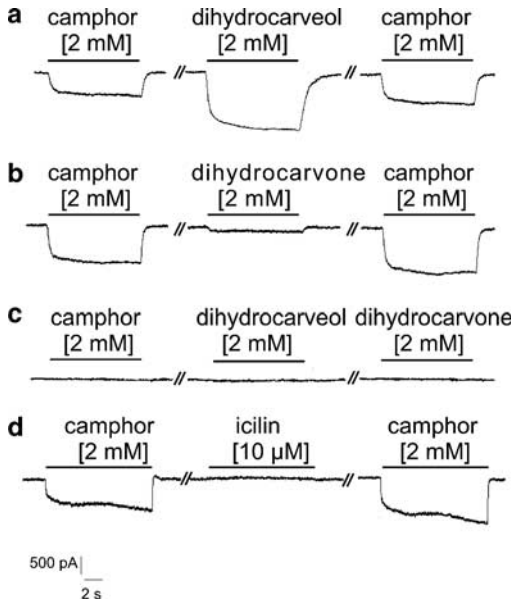


Figure 1 Representative current traces illustrating the screening of monoterpenes for TRPV3 activation. HEK293 cells transfected with TRPV3 were held in the whole-cell voltage-clamp mode at −40 mV and superfused as indicated. (**a**) Representative response of one cell to dihydrocarveol and camphor. (**b**) Representative response of the same cell to (+)-dihydrocarvone. (**c**) Assay in an untransfected cell. Note that neither camphor nor the other two monoterpenes evoked any current. (**d**) TRPV3 expressing cell superfused with icilin.

6-tert-butyl-*m*-cresol to 135±17%, carvacrol to 127±10%, thymol to 132±15%, dihydrocarveol to 129±13% and carveol to 120±28% (mean±s.e.m; $n=4$ each). Like camphor, all six compounds were able to activate the naïve receptor (without prior sensitization by 2-APB) when applied at a concentration of 10 mM (data not shown).

To examine TRPV3 activation by these compounds in more detail, dose–response curves were created. This was done in *Xenopus* oocytes, as TRPV3 is known to show no apparent sensitization in this system (Xu *et al*., 2002; Hu *et al*., 2006), making the comparison of elicited current amplitudes more reliable. Typical recordings are shown in Figure 2. Camphor activated TRPV3 with an EC_{50} of about 6 mM (see Table 1). The synthetic agonist 2-APB was also tested and showed an EC_{50} of 0.515±0.005 mM, which is in fair agreement with an EC_{50} of 1.06 mM reported by others (Hu *et al*., 2006).

Consistent with the results obtained in HEK293 cells, all six compounds activated the receptor with a lower EC_{50} than camphor (Table 1). Also in line with the results obtained in HEK293 cells, is the relative order of agonist potency: 6-tert-butyl-*m*-cresol and carvacrol have the highest potency, followed by thymol. The remaining compounds have markedly lower potencies, with EC_{50} values between 2 and 4 mM.

Structure–activity relationship

To determine the importance of different features in the chemical structure, compound pairs differing by type or position of substituents were compared.

While thymol, a monocyclic aromatic monoterpene which carries a hydroxyl group as a substituent, activated more than twice the camphor-induced current, *p*-cymene lacking this group activated only negligible currents (15% of camphor). To determine the effect of the oxidation of the hydroxyl to a keto group, a number of compound pairs belonging to monocyclic as well as bicyclic monoterpenes were compared. Consistently, all ketones activated smaller currents than the corresponding alcohols (carveol > carvone, dihydrocarveol > dihydrocarvone, menthol > menthone, borneol > camphor).

Furthermore, the position of the hydroxyl group was varied in aromatic and non-aromatic monocyclic compounds. In non-aromatic compounds, larger currents were activated by compounds with the hydroxyl group in the meta position to the isopropyl residue rather than the ortho position. For instance, dihydrocarveol evoked 255% of the standard camphor current, whereas isopulegol only evoked 90% of the response to camphor. In aromatic monoterpenes, however, activation was similar irrespective of the position of the hydroxyl group (carvacrol 265%, thymol 245% of camphor). Compounds in which the hydroxyl group was not located on the ring but rather on side chains induced only minor responses (terpineol, (−)-α-bisabolol, mugetanol). The importance of the hydroxyl group for TRPV3 activation is further supported by the finding that esterification of the hydroxyl group in isoborneol to isobornyl acetate or methylation of carvacrol to carvacrol methylether abolished the activity.

Overlap of TRPV3 and TRPM8 agonists

The first described agonist for the cool sensitive channel TRPM8, (−)-menthol, also belongs to the monoterpenes (Peier *et al*., 2002a, b). As (−)-menthol also activated TRPV3 (albeit to a relatively minor extent, 65% of the camphor amplitude), it appeared interesting to determine whether other structurally related TRPV3 agonists could activate TRPM8.

We therefore tested the most potent TRPV3 agonists for activation of murine TRPM8. In addition, (−)-menthone was included as a derivative of (−)-menthol and (+)-limonene as an example for a compound without an oxygen moiety.

As in the experiments described above, currents evoked by 2 mM of the respective substance were compared with those evoked by 2 mM (−)-menthol, which is a saturating concentration on TRPM8 (Bandell *et al*., 2006). As shown in Figure 3, the responses elicited by all of the tested compounds were well below that of (−)-menthol, being maximally 35% of the (−)-menthol-activated current for thymol and dihydrocarveol. The drastically reduced amplitude at 2 mM for all tested compounds indicates that neither compound is a potent TRPM8 agonist as potency and/or efficacy are markedly weaker than that of (−)-menthol.

To further investigate the possibility of common agonists of TRPV3 and TRPM8, the high-potency synthetic TRPM8 agonist icilin was tested for TRPV3 activation at 10 µM, a concentration that is saturating on TRPM8. As shown in Figure 1d, icilin, which shares no structural similarity with monoterpenes, did not activate TRPV3.

Table 1 Chemical structure and activity on TRPV3 channels for all tested monoterpenes. Activation of TRPV3 was assessed by the current evoked by the compound relative to that evoked by 2 mM camphor in HEK293 cells

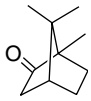
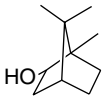
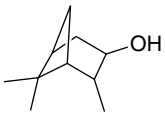
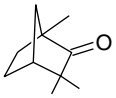
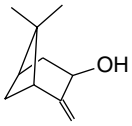
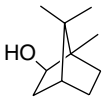
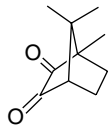
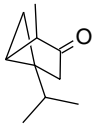
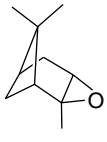
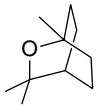
Compound	*Structure*	*V3 activation (% to camphor)*	n	*EC_{50} (mM)*
Bicyclic monoterpenes				
Camphor		100	315	6.03±1.47
(+)-Borneol		150±35*	24	3.45±0.13
(−)-Isopinocampheol		140±35	3	
(−)-Fenchone		95±30	17	
(−)-*Trans*-pinocarveol		85±20	4	
Isoborneol		65±10*	5	
(+)-Camphorquinone		55±15*	8	
(−)-α-Thujone		50±45*	6	
α-pinene oxide		35±15*	3	
1,8-Cineole		25±10*	3	

Table 1 *Continued*

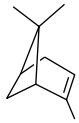
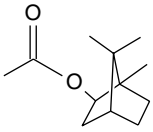
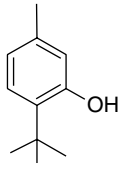
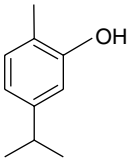
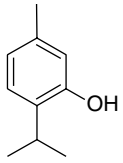
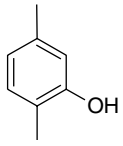
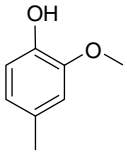
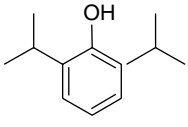
Compound	*Structure*	*V3 activation (% to camphor)*	n	*EC_{50} (mM)*
(−)-α-Pinene		15±10*	3	
Isobornyl acetate		0±0	3	
Aromatic monocyclic monoterpenes				
6-tert-butyl-*m*-cresol		290±120*	7	0.37±0.1
Carvacrol		265±60*	6	0.49±0.07
Thymol		245±90*	12	0.86±0.07
p-xylenol		100±45	3	
Kreosol		70±50	5	
Propofol		55±15*	4	

Table 1 *Continued*

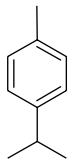
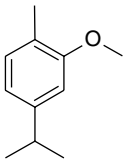
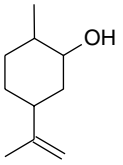
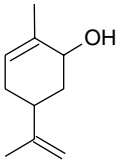
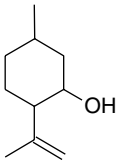
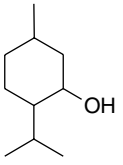
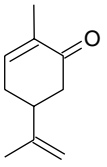
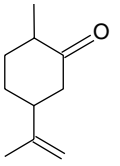
Compound	*Structure*	*V3 activation (% to camphor)*	n	*EC_{50} (mM)*
p-cymene		15±10*	3	
Carvacrol methylether		5±10*	5	
Non-aromatic monocyclic monoterpenes				
Dihydrocarveol		255±100*	24	2.57±0.42
(−)-Carveol		150±15*	3	3.03±1.16
(−)-Isopulegol		90±10	5	
(−)-Menthol		65±10	3	
(−)-Carvone		25±15*	16	
(+)-Dihydrocarvone		25±15*	6	

Table 1 *Continued*

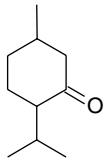
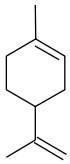
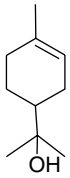
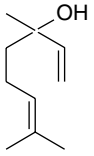
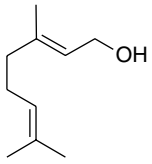
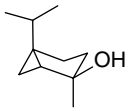
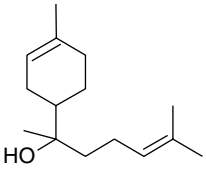
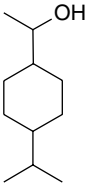
Compound	*Structure*	*V3 activation (% to camphor)*	n	*EC_{50} (mM)*
(−)-Menthone		20±2*	5	
(+)-Limonene		0±0	7	
Terpineol		0±0	4	
Acyclic monoterpenes				
(+)-Linalool		75±20	3	
Geraniol		35±15*	3	
Other compounds				
1-Isopropyl-4-methyl-bicyclo[3.1.0]hexan-4-ol		35±25*	4	
(−)α-Bisabolol		5±2*	3	

Table 1 *Continued*

Compound	*Structure*	*V3 activation (% to camphor)*	n	*EC_{50} (mM)*
mugetanol		2±4*	5	

EC_{50} values were determined in *Xenopus* oocytes.
Values shown are means±s.d.
*Significantly different from camphor (*t*-test; $P<0.05$).

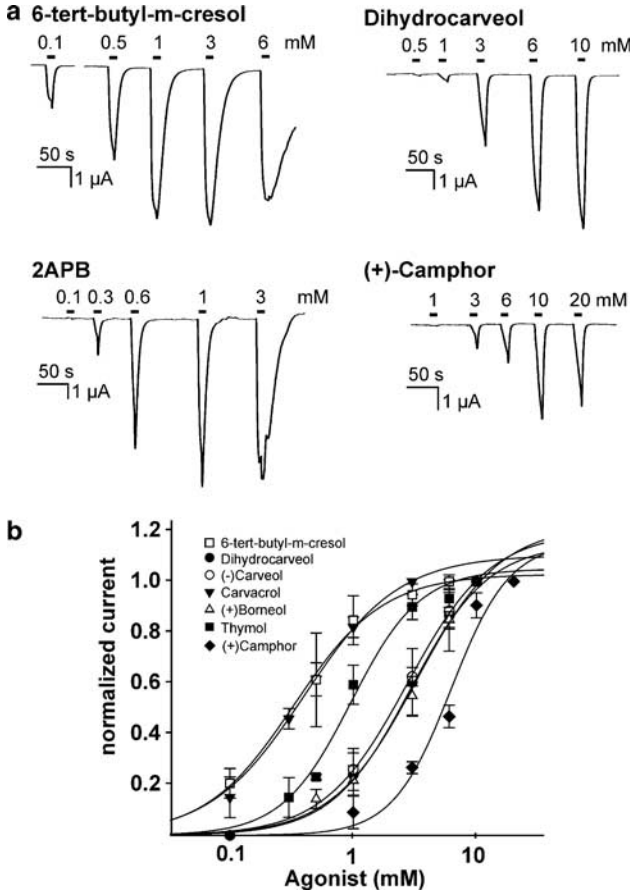


Figure 2 (**a**) Representative recordings in *Xenopus* oocytes for the establishment of dose–response curves. (**b**) Dose–response curves constructed from mean values of three to five experiments fitted by the Hill equation.

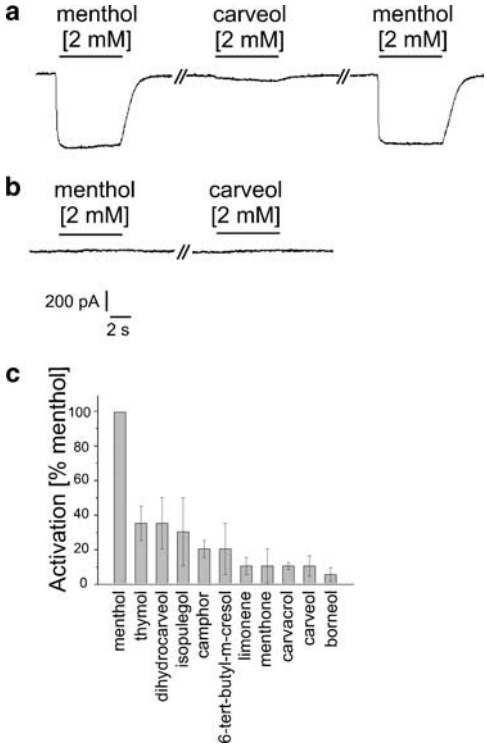


Figure 3 Activation of TRPM8 by potent TRPV3 monoterpenoid agonists. (**a**) Representative current trace of a TRPM8 expressing HEK293 cell. The cell was held in the voltage clamp mode at −40 mV and transiently superfused with (−)-menthol (2 mM) and carveol (2 mM) as indicated. (**b**) Equivalent stimulation of an untransfected cell did not result in any response to either (−)-menthol or carveol (**c**) Summary of the evoked currents relative to that evoked by (−)-menthol; all compounds were applied at a concentration of 2 mM. Values shown are means±s.d.

Discussion and conclusions

The recent identification of thermosensitive TRP ion channels has been a major advance in our understanding of thermosensation. Interestingly, members of this ion channel family can also be activated or inhibited by chemical agonists. The ability of compounds to elicit warm, cool, hot or cold sensations is therefore currently attributed to the activation or inhibition of thermosensitive TRP channels. However, owing to the quite recent identification of most members of this ion channel family, relatively few agonists are known for most of them and little is known about the structural basis for activation.

While the general pharmacology of TRPM8 has been characterized in detail (Behrendt *et al*., 2004), chemical agonists for TRPV3 are only slowly emerging. The first chemical agonist identified for TRPV3 was the synthetic compound 2-APB (Hu *et al*., 2004). Shortly afterwards, the

monoterpene camphor was found to be an agonist, albeit only at rather high concentrations (Moqrich *et al*., 2005). In addition, thymol, carvacrol and menthol have recently been described to activate TRPV3 (Xu *et al*., 2006; Macpherson *et al*., 2006), although no quantitative comparison was performed.

We therefore examined a number of terpenoid compounds in which different structural elements were systematically varied to identify features required for channel activation. Out of the 33 tested terpenes and related compounds, six agonists significantly stronger than camphor were identified. All of these activate TRPV3 with an EC_{50} substantially lower than that of camphor and the two most potent ones – 6-tert-butyl-*m*-cresol and carvacrol, also show a higher potency than the synthetic agonist 2-APB.

While camphor belongs to the group of the bicyclic monoterpenes, the best agonists were found among the monocyclic group. Interestingly, highly active agonists were encountered both among the aromatic and the non-aromatic compounds. Both tested acyclic monoterpenes activated the channel to a much lesser extent than camphor, which may indicate that a cyclic structure is required for activation.

It is striking that all of the six compounds more potent than camphor carry a secondary hydroxyl group. Oxidation to a carbonyl group reduced the activity of the substance drastically, arguing that a hydroxyl group is a structural requirement for efficient activation of TRPV3. In line with this, none of the compounds without an oxygen moiety (*p*-cymene and (+)-limonene) activated the channel to a significant extent.

While the position of the hydroxyl group on the ring does not appear to be critical for TRPV3 activation in aromatic substances, it is relevant for non-aromatic compounds. Here, much stronger activation is achieved with the hydroxyl group in the meta position to the isopropyl residue as in dihydrocarveol and (−)-carveol, rather than in the ortho position as in (−)-isopulegol and (−)-menthol. No significant activation can be seen by compounds in which the hydroxyl group is not located on the ring itself (as in terpineol, mugetanol or (−)-α-bisabolol).

A similar requirement for a ring-located hydroxyl group has also been described for the modulation of $GABA_A$ receptors by terpenoids (Mohammadi *et al*., 2001). Among the investigated compounds are several monoterpenes such as thymol and menthol, accounting for their ability to serve as anxiolytic or sedative agents (Priestley *et al*., 2003; Hall *et al*., 2004). Thymol was also identified as one of the most potent TRVP3 agonists in the presented work. In contrast, the high affinity $GABA_A$ ligand propofol did not activate TRPV3 to a significant extent, indicating differences in the structural requirements for activation and that the overlap between these unrelated receptors is probably confined to a small group of substances.

Like odorant receptors, thermoTRP channels are not highly specific for one given ligand, but are activated by a number of chemically similar agonists. Recent studies identified overlaps in the agonist profiles of different thermoTRPs, such as TRPV1, TRPV3, TRPM8 and TRPA1 (Macpherson *et al*., 2005, 2006), which complicates the prediction of the sensory effects elicited by a given compound. While the activation of TRPV1 and TRPA1, which both localize to nociceptors, by the same compounds offers a comprehensible explanation of the pungent sensation evoked by these substances, the concomitant activation of cool-activating TRPM8 and warm-activating TRPV3 offers more of a puzzle. In the case of (−)-menthol, activation of both receptors has been correlated with a paradoxical feeling of warmth at higher temperatures in addition to the well-known cooling sensation elicited by this compound when applied at lower temperatures (Macpherson *et al*., 2006).

To further investigate whether additional substances are shared agonists on these two receptors and whether structural requirements for activation are similar, we applied the most potent identified agonists of TRPV3 on TRPM8. As seen with TRPV3, monoterpenes without a substituent only weakly activated TRPM8. Also as for TRPV3, oxidation of the hydroxyl group in (−)-menthol to yield (−)-menthone drastically reduced M8 activation.

However, neither of the potent TRPV3 agonists could activate TRPM8 as effectively as (−)-menthol and even small changes in the structure of this ligand resulted in a much reduced activation: Both the introduction of a double bond in the isopropyl residue, as in (−)-isopulegol, or the change to an aromatic ring, as in thymol, reduced the activity by a factor of at least three. While a number of (−)-menthol derivatives are known to be potent TRPM8 activators, such as Frescolat ML, WS-3 or cooling- agent- 10, all of these compounds share the (−)-menthol structure and stereochemistry and carry different substituents at the position of the hydroxyl group only.

Interestingly, the non-terpenoid TRPV3-ligand 2-APB is known to inhibit TRPM8 (Hu *et al*., 2004) and thus has opposing effects on the two channels. Therefore, although both channels are activated by members of the monoterpene family, their pharmacological profiles appear to overlap only marginally.

It has been suggested that TRPV3 might be a molecular target for skin sensitizers (Xu *et al*., 2006). However, our data do not provide evidence for a correlation of TRPV3 agonism and skin sensitization. Thus, the potent TRPV3 agonists carvacrol and thymol are not sensitizers (Andersen, 2006), borneol is a weak skin sensitizer and allergic reactions to camphor are rare. In contrast, the known skin sensitizers, geraniol (Frosch *et al*., 1995) and carvone (Paulsen *et al*., 1993) are only poor TRPV3 agonists. While our study did not address the human receptor or test the behaviour of TRPV3 in the native system, a direct relationship between TRPV3 agonism and skin sensitization is not supported by our results, leading us to suggest that the role of TRPV3 in skin sensitization still needs to be clarified.

Terpenoids have been used in essential oils for centuries as medically and cosmetically relevant compounds, but still little is known about their mechanism of action. TRP channels as targets for terpenoids can potentially explain several of the described effects. For instance, the desensitization of nociceptive TRPV1 and/or the activation of TRPM8 have been suggested to underlie the analgesic effect of naturally occurring compounds like (−)-menthol or camphor (Xu *et al*., 2005; Macpherson *et al*., 2006; Proudfoot

et al., 2006). Consequently, activation, desensitization or inhibition of particular TRP channels by natural or synthetic compounds is a promising tool in medicine and pharmacology. Moreover, a more comprehensive knowledge of the pharmacology of different thermoTRPs may offer the possibility to alter chemical structures to achieve activation or inhibition of these channels. In this context, the investigation of ligands for members of the TRP channel family is not only relevant for the understanding of this protein family, but also has implications for target-directed drug design.

Acknowledgements

We thank Harry Bartel for excellent technical support. MAS is supported by a DAAD fellowship.

Conflict of interest

The authors state no conflict of interest. Two of the authors (GV and JP) are employees of Symrise GmbH.

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