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Borneol exerts its antipruritic effects by inhibiting TRPA1 and activating TRPM8

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ABSTRACT

Ethnopharmacological relevance: Borneol is a long-established traditional Chinese medicine that has been found to be effective in treating pain and itchy skin. However, whether borneol has a therapeutic effect on chronic itch and its related mechanisms remain unclear.

Aim of the study: To investigate the antipruritic effect of borneol and its molecular mechanism.

Materials and methods: DrugBAN framework and molecular docking were applied to predict the targets of borneol, and the calcium imaging or patch-clamp recording analysis were used to detect the effects of borneol on TRPA1, TRPM8 or TRPV3 channels in HEK293T cells. In addition, various mouse models of acute itch and chronic itch were established to evaluate the antipruritic effects of borneol on *C57BL/6J* mice. Then, the borneol-induced pruritic relief was further investigated in *Trpa1*$^{-/-}$, *Trpm8*$^{-/-}$, or *Trpa1*$^{-/-}$/*Trpm8*$^{-/-}$ mice. The effects of borneol on the activation of TRPM8 and the inhibition of TRPA1 were also measured in dorsal root ganglia neurons of wild-type (*WT*), *Trpm8*$^{-/-}$ and *Trpv1*$^{-/-}$ mice. Lastly, a randomized, double-blind study of adult patients was conducted to evaluate the clinical antipruritic effect of borneol.

Results: TRPA1, TRPV3 and TRPM8 are the potential targets of borneol according to the results of DrugBAN algorithm and molecular docking. Calcium imaging and patch-clamp recording analysis demonstrated that borneol activates TRPM8 channel-induced cell excitability and inhibits TRPA1 channel-mediated cell excitability in transfected HEK293T cells. Animal behavior analysis showed that borneol can significantly reduce acute and chronic itch behavior in *C57BL/6J* mice, but this effect was eliminated in *Trpa1*$^{-/-}$, *Trpm8*$^{-/-}$ mice, or at least in *Trpa1*$^{-/-}$/*Trpm8*$^{-/-}$ mice. Borneol elicits TRPM8 channel induced $[Ca^{2+}]_i$ responses but inhibits AITC or SADBE-induced activation of TRPA1 channels in dorsal root ganglia neurons of *WT* and *Trpv1*$^{-/-}$ mice, respectively. Furthermore, the clinical results indicated that borneol could reduce itching symptoms in patients and its efficacy is similar to that of menthol.

Conclusion: Borneol has therapeutic effects on multiple pruritus models in mice and patients with chronic itch, and the mechanism may be through inhibiting TRPA1 and activating TRPM8.

1. Introduction

Itching is defined as an unpleasant sensation that makes one want to scratch. Chronic itch is a cardinal manifestation of both dermatologic diseases and non-dermatologic conditions which still lacks effective treatments (Yosipovitch et al., 2018). Transient receptor potential (TRP)

Abbreviations: Cap, capsaicin; DRG, dorsal root ganglia; GRP, gastrin-releasing peptide; IMQ, Imiquimod; SADBE, squaric acid dibutylester; TRP, transient receptor potential.

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channels are molecular sensors of mechanical, chemical, and thermal environmental stimuli. In the past two decades, more and more studies have focused on the therapeutic strategies targeting TRP channels, and even proposed that TRP could refer to "targeted relief of pain" (Moore et al., 2018; Xie and Hu, 2018). TRPA1 and TRPV1 are essential for itch signal generation and transduction, even though it is still contentious, it is believed that TRPA1 is required for histamine-independent itch whereas TRPV1 is more sensitive to histamine-dependent itch (Moore et al., 2018; Wilson et al., 2013; Xie and Hu, 2018). TRPM8 is the major cold sensor and was recently reported as the molecular basis of cooling, acting as a counter stimulus to ameliorate behavioral changes in itch (Palkar et al., 2018). The lack of commonly used mouse itch models and stringent evidence-based clinical studies cloud the issue of the exact role of TRPM8 in itch, but is currently unresolved. It is important to explore new antipruritic agents targeting TRPM8 or other TRPs.

Natural borneol ((+)borneol) originally from *Cinnamomum camphora* (L.) Presl., also known as Longnao, possesses the effects of clearing heat, resuscitating, relieving pain and itch as a traditional Chinese medicine with a long history (Wang et al., 2017; Tian et al., 2023). The structure of borneol is similar to that of menthol, belonging to monoterpenes, and both of them are widely used as the components in the traditional Chinese medicine prescription recorded in "Chinese Pharmacopoeia Commission (2020)". Although more and more pharmacological studies have demonstrated that borneol has analgesic, antipruritic and anti-inflammatory effects (Jiang et al., 2015; Nguyen et al., 2020; Tian et al., 2023; Wang et al., 2017; Zhou et al., 2016), it still lacks verifiable scientific basis for the traditional effects. In contrast to borneol, menthol has been proved to elicit a cooling sensation and activate TRPM8 to induce antipruritic effects (Palkar et al., 2018), and exhibit paradoxical effects on the TRPA1 at different concentrations (Xiao et al., 2008). Most recent studies have demonstrated that borneol targets TRPM8 channel rather than TRPA1 to relieve pain with an effect even better than menthol (Wang et al., 2017), and both TRPA1 and TRPM8 are required for borneol-mediated acute nonhistaminergic itch relief (Tian et al., 2023). However, whether the borneol can treat chronic itch and the possible mechanisms are unknown.

To predict the targets of borneol, in this study DrugBAN algorithm (Bai P et al., 2023) as an artificial intelligence (AI)-based methodology and molecular docking were performed. And then using pharmacological and behavioral experiments on knockout mice, with analysis by calcium imaging method and patch-clamp recording analysis, we demonstrated that borneol can relieve itch through activating TRPM8 and inhibiting TRPA1 channel. Furthermore, we provided the clinical evidence that borneol can reduce chronic itch in patients with an efficacy similar to menthol for the first time.

2. Materials and methods

2.1. Reagents

Borneol ((+) borneol), menthol ((−) menthol), and chloroquine phosphate (CQ) were purchased from Yuanye Biotechnology Co., Ltd (Shanghai, China). Allyl-isothiocyanat (AITC) was purchased from Sigma-Aldrich (Shanghai, China). Squaric acid dibutyl ester (SADBE) and Imiquimod cream (IMQ) were purchased from J&K Scientific Ltd (Beijing, China), and Mingxinlidi Co., Ltd (Chengdu, China), respectively. Forsythoside B, RQ-00203078 and AMTB were purchased from MedChemExpress Co., Ltd (Shanghai, China).

2.2. Target prediction for Borneol through AI-based methodologies

The compound borneol (CAS: 464-43-7) with the character set of canonical SMILES from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), and the binding data with target protein sequences available in the BindingDB database (https://www.bindingdb.org/bind/index.jsp) were collected. The DrugBAN algorithm was applied, with the SMILES string of borneol and the sequences of target proteins as input data (Bai P et al., 2023). Then the confidence scores of borneol with various targets to shed light on its binding effect were assessed. Targets with prediction confidence scores exceeding 0.8 were considered as effective target candidates. Moreover, the STRING tool was used for visualization of the protein-protein interactions (https://string-db.org/).

2.3. Docking with Surflex-Dock

Borneol was prepared by a molecule minimization computational module and docked into the prepared TRPM8 (PDB Code: 6NR2), TRPA1 (PDB Code: 3J9P) and TRPV3 (PDB Code: 6LGP) structures by using Surflex-Dock in Sybyl-X 1.0. The Surflex-Dock algorithm was used following the empirical scoring system (Song H et al., 2019).

2.4. HEK293T cell transfection

HEK293T cells were cultured at 37 °C, 5% CO_2 in DMEM (Life Technologies, Carlsbad, CA) containing 10% FBS and 1% antibiotics. The cells were transiently transfected with cDNAs for human TRPM8, human TRPA1, mouse TRPV1, or human TRPV3 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After transfection, the cells were maintained in DMEM for 24 h before use (Feng et al., 2017).

2.5. Isolation and culture of mouse DRG neurons

Dorsal root ganglia (DRGs) in mice were isolated and dissected. After removal of connective tissue, the DRGs were rinsed with Hank's buffer (Gibco, New York, USA) containing 1 μL saturated $NaHCO_3$, 0.35 mg L-cysteine, and 20 U papain (Worthington Biochemical, Lakewood, NJ, USA) for 10 min in a 37 °C water bath. After centrifugation, the supernatant was removed and 1 mL HBSS (Ca^{2+}/Mg^{2+}-free), which contained 3.75 mg collagenase type II (Worthington Biochemical) and 7.5 mg dispase type II (Worthington Biochemical), was added and incubated at 37 °C for 10 min. After digestion, neurons were gently triturated, pelleted, and then resuspended in Neurobasal-A culture medium containing 2% B-27 supplement (ThermoFisher Scientific, Waltham, MA, USA), 100 U/mL penicillin plus 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA), 100 ng/mL nerve growth factor (Livzon Pharmaceutical Group Inc., Zhuhan, China), 20 μg/mL glial cell-derived neurotrophic factor (Sigma-Aldrich) and 10% heat-inactivated FBS (Sigma-Aldrich), and cultured in a humidified incubator at 37 °C for 18–24 h before use (Feng et al., 2017).

2.6. Ca^{2+} imaging of transfected HEK293T cells and DRG neurons

The short-term cultured TRP channel-expressing HEK293T cells or DRG neurons were loaded with 4 μM Fura-2 AM (Life Technologies) at 37 °C for 60 min. Before using, Fura-2-loaded cells were washed 3 times with HBSS at room temperature. The fluorescence ratios of F340/F380 were measured using a fluorescence microscope system (Nikon Instruments, Inc., Melville, NY). The ratios (F340/F380) reflect the changes in intracellular Ca^{2+} upon stimulation. Cells were considered responsive if they demonstrated a change in fluorescence ratio >10% of baseline (Feng et al., 2017).

2.7. Animals

Male *C57BL/6J* mice as the wild type mice (*WT*) were purchased from the Liaoning Changsheng Biotechnology Co., Ltd. (Shenyang, Liaoning, China). Breeder pairs of *Trpm8*$^{-/-}$, *Trpa1*$^{-/-}$, *and Trpv1*$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were housed in a 12-h light/dark cycle animal rooms with free access to standard food and water. All experimental procedures were conducted in accordance with the guidelines of the National Institutes of

Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Experimental Ethics Committee of South-Central Minzu University (No. 2018-SCUEC-AEC-024 and 2023-SCUEC-012). All mouse behavioral tests were videotaped, and behavioral assessments were done by those not aware of the experimental grouping.

2.8. Measurement of scratching behavior

Unless otherwise stated, all animals used in behavioral experiments are male mice aged 8–12 weeks. Briefly, mice were shaved on the nape 2 days before the tests. Then mice were randomly divided into five groups, including a normal group, a model group, borneol administration groups (25 or 50 mg/kg) and a positive group using menthol (n = 6–7 mice, per group). For the allergic contact dermatitis model mice were topically applied 20 μL of 0.5% SADBE in acetone to shaved abdominal skin once a day for 3 consecutive days (Feng et al., 2017). Five days later the mice were challenged with another topical application of 20 μL of 0.5% SADBE to the nape skin or ear once a day for 3 consecutive days. The normal group was treated topically with acetone at the time. On day 13, borneol group was subcutaneously injected with 25 or 50 mg/kg borneol. After 60 min, their spontaneous scratching behavior was recorded for 60 min. To establishing a psoriasis-like dermatitis model, imiquimod (IMQ) was used and mice were randomly divided into a normal group, a model group, borneol administration groups (25 or 50 mg/kg) and the positive control (menthol administration) group (n = 6–7 mice per group). Then IMQ-treated mice were sensitized once per day for 4 days (from day 0 to day 3) by applying 40 mg ointment of 5% IMQ topically to the shaved back neck skin (Sakai et al., 2016). On day 4, borneol groups were treated by administration of borneol (25 or 50 mg/kg). After 60 min, the scratching behavior was recorded for 60 min.

For the acute itch model, mice were randomly divided into the normal group, the model group, the borneol administration group (250 mg/kg) and the positive group of menthol (n = 6–11). Then borneol (250 mg/kg) was injected subcutaneously, and 30 min later, chloroquine (CQ, 100 μg) was intradermally injected into the nape (Palkar et al., 2018), or Gastrin-releasing peptide (GRP, 0.10 nmol) was intrathecally injected (Palkar et al., 2018). Immediately after the CQ or GRP injection, mice were videotaped for 30 min. The normal group were administrated with normal saline intradermally or intrathecally.

A scratching bout was defined as the mouse lifted its hind paws and scratched the shaved area of the neck before returning it to the ground (Sakai et al., 2016). Two trained observers (not knowing the grouping) counted the number of scratch bouts.

2.9. Clinical study

This clinical study is a randomized, double-blind study of adult patients at the Dermatology Hospital of Jiangxi Province. The study was approved by the Research Ethics Committee of the Dermatology Hospital of Jiangxi Province (Approval No. 201801) and was conducted in accordance with the ethical principles of the Declaration of Helsinki and the Council for International Organizations of Medical Sciences (CIOMS). After receiving oral and written explanations of the study protocol, the patients provided written informed consents. A total of 120 patients from the ages 18–70 years, both men and women, were randomly assigned to either the borneol group or menthol group. All patients suffer from skin diseases such as eczema, atopic dermatitis, and urticaria diagnosed by doctors, accompanied by itching symptoms. Six patients withdrew from the study without providing detailed reasons. A total of 114 patients completed the study and were included in the analysis. Trained hospital staff prepared 4% borneol solution and 4% menthol solution, and supervised patients to apply borneol solution or menthol solution to the skin with itching symptoms three times a day for one week. The pruritus VAS (visual analogue scale, which measures pruritus intensity on a scale from 0 [no itch] to 10 [very severe itch]), sleep quality VAS (ranging from 0 [sleep well] to 10 [completely unable to sleep]), and Dermatology Life Quality Index score (DLQI; measured on a scale of 0–30, with higher scores representing greater impairment) were evaluated for the exploratory efficacy outcomes (Kabashima et al., 2018; Wang et al., 2017). Eight patients in borneol group and six patients in menthol group failed to provide complete and compliant reports. Therefore, in total 48 patients in borneol group and 52 patients in menthol group completed the clinical study and their results were analyzed.

2.10. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6. All data were expressed as mean ± SEM. Statistical significance was evaluated using Two-tailed unpaired Student *t*-test for two-group comparisons or one-way analysis of variance (ANOVA) followed by Tukey's test for multi-group comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Target prediction for Borneol

To explore the potential protein targets for borneol, we applied the DrugBAN algorithm and assessed the confidence scores of borneol with various targets. Eighty-seven targets with confidence scores exceeding 0.8 were predicted (Supplementary Table S1). Among the targets, there were 32 targets with the prediction confidence exceeding 0.9 mainly including TRPA1, TRPM8 and TRPV3, etc. (Fig. 1A). The top 10 items of enriched KEGG pathways of the predicted target of borneol were presented (Fig. 1B). Moreover, Gene ontology (GO) enrichment analysis for key targets also demonstrated that 'calcium ion transport', 'response to cold/heat' and 'thermoception', etc. (Fig. 1C) were associated with the pharmaceutical function of borneol. The data combined the itch mechanism (Moore et al., 2018; Xie and Hu, 2018) indicated that 'Calcium signaling pathway' and TRPs channels containing TRPA1, TRPM8 and TRPV3 may be involved in the antipruritic effect of borneol.

According to the result of drug-target prediction using DrugBAN algorithm, the molecular docking simulation was also used to characterize the binding strength between borneol and TRPA1, TRPM8 or TRPV3. From the predicted binding sites, borneol showed a C-score of 3.824 with the formation of a hydrogen bond at the LYS-974 site of TRPA1 and three hydrophobic bonds at VAL-690, LEU-973 and ALA-977 of TRPA1 (Fig. 1D). The docking score of borneol towards the TRPM8 target was 3.419. The result of Fig. 1E displayed that borneol bonds three residues of TRPM8 (GLN-785, ARG-1007 and GLU-1003) through three hydrogen bonds and contacts a residue HIS-844 of TRPM8 through a hydrophobic bond. Besides, the docking score of borneol towards the TRPV3 target was 3.713. And the predicted binding sites showed that borneol forms a hydrogen bond with TRP-559 and has hydrophobic interaction forces with residues LEU-563, ILE-579, VAL-593, VAL-596, and PHE-597 (Fig. 1F).

3.2. The effect of borneol on TRPA1, TRPM8 or TRPV3

Then we applied borneol to HEK293T cells transfected with TRPA1, TRPM8 or TRPV3, and used Ca^{2+} imaging and patch-clamp recording to detect the effect of borneol on TRP channels. Borneol (2.5 μM) exhibited a strong inhibitory effect on AITC (0.1 mM) induced $[Ca^{2+}]_i$ responses in TRPA1-transfected HEK293T cells (Fig. 2A). And Ionomycin (Ion, 1.0 μM) included Ca^{2+} influx, suggesting good condition of HEK293T cells. Considering the activation of IMQ on TRPA1 channel (Esancy et al., 2018), additional studies were performed and exhibited that borneol could suppress IMQ (0.1 mM)-elicited $[Ca^{2+}]_i$ responses (Fig. 2B). The above results indicated that borneol can indeed inhibit TRPA1 function. Moreover, consistently borneol significantly inhibited the membrane

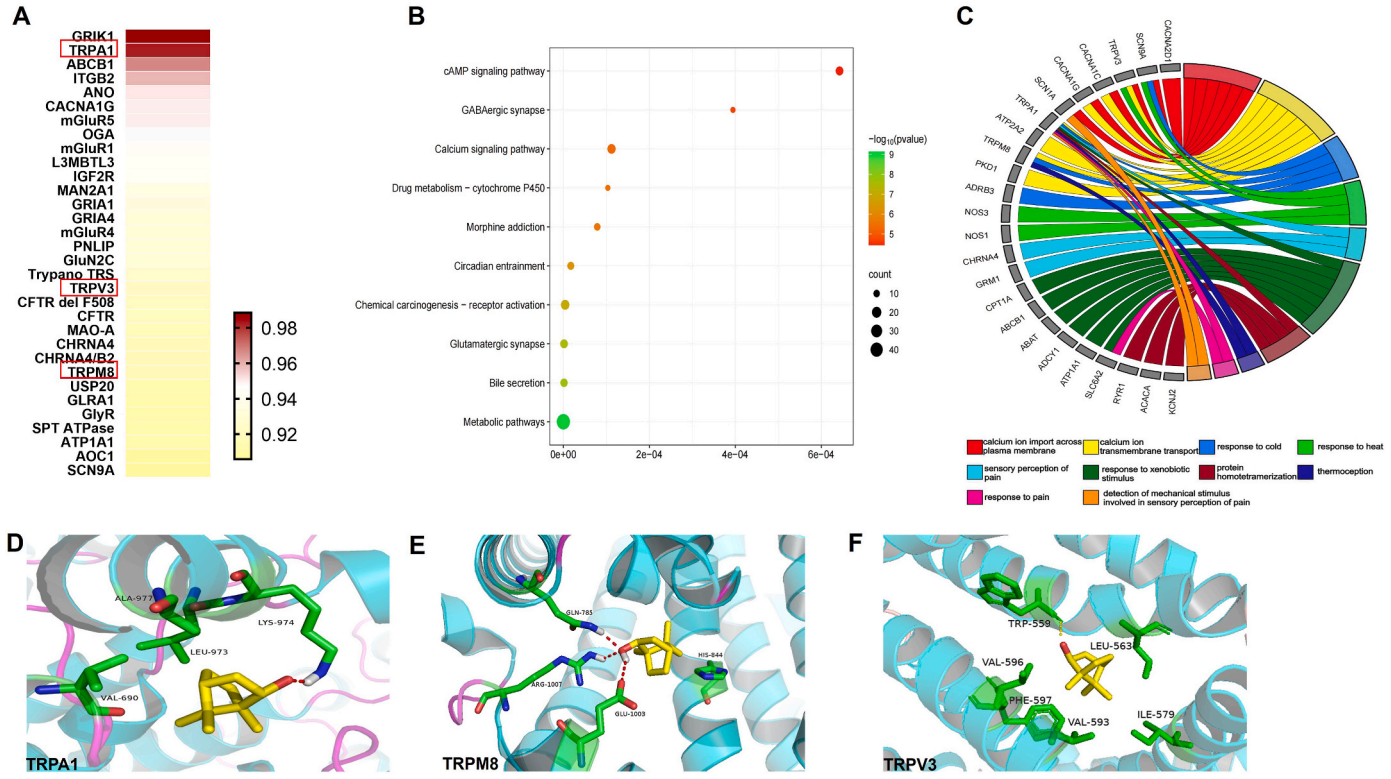

Fig. 1. Target prediction for Borneol through DrugBAN algorithm and modeling the interactions between borneol and TRPA1, TRPM8 or TRPV3. (**A**–**C**) The confidence scores of targets (**A**), KEGG pathway enrichment analysis (**B**) and Gene ontology (GO) enrichment analysis (**C**) using DrugBAN algorithm. (**D**–**F**) Docking pose of borneol in the active sites of TRPA1 (**D**), TRPM8 (**E**) or TRPV3 (**F**). Borneol is rendered in yellow in the center, and the surrounding key residues are rendered in green and labeled. Hydrogen bonds are depicted as dashed red or yellow lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

current activated by subsequent application of AITC in TRPA1-expressing HEK293T cells. However, menthol (3.0 mM) failed to eliminate the AITC induced responses (Supplementary Fig. S1A).

Application of borneol (2.5 mM) and menthol (0.1 mM) induced robust $[Ca^{2+}]_i$ responses in TRPM8-transfected HEK293T cells (Fig. 2C), and both were blocked by the co-application of the selective TRPM8 antagonist AMTB (2.0 μM). We also tested the effects of borneol and menthol on whole-cell currents of recombinant TRPM8-transfected HEK293T cells. Unlike a large rectification induced by menthol (0.3 mM), current-voltage relationship after bath application of 3.0 mM borneol displayed a weak response (Supplementary Fig. S1B), indicating that borneol can activate TRPM8 but not as potent as menthol. Then we determined the amino acid residues in TRPM8 proteins critical for the responses of borneol by using Ca^{2+} imaging analysis (Jiang et al., 2015). Borneol barely displayed any stimulation to TRPM8-Y745H, TRPM8-Y1005F, and TRPM8-L1009R mutants (Fig. 2D), suggesting that borneol uses these interaction sites for electrophiles to activate TRPM8.

The application of borneol also evoked robust $[Ca^{2+}]_i$ responses in TRPV3- transfected HEK293T cells (Supplementary Fig. S2A and Fig. 2E). And TRPV3 inhibitor, Forsythoside B (0.1 mM) significantly inhibited the membrane current activated by application of borneol in TRPV3-expressing HEK293T cells (Fig. 2E), indicating that borneol could active TRPV3 channels but not inhibit them. In addition, another itch related TRP channel, TRPV1, was also measured. We applied borneol to HEK293T cells transfected with TRPV1 using Ca^{2+} imaging to detect the effect of borneol on TRPV1 channels (Li and Wang, 2021). Application of borneol did not activate TRPV1, nor did it inhibit the capsaicin-induced $[Ca^{2+}]_i$ responses in TRPV1-expressing HEK293T cells (Supplementary Fig. S2B).

3.3. Borneol displayed antipruritic effects on chronic and acute itch models in mice

We next evaluated the antipruritic effect of borneol on mice. The schematic diagram of borneol on the treatment of chronic and acute itch models in mice is showed in Fig. 3A. In fact, as shown in Fig. 3B, in the chronic itch mice induced by SADBE or IMQ, treatment with borneol at the doses of 25 and 50 mg/kg significantly reduced scratching as compared with the model group of chronic itch.

Then the inhibitory effect of borneol was evaluated on acute itch models including chloroquine and GRP-induced acute itch. In Fig. 3C, after 30 min of subcutaneous injection of borneol (250 mg/kg), scratching caused by intradermal injection of chloroquine or intrathecal injection of GRP was significantly inhibited. Therefore, borneol shows antipruritic effects on acute and chronic itch models.

3.4. Borneol activated TRPM8 and inhibited AITC induced responses in DRG neurons

We further examined the effects of borneol on peripheral sensory neurons to investigate the mechanism of borneol on relieving itch. Borneol (1.0 mM and 2.0 mM) and menthol (0.1 mM) activated a small portion of DRG neurons (2.90%, 3.99% and 4.40%, respectively) (Fig. 4A), which was significantly blocked by AMTB (Fig. 4B). However, both menthol (0.1 mM) and borneol (2.0 mM) failed to induce Ca^{2+} responses in the DRG neurons isolated from *Trpm8*$^{-/-}$ mice (Fig. 4C), suggesting that TRPM8 is a molecular target of borneol (Wang et al., 2017; Zhou et al., 2016). To clarify further whether TRPA1 is required in the effect of borneol, we also compared AITC-evoked $[Ca^{2+}]_i$ responses in cultured mouse DRG neurons with or without treatment of borneol. Upon administration of borneol, the number populations of neurons

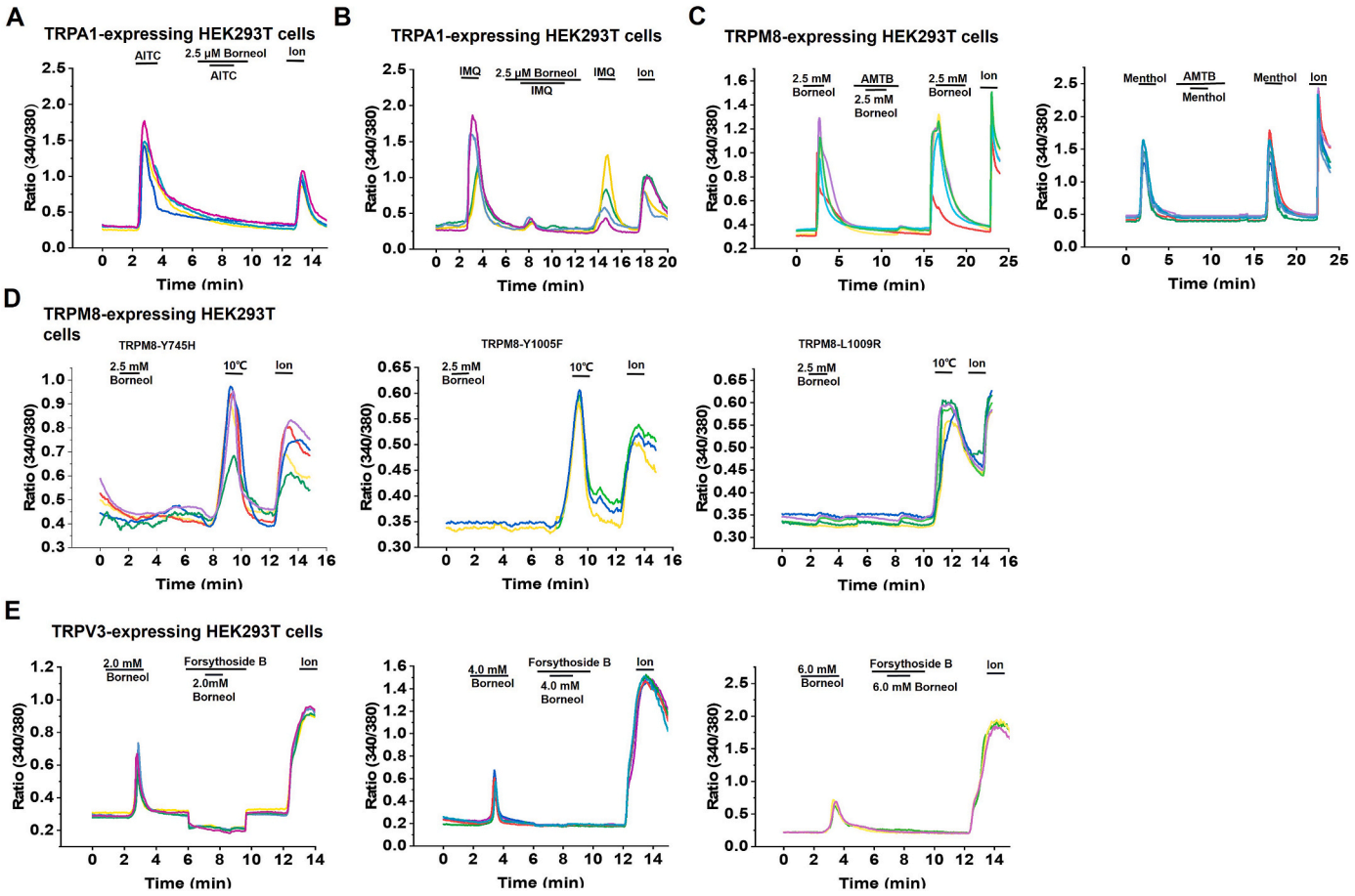

Fig. 2. The effect of borneol on TRPA1, TRPM8 and TRPV3 in HEK293T cells. **(A–B)** Representative time-lapse traces of borneol (2.5 μM) on AITC (0.1 mM, **A**) or IMQ (0.1 mM, **B**)-elicited $[Ca^{2+}]_i$ responses in TRPA1-transfected HEK293T cells. (**C**) Representative time-lapse traces illustrated borneol (2.5 mM) or menthol (0.1 mM) -elicited $[Ca^{2+}]_i$ responses inhibited by AMTB (2.0 μM) in TRPM8-transfected HEK293T cells. (**D**) Borneol (2.5 mM) lost effects on $[Ca^{2+}]_i$ responses in HEK293T cells transfected with TRPM8-Y745H, TRPM8-Y1005F, and TRPM8-L1009R mutants. (**E**) Representative time-lapse traces illustrated borneol (2.0, 4.0, 6.0 mM)-elicited $[Ca^{2+}]_i$ responses in TRPV3-transfected HEK293T cells were inhibited by the TRPV3 inhibitor, Forsythoside B.

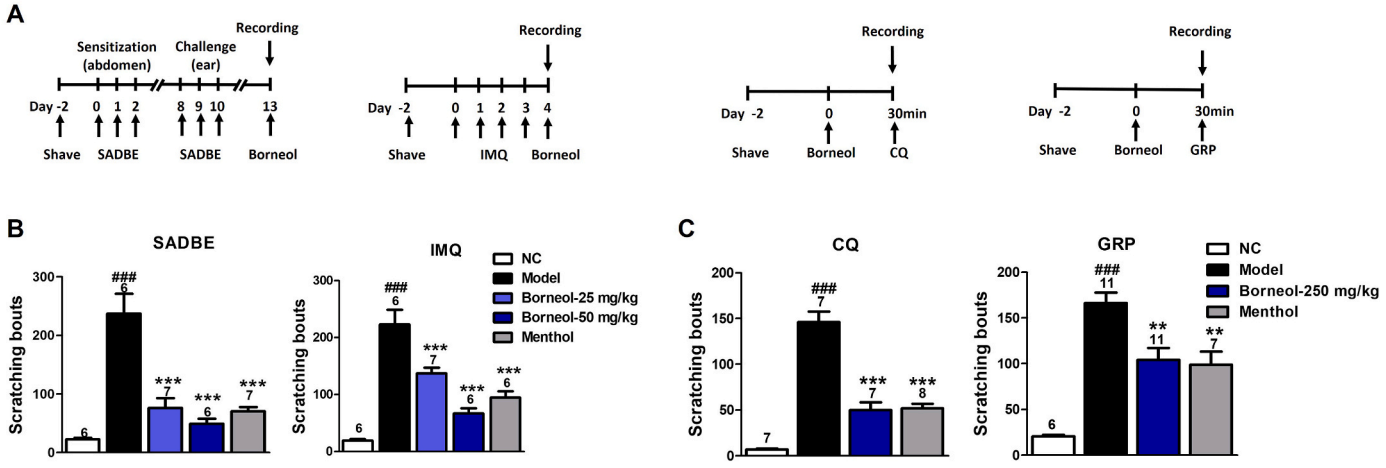

Fig. 3. Borneol inhibits acute and chronic itch in *WT* mice. **(A)** The schematic experimental schedule of borneol on the treatment of SADBE or IMQ induced chronic and chloroquine and GRP-induced acute itch models in mice. (**B–C**) Effects of borneol on scratching behavior of *WT* mice in chronic (**B**) and acute itch (**C**) models. All data are expressed as mean ± SEM, n = 6–11 mice for each group. Statistical significance was evaluated using one-way analysis of variance (ANOVA) followed by Tukey's test for multi-group comparisons. $^{###}P < 0.001$, model versus blank; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$; borneol or menthol versus model.

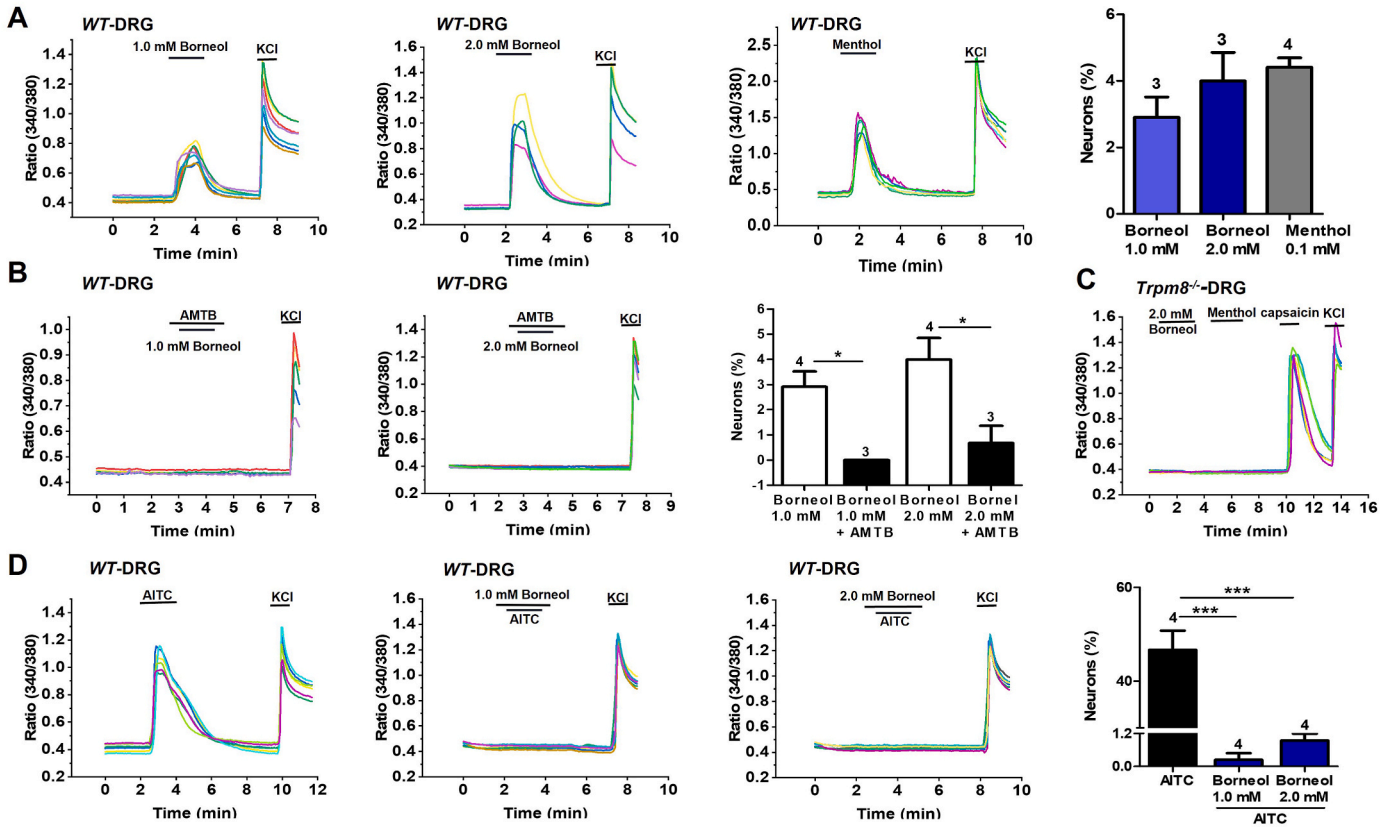

Fig. 4. Borneol activated TRPM8 and inhibited AITCinduced responses in DRG neuron. (**A**–**B**) Representative time-lapse traces illustrated borneol or menthol induced $[Ca^{2+}]_i$ responses in DRG neurons of *WT* mice (**A**), whereas blocked by AMTB (**B**), n = 3–4. All data are expressed as mean ± SEM. Statistical significance was evaluated using Two-tailed unpaired Student *t*-test for two-group comparisons. $^*P < 0.05$, treated with AMTB *versus* only treated with borneol. (**C**) $[Ca^{2+}]_i$ responses in DRG neurons of *Trpm8*$^{-/-}$ mice were induced by capsaicin but not borneol and menthol. (**D**) The inhibitory effect of borneol (1.0 and 2.0 mM) on AITC-evoked $[Ca^{2+}]_i$ responses in DRG neurons of *WT* mice, n = 4. All data are expressed as mean ± SEM. Statistical significance was evaluated using Two-tailed unpaired Student *t*-test for two-group comparisons. $^{***}P < 0.001$, borneol-*versus* AITC- treated.

responded to AITC was significantly reduced (Fig. 4D).

3.5. Borneol directly inhibited SADBE induced activation of TRPA1

SADBE elicited increased intracellular $[Ca^{2+}]_i$ responses in DRG neurons from both *WT* (Fig. 5A and B) and *Trpv1*$^{-/-}$ mice (Fig. 5C and D). Upon administration of borneol (1.0 mM), induced a sustained inhibition of the $[Ca^{2+}]_i$ response (Fig. 5A). In contrast, menthol induced a transitory reduction and delayed the SADBE induced $[Ca^{2+}]_i$ responses, but did not inhibit them (Fig. 5B). Furthermore, administration of borneol at the 0.5 or 1.0 mM, completely abolished the SADBE induced $[Ca^{2+}]_i$ response in DRG neurons of *Trpv1*$^{-/-}$ mice (Fig. 5D). However, menthol (1.0 mM) did not show similar effects on the SADBE induced $[Ca^{2+}]_i$ response (Fig. 5C).

3.6. Borneol induced itch relief is dependent on both TRPM8 and TRPA1

Previous studies have demonstrated that TRPA1 is an essential part in the signaling pathways for both acute and chronic itch (Feng et al., 2017; Moore et al., 2018; Wilson et al., 2011; Xie and Hu, 2018). Consistent with this, we found that *Trpa1*$^{-/-}$ mice have significantly fewer scratches in acute and chronic models than that in *WT* controls (Fig. 6A). Since it is still uncertain whether TRPA1 is the target molecule of borneol (Takaishi et al., 2014; Wang et al., 2017), we evaluated the scratching phenotype of *Trpa1*$^{-/-}$ mice after borneol treatment. Except for acute itch behavior caused by chloroquine and GRP, the scratching behavior of *Trpa1*$^{-/-}$ mice was not significantly attenuated by borneol in all other models as tested. The above results indicate that TRPA1 is required for the antipruritic effect of borneol, but other molecular mechanisms may also be involved.

TRPM8 has been shown as a target of borneol to relieve pain, which even exhibits advantages over menthol in mice (Wang et al., 2017). To assess the role of TRPM8 in itch signaling, we examined the scratching behavior of *Trpm8*$^{-/-}$ mice in acute and chronic itch models. Unlike that in *WT* mice, scratching behavior in *Trpm8*$^{-/-}$ mice was not attenuated after injection borneol (except for that of GRP-induced acute itch) (Fig. 6B), indicating that borneol treatment of itching depends on TRPM8.

To determine whether there is a TRPM8 and TRPA1 synergistic effect and other mechanisms mediate the borneol effect, we then pretreated *Trpa1*$^{-/-}$ mice with a specific TRPM8 antagonist RQ-00203078 (RQ, 1.0 mg/0.1% DMSO) 1 h before borneol administration. RQ pretreatment had no effect on borneol in SADBE and IMQ-induced itching in *Trpa1*$^{-/-}$ mice (Supplementary Figs. S3A and B), showing that the role of borneol in these two models mainly depends on TRPA1. Interestingly, the remission induced by borneol's itching in *Trpa1*$^{-/-}$/*Trpm8*$^{-/-}$ mice was reversed in the chloroquine model but not in the GRP model (Fig. 6C). The above evidence indicates that TRPM8 and TRPA1 are required for borneol induced itch relief in the chloroquine model, but TRPA1 and other molecules are required in GRP induced itch as well as for borneol induced itch relief in this model.

3.7. Borneol-induced itch relief and clinically showed similar effects as menthol

To verify the antipruritic effect of topical borneol on humans, we

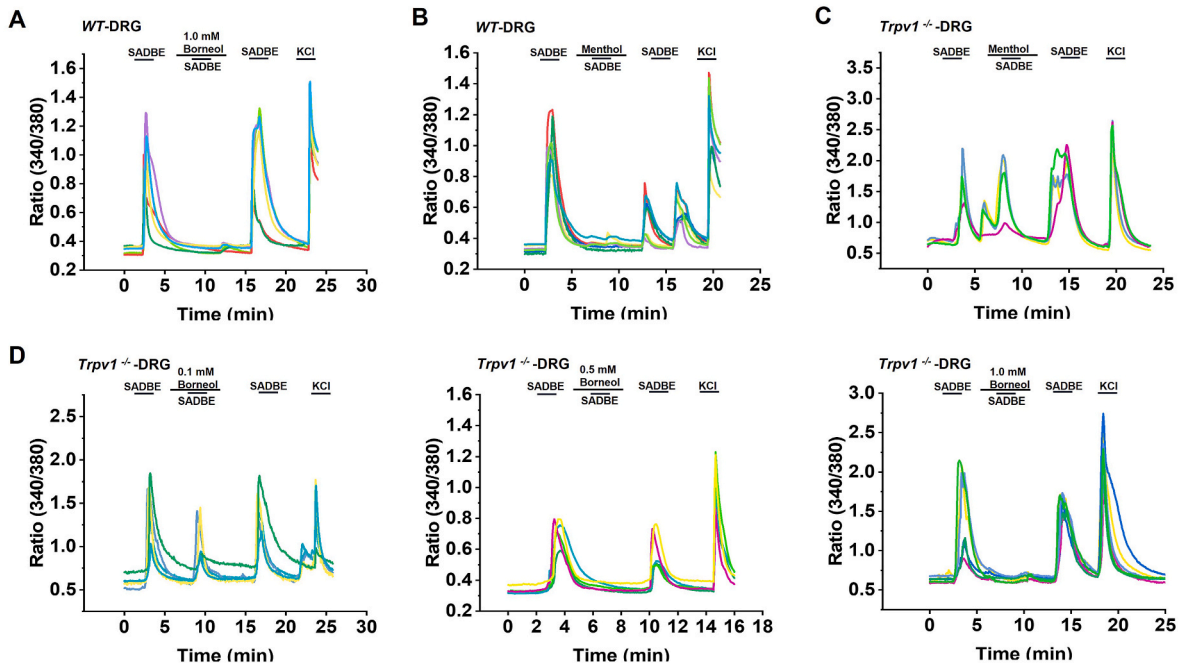

Fig. 5. The effects of borneol on SADBE induced $[Ca^{2+}]_i$ responses in both *WT* and *Trpv1*$^{-/-}$ mice. (**A**) Representative time-lapse traces illustrated SADBE-evoked $[Ca^{2+}]_i$ responses were inhibited by borneol (1.0 mM) in DRG neurons of *WT* mice. Menthol (1.0 mM) showed little effect on SADBE-evoked $[Ca^{2+}]_i$ responses in DRG neurons from both *WT* (**B**) and *Trpv1*$^{-/-}$ (**C**) mice. (**D**) The inhibitory effects of borneol at the 0.1, 0.5 and 1.0 mM on SADBE-evoked $[Ca^{2+}]_i$ responses in DRG neurons of *Trpv1*$^{-/-}$ mice.

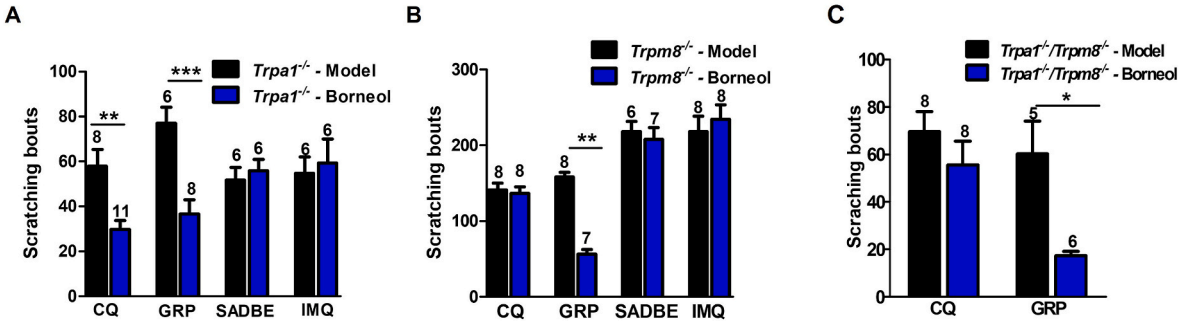

Fig. 6. Effects of borneol on acute and chronic itch models in *Trpa1*$^{-/-}$, *Trpm8*$^{-/-}$ and *Trpa1*$^{-/-}$/*Trpm8*$^{-/-}$mice. (**A**–**B**) The effects of borneol on the acute itch behavior caused by CQ and GRP, and chronic itch behavior by SADBE and IMQ in *Trpa1*$^{-/-}$ mice (**A**) or *Trpm8*$^{-/-}$ mice (**B**). (**C**) The effects of borneol on CQ or GRP-induced acute itch behavior in *Trpa1*$^{-/-}$/*Trpm8*$^{-/-}$ mice. All data are expressed as mean ± SEM. Statistical significance was evaluated using Two-tailed unpaired Student *t*-test for two-group comparisons, n = 6–11 mice. $^{###}P < 0.001$, model versus blank; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ borneol or menthol versus model.

firstly conducted a randomized, double-blind clinical study. Demographics and baseline information for patients participating in the clinical study were presented in Fig. 7A. After treatment of borneol or menthol for 7 days, the patients in the borneol group were significantly reduced in dermatology life quality index (DLQI) and pruritus visual analogue scale (VAS) score ($P < 0.001$, Fig. 7B). Furthermore, among these patients, 66.67% of patients' DLQI and pruritus VAS scores were reduced. A similar trend was observed in the menthol group (67.30%). In addition, the sleep quality VAS score had a greater improvement than that of before the treatment of borneol ($P < 0.001$, Fig. 7B). The results provided the first clinical evidence that borneol is able to reduce itch with an efficiency similar to menthol.

4. Discussion

Borneol is a traditional Chinese medicine that has long been used as a messenger medicine, which can effectively promote the drug permeability through corneal, intestinal mucosa, and nasal cavity mucosa, and even the blood–brain barrier for a greater access of drug to the brain (Zheng et al., 2018). Moreover, animal, and preclinical studies have shown that borneol has vasorelaxant, anti-inflammatory neuroprotective, and the analgesic effects, as well as relieving acute itching (Tian et al., 2023; Wang et al., 2017; Zhou et al., 2016). However, whether borneol is effective in treating itch and its mechanism of action remain unclear.

Through the DrugBAN framework, we obtained 32 targets including TRPA1, TRPM8, and TRPV3 as potential targets of borneol. Investigating the interaction between chemical compounds and proteins and predicting the targets through AI-based methodologies has become a viable endeavor (Shen C et al., 2020; Dhakal A et al., 2022). The DrugBAN framework, as a deep bilinear attention network (BAN) framework, is based on the chemical structure of drug and the protein sequences and utilizes conditional domain adversarial learning to align learned interaction representations across distinct data distributions, thus investigating the compound-protein associations. Previous studies have reported that borneol could act on TRPA1, TRPM8, and TRPV3 channels

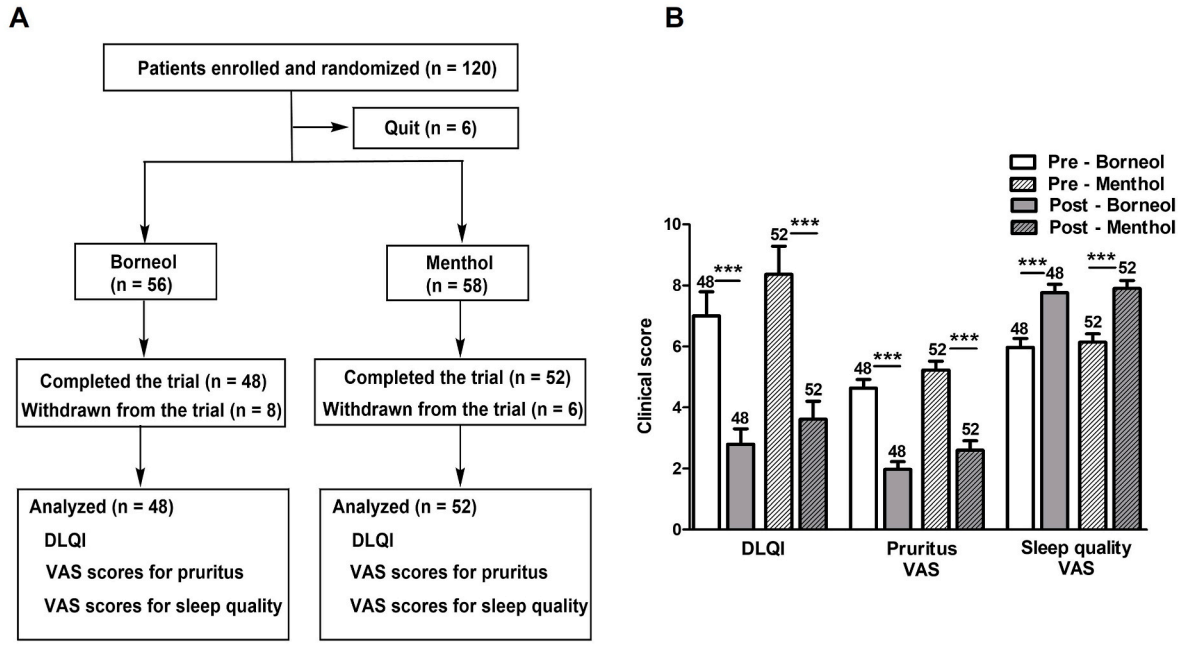

Fig. 7. Borneol relieves pruritus in humans. **(A)** Patient flow through the clinical study. Eligible participants are 120 patients with itching symptoms between the ages 18–70 years, and have skin diseases including skin eczema, atopic dermatitis and urticaria, etc. confirmed by physicians. They were enrolled in either the borneol group or the menthol group by computer randomization. A total of 114 patients completed the study and were included in the analysis. Patients in both groups received 4% borneol solution or 4% menthol solution three times a day for one week, respectively. 48 patients in borneol group and 52 patients in menthol group provided completed reports, including DLQI and VAS pruritus, sleep quality, and follow-up. **(B)** Borneol induced itch relief in patients. n = 48 for borneol treatment, and n = 52 for menthol treatment. All data are expressed as mean ± SEM. Statistical significance was evaluated using Two-tailed unpaired Student *t*-test for two-group comparisons. ***$P < 0.001$, post versus pretreatment.

(Mahmoud et al., 2022; Tian et al., 2023; Wang et al., 2017), which are receptors related to itch (Xie and Hu, 2018). Molecular docking results further supported the effective binding between borneol and TRPA1, TRPM8, or TRPV3 (Fig. 1D–F). In addition, the results of calcium imaging exhibited that borneol could active but not inhibit TRPV3 (Fig. 2E). However, TRPV3 activation helps induce itch rather than relieve it (Yamamoto-Kasai et al., 2012). Thus, TRPV3 is the target of borneol, but not contribute to borneol-induced itch relief.

TRPA1 is expressed in peripheral and spinal terminals of sensory neurons and plays a pivotal role in the process of itch or pain (Dong and Dong, 2018; Sun and Dong, 2016; Xie and Hu, 2018). Borneol has been reported that could directly inhibit hTRPA1 *in vitro* (Takaishi et al., 2014). However, it is still equivocal that TRPA1 is the target molecule of borneol in *vivo* (Chen et al., 2016; Tian et al., 2023; Zheng et al., 2018; Zhou et al., 2016). In the current study, our results showed borneol inhibits AITC induced $[Ca^{2+}]_i$ responses in TRPA1-transfected HEK293T cells (Fig. 2A) and subsets of DRG cells (Fig. 4D), as well as the membrane current (Supplementary Fig. S1A) in TRPA1-transfected HEK293T cells. Moreover, a previous study has demonstrated that SADBE could active both of TRPA1 and TRPV1(Feng et al., 2017). Our results showed that borneol could abolish the SADBE elicited $[Ca^{2+}]_i$ responses in DRG neurons of *C57BL/6J* mice. Borneol at the 0.5 mM and 1.0 mM exhibited a satisfactory inhibitory activity on SADBE-induced Ca^{2+} influx in DRG neurons of *Trpv1*$^{-/-}$ mice (Fig. 5D). An increasing number of studies are currently using mouse models of chronic itch induced by SADBE or IMQ. Studies have shown that IMQ could directly activate TRPA1 to evoke pruritic behavior in mice (Esancy et al., 2018). TRPA1 plays a key role in psoriasis, which raises its potential as a target for therapeutic intervention. TRPA1 is also required for generating the SADBE-induced persistent itch (Feng et al., 2017). A previous study reported the antipruritic effect of borneol on CQ-induced acute itch (Tian et al., 2023). In the current study, our results demonstrated that borneol significantly decreased the scratching bouts in both SADBE- and IMQ-induced chronic itch (Fig. 3B). Although previous studies have shown that TRPV3, TRPA1 and TRPM8 have been identified as the molecular targets of borneol, our results showed that the beneficial effects of borneol on SADBE or IMQ induced itch were absent in *Trpa1*$^{-/-}$ mice (Figs. 3B and 6A), suggesting that the antipruritic activity of borneol depends on TRPA1.

TRPM8, a cold-activated ion channel, is essential for cooling to relieve itch and has a potential clinical value for itch treatment (Liu et al., 2020; Palkar et al., 2018). Empirical observations and human trials support cooling of the skin or cold-induced counter-irritation may attenuate itch in patients or healthy volunteers (Andersen et al., 2017; Mochizuki et al., 2014; Xie and Hu, 2018). Especially cooling the skin, or using TRPM8 activators, like menthol, icillin have also been shown to inhibit different acute or chronic itch in animal models (Sanders et al., 2018; Xie and Hu, 2018). Then how itch signaling triggered upon acute induction or chronic dysregulation is inhibited by TRPM8 activation? Recently, Palkar et al. reported that cooling and menthol can inhibit both histaminergic and nonhistaminergic itch (Palkar et al., 2018). Previously it was proposed that either through spinal inhibitory interneurons or directly to nociceptors for cold- and menthol-gated TRPM8 channel to induce cooling analgesia (Knowlton et al., 2013), therefore identification of what an exactly specific neural circuit for borneol- and menthol-gated TRPM8 inhibiting itch would be a logical next step to investigate. Besides, the effects of borneol on the CQ-induced itch was also disappeared in *Trpm8*$^{-/-}$/*Trpa1*$^{-/-}$ mice (Figs. 3C and 6C). These results suggest that borneol targets both TRPA1 and TRPM8 to exert its antipruritic activity.

GRP is a key neuropeptide for transmitting itch information from DRG neurons to the spinal cord and key supraspinal regions (Dong and Dong, 2018; Liu et al., 2020; Pagani et al., 2019). The spinal itch requires sustained repetitive activity of presynaptic GRP neurons and postsynaptic GRP signaling to drive GRPR neuron output (Liu et al., 2020; Pagani et al., 2019). Previous research indicated that borneol induced analgesic effect interfering a central mechanism involving the mGluRs, of which may co-express GRPR in a subpopulation of spinal

excitatory interneurons (Pagani et al., 2019; Wang et al., 2017). In the current study, we demonstrated that borneol ameliorated the spinally administrated GRP-induced acute itch in all *WT*, *Trpa1*$^{-/-}$, and *Trpm8*$^{-/-}$ mice. In addition, borneol was able to further induce relief in the GRP model of *Trpa1*$^{-/-}$/*Trpm8*$^{-/-}$ mice (Fig. 6C), which suggesting at least a portion of GRP-induced itch by borneol may be independent of TRPA1 and TRPM8. As mentioned above, borneol has a unique pharmacological property of simultaneously stimulating TRPM8 and inhibiting TRPA1 and has promising blood–brain barrier permeability. Further research on how to develop suitable borneol and its combined application with other drugs, such as GRPR inhibitors, will be a very meaningful work.

Furthermore, we established a randomized, double-blind, clinical study that demonstrated application of 4% borneol solution three times a day for one week produced a significantly greater decrease in DLQI and pruritus VAS score, and a greater improvement of the sleep quality VAS score (Fig. 7B). Borneol showed a similar trend to that observed in the menthol group (Fig. 7B). To the best of our knowledge, our results should be the first to report that borneol can effectively relieve the chronic itch in both animals and human beings, and it exhibits similar effects to menthol.

5. Conclusions

The current study provides the first clinical evidence that borneol can attenuate itching symptoms in patients with similar efficacy to menthol. Furthermore, borneol could significantly reduce acute and chronic itch behavior in *WT* mice, but this effect was eliminated in *Trpa1*$^{-/-}$, *Trpm8*$^{-/-}$ mice, or at least in *Trpa1*$^{-/-}$/*Trpm8*$^{-/-}$ mice. Also, calcium imaging and patch-clamp recording analysis revealed that borneol elicits TRPM8 channel induced $[Ca^{2+}]_i$ responses but inhibits AITC or SADBE-induced TRPA1 activation in transfected HEK293T cells or dorsal root ganglia neurons, respectively. Taken together, our results suggested that borneol has therapeutic effects on multiple pruritus models in mice and patients, and the mechanism may be through inhibition of TRPA1 and activation of TRPM8.

CRediT authorship contribution statement

Miao Luo: Data curation, Methodology, Software, Writing – original draft, Writing – review & editing. **Jinfeng He:** Data curation, Methodology, Software, Writing – original draft, Writing – review & editing. **Liang Yin:** Methodology, Software. **Ping Zhan:** Methodology, Software. **Zhongqiu Zhao:** Writing – review & editing. **Hui Xiong:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Software, Writing – original draft, Writing – review & editing. **Zhinan Mei:** Conceptualization, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2023.117581.

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Supplementary Material

Table S1. The confidence scores of predictive targets

Score	Gene	Score	Gene	Score	Gene
0.988321364	GRIK1	0.906440973	AOC1	0.860509217	ABAT
0.982382894	TRPA1	0.905816972	SCN9A	0.854410589	HTR3A
0.966790497	ABCB1	0.89713192	15-LOX	0.853663743	UGT2B7
0.959157884	ITGB2	0.896860421	SELP	0.850843847	ClC-1
0.950878441	ANO	0.892071068	CYP1B1	0.846879482	TBXA2R
0.950047076	CACNA1G	0.892053127	Bact glmS	0.843780696	MAO-B
0.949767411	mGluR5	0.89165014	MPIP2	0.842129171	RAPGEF3
0.947646141	OGA	0.88947469	PDE8	0.840447307	GAA
0.943918645	mGluR1	0.889051676	LCT	0.838784873	SLC5A2
0.94335413	L3MBTL3	0.888335407	HTR5A	0.838784873	SGLT2
0.942702293	IGF2R	0.887615085	PCSK4	0.836641788	SLC15A1
0.935581386	MAN2A1	0.886751711	CACNA2D1	0.832315624	SLCO1B3
0.933417618	GRIA1	0.882472694	CYP2B6	0.831515789	H2R
0.930744052	GRIA4	0.880970001	ALDH5A1	0.821484923	GOAT
0.930286586	mGluR4	0.880954862	UGT1A1	0.821180642	KDM2A
0.930174649	PNLIP	0.880455077	ADRB3	0.819951475	GSTP1
0.929842472	GluN2C	0.880421281	MANA	0.816799521	CTSF
0.924964726	Trypano TRS	0.878059566	GABBR	0.816748619	LIPA
0.921314359	TRPV3	0.875807822	ENPEP	0.815272272	CBX7
0.920362592	CFTR del F508	0.872775733	HSV UL30	0.813055456	PLCB1
0.920362592	CFTR	0.872222424	GluK4	0.812972009	PDE4D
0.918232739	MAO-A	0.871759355	DPYD	0.812410295	ITGA2
0.917341352	CHRNA4	0.870608866	HPTP	0.812184095	GABRA3
0.917341352	CHRNA4/B2	0.870161891	CACNA1C	0.807244062	CHRNA2/A3
0.916699994	TRPM8	0.869282842	KIF11	0.807244062	CHRNA2
0.912807345	USP20	0.868180931	PRKCA	0.803800523	MGAM
0.912125945	GLRA1	0.865227044	JMJD1C	0.802310109	VZV ORF28
0.912125945	GlyR	0.863158107	PCSK1		
0.911659241	SPT ATPase	0.862120807	SCN1A		
0.911659241	ATP1A1	0.861671746	GRIA3		

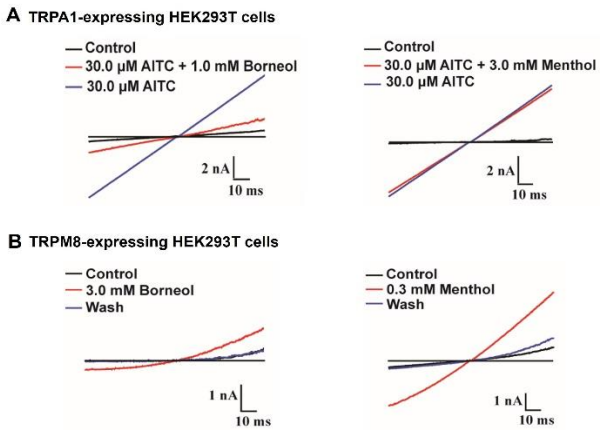


Fig. S1. The effects of borneol or menthol on whole-cell currents in TRPA1 (**A**) and TRPM8 (**B**) transfected HEK293T cells.

(A) Borneol significantly inhibited the membrane current activated by subsequent application of AITC in TRPA1-expressing HEK293 cells, but menthol (3.0 mM) failed to eliminate the AITC induced response. **(B)**Menthol (0.3 mM) induced a large rectification in TRPM8 transfected HEK293T cells, while application of 3.0 mM borneol displayed a weak response.

Whole-cell patch-clamp recordings were performed using an EPC 9 amplifier (HEKA Elektronik, Germany) at room temperature (22–24°C) on a platform equipped with an inverting microscope with a filter set for green fluorescent protein visualization. Pipettes pulled from borosilicate glass (BF 150-86-10; Sutter Instrument Company, Novato, CA) equipped with a Sutter P-97 pipette had resistances of 2–4 MΩ when filled solution containing 140 mM CsCl, 2 mM EGTA, and 10 mM HEPES with pH 7.3 and 315 mOsm·L^{-1}. Ca^{2+} -free extracellular solution for whole-cell recording contains (in mM): 140 NaCl, 5 KCl, 0.5 EGTA, 1 $MgCl_2$, 10 glucose and 10 HEPES (pH was adjusted to 7.4 with NaOH, and the osmolarity was adjusted to ≈340 mOsm·L^{-1} with sucrose). At holding potential of 0 mV, a whole-cell membrane current was recorded using voltage ramp from -100 to +100 mV for 500 ms. Data were acquired using the PatchMaster software (HEKA Elektronik). Currents were filtered at 2 kHz and digitized at 10 kHz (*Sensory TRP channels contribute differentially to skin inflammation and persistent itch. Nat Commun 2017, 8, 980.*).

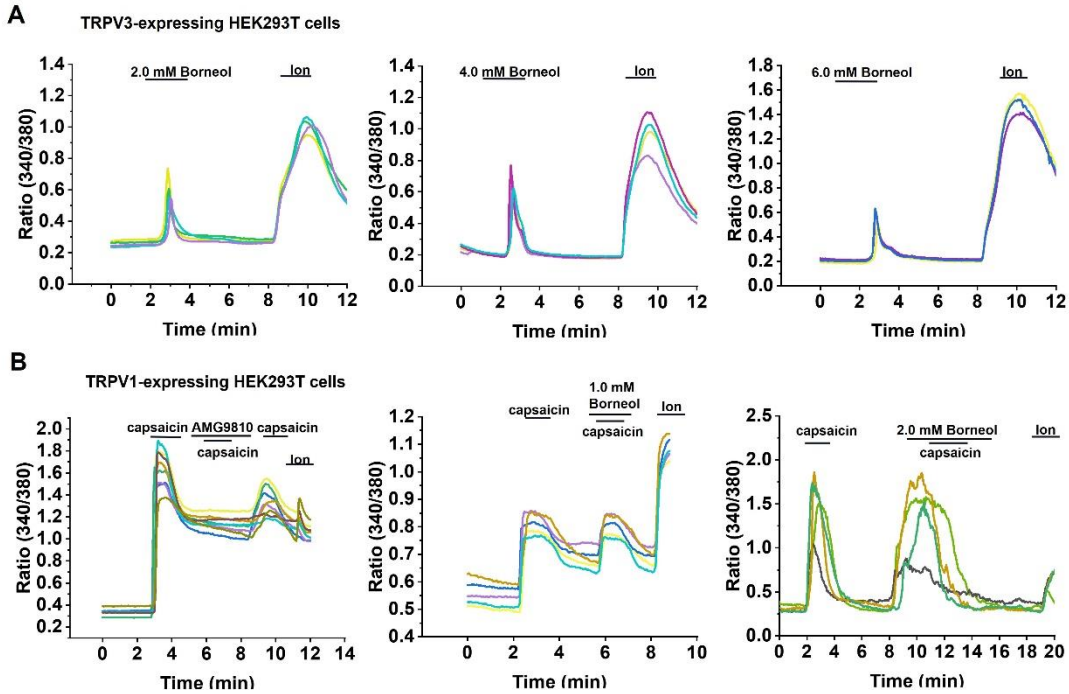
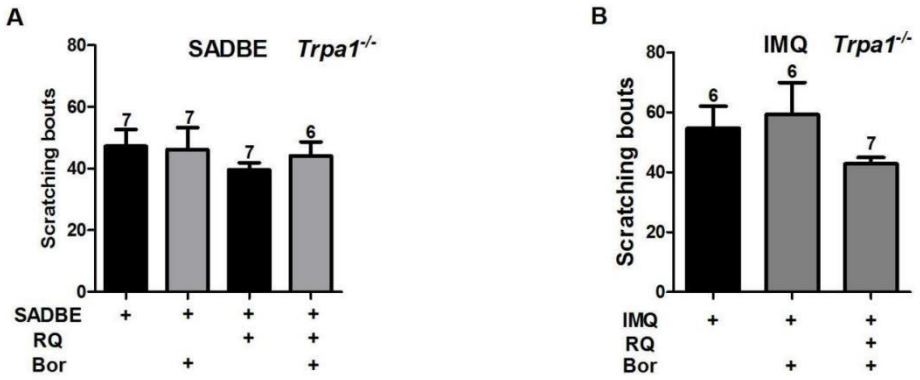


Fig. S2. The effects of borneol on TRPV3 or TRPV1

(**A**) Representative time-lapse traces illustrated borneol (2.0, 4.0 and 6.0 mM) elicited $[Ca^{2+}]_i$ responses in TRPV3 transfected HEK293T cells. (**B**) Representative time-lapse traces illustrated borneol (1.0 and 2.0 mM) could not inhibit the capsaicin-elicited $[Ca^{2+}]_i$ responses in TRPV1 transfected HEK293T cells.



Supplementary Fig. S3. The effects of borneol on SADBE (**A**) or IMQ (**B**)-induced scratching behavior in *Trpa1*$^{-/-}$ mice

The effects of borneol on scratching behavior in SADBE (**A**) or IMQ (**B**) treated *Trpa1*$^{-/-}$ mice in the absence or presence of the TRPM8 inhibitor RQ-00203078 (1.0 mg/kg).

All data are expressed as mean ± SEM, n = 6 - 7 mice for each group. Statistical significance was evaluated using one-way analysis of variance (ANOVA) followed by Tukey's test for multi - group comparisons. Bor, borneol; RQ, RQ-0020307