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Cimifugin relieves pruritus in psoriasis by inhibiting TRPV4

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ABSTRACT

Psoriasis is an immune-mediated chronic inflammatory skin disease characterized by erythema, scales, and infiltration of the skin, which causes deleterious effects on patient quality of life. TRP channel played important roles in the generation and conductance of itch signal . According to our results, psoriasis induced itch was TRPV4 dependent, and TRPV4 expression in both epidermis and DRG were up-regulated in psoriasis. Thus, TRPV4 is an attractive candidate for treating psoriasis induced itch. Cimifugin is a common compound in antipruritic Chinese medicine. In our study, GSK1016790A, a TRPV4 channel specific agonist, induced acute itch was inhibited by cimifugin in a dose-dependent manner. Furthermore, cimifugin treatment reduced the scratching behavior and reversed the TRPV4 up-regulation induced by psoriasis. In particular, cimifugin decreased GSK1016790A induced calcium response both in HaCaT cells and DRG neurons. Importantly, in TRPV4 transfected HEK293 cells, GSK101 induced calcium response was also significantly inhibited by cimifugin pretreatment. Consistent with our calcium imaging result, cimifugin pretreatment also inhibited GSK101 induced inward currents. Our study delineated a new role of TRPV4 in psoriasis and emphasized the antipruritic effect of cimifugin, which opened a new avenue to itch management in psoriasis.

1. Introduction

Psoriasis is an immune-mediated chronic inflammatory skin disease characterized by erythema, scales, and lymphocytic infiltration of the skin that affects more than 125 million people worldwide [1, 2]. Because of the limitations of various treatments, patients with psoriasis still suffer from the itch of the disease [3, 4]. Psoriasis patients have many clinical symptoms including pruritus. It is reported that about 60% ~90% of patients with psoriasis have clinical manifestations of pruritus, and this kind of itch caused by sensitizing signaling pathways [5]. Compared with other chronic pruritus, psoriatic pruritus is more difficult to treat, and it is easy to relapse [6]. Therefore, it is the direction of many clinicians and basic researchers to seek effective drug for treatment of psoriasis pruritus.

Antihistamine drugs, such as loratadine, cetirizine, etc, are commonly used for the treatment of psoriasis, but most patients are not satisfied with the treatment effect [5]. Thalidomide can relieve the itching of psoriasis and improve the quality of life of patients, but it has strong side effects [7]. Corticosteroids, menthol, anesthetics and some immunosuppressive agents have a faster antipruritic effect, but the effect is weak, the duration is short, and is easy to relapse [8, 9]. Some biological agents, such as etanercept, afacet and infliximab, have obvious therapeutic effect on psoriasis pruritus, especially severe pruritus, but there are still some problems, such as quicker but weaker antipruritic effect and short duration, so that it is easy to relapse and cannot be popularized in clinical [10]. In addition, phototherapy such as ultraviolet light, percutaneous electronic nerve therapy, traditional Chinese medicine bath therapy and external moisturizing emollient can

Abbreviations: trp, transient receptor potential; Trpv4, transient receptor potential cation channel subfamily v member 4; Trpv1, transient receptor potential cation channel subfamily v member 1; Trpa1, transient receptor potential cation channel subfamily a member 1; DRG, Dorsal Root Ganglion; TG, Trigeminal Ganglia; TSLP, Thymic Stromal Lymphopoietin; IL-13, Interleukin 13; AD, Atopic Dermatitis.

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alleviate the itching symptoms of psoriasis, which is an effective choice for the treatment of psoriasis itching [11]. Based on the plight of psoriasis pruritus treatment, the pathogenesis of psoriasis pruritus and the study of safe and effective antipruritic drugs may become an ideal strategy for the treatment of psoriasis pruritus.

Transient Receptor Potential (TRP) channels, which mediate cation influx to many chemical or physical stimuli (temperature, mechanical pressure), are expressed in different cell types in skin and nervous system, such as keratinocytes and dorsal root ganglion (DRG) neurons [12]. There are many documents implicating that TRP channel dysfunction under pathological skin conditions such as chronic pain and itch, dermatitis, and skin barrier damage [13]. Of note, as a member of TRPs family, TRPV4 is highly expressed in DRG, trigeminal ganglia (TG) as well as keratinocytes, and plays an important role in epidermal barrier homeostasis, inflammation, and photosensitivity dermatitis [14, 15]. It has been reported that histamine, compound 48/80, serotonin, and ET-1-induced pruritus are significantly decreased in TRPV4 KO mice [16]. TRPV4 is also involved in a variety of chronic itch [17-19]. Previous studies have shown that TRPV4 gene expression is increased in DRG of the imiquimod-induced psoriasis model, and *TRPV4* mRNA is firstly up-regulated then slowly down-regulated in psoriasis skin [20, 21]. Thus, whether TRPV4 involved in psoriasis is still unclear. Our results showed that the strength of $TRPV4^{+}$ fluorescence is well correlated with the skin damage score of psoriasis patients, and the expression of TRPV4 is up-regulated in both skin and DRG of psoriasis mice model. Further study showed that psoriasis model induced scratching behavior is significantly decreased in TRPV4 KO mice. Thus, TRPV4 acts an important participant in the formation of psoriasis pruritus and can be a key target for the treatment of psoriasis induced pruritus.

In Chinese medicine, *Saposhnikovia divaricate* and *Rhizoma cimicifugae* are often used to treat with pruritus [22]. As an important active component of them, cimifugin has obvious antiinflammatory effect [23], however its antipruritus effect and mechanism were unknown in psoriasis. The results of our study showed that cimifugin significantly decreased the scratching behavior of psoriasis mice model by inhibiting the activity of TRPV4 channel.

2. Materials and methods

2.1. Animals

C57BL/6 male mice (8–10 weeks) and $TRPV4^{-/-}$ male mice (C57BL/6 background) (8–10 weeks) were used for behavioral testing. Mice were placed in a temperature controlled animal room (22 ± 2 °C) and food and water were freely obtained under a 12-hour light/dark cycle. $TRPV4^{-/-}$ mice were generated as previous description [17, 24].

Animal experiments were conducted in accordance with the relevant guidelines and regulations of the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine (Ethics licence ACU190601, 20,190,605). All protocols were approved by the International Association for the Study of Pain, and every effort was made to minimize the number and suffering of the included animals, including providing standard living condition and no additional damage appearing during the study.

2.2. The skin of psoriasis patients

Psoriasis patients' skin was provided by the Department of Dermatology, the First Affiliated Hospital of Nanjing Medical University, and this study was approved by the hospital ethics committee.

2.3. Drugs

GSK1016790A (G0798), GSK2193874 (SML0942), serotonin (H9523), histamine (H7250), chloroquine (C6628), capsaicin (12,084) and mustard oil (MO) (476,013, Sigma, USA). Cimifugin (B21156, Yuanye, China) and 5% imiquimod Cream (H20030128, Mingxin, China). All drugs were dissolved in DMSO (34,869, Beijing Soleibao, China). When the drug was used in behavioral experiments, the drug was diluted in saline, the final concentration of DMSO do not exceed 1%. When the drug was used in the calcium imaging experiment, the drug was diluted in calcium imaging buffer, the final concentration of DMSO do not exceed 0.1%.

2.4. Establishment of psoriasis model

Three days before the modeling, the mouse was shaved (3 × × 3 cm) to expose the skin of the neck, and then 62.5 mg of aldara cream (5% imiquimod) was applied to the shaved skin (2× 2 cm) for 5 consecutive days [20]. All the experimental procedures were conducted on imiquimod mice by an investigator who was unaware of the experimental treatment.

2.5. Behavioral tests

Animals were acclimated to the testing environment for 30 min before the initiation of behavior tests. For acute itch test, mice were pretreatment (30 min) with subcutaneous injection of cimifugin (1.625 mM, 4.875 mM, 8.125 mM, 25μL) before GSK101 (60 μM, 25μL) injection in the mouse nape. The behavior of mice was recorded for 30 min after GSK101 injection. For chronic itch test, mice were divided into 2 groups ($n = 5$ per group) randomly (control group and model group), the behavior of the mice was recorded for 60 min before treatment every day. $TRPV4^{-/-}$ mice ($n = 5$ per group) were applied to identify the function of TRPV4 in imiquimod induced psoriasis by compared with the itch behavior of *WT* mice ($n = 5$ per group). To study the effect of cimifugin on psoriasis, cimifugin (75 mg/kg, 100μL) was used to treat model group ($n = 5$ per group) by intragastric administration. A bout of scratching was defined as a continuous scratching movement with a hindpaw directed at the treated site or drug injection site. All behavioral experiments were performed with observers blinded, randomized, controlled to treatment.

2.6. Isolation of drg neuron

The mice were sacrificed with isoflurane and decapitated. The back of the mice was exposed by cutting along the midline of the back with ophthalmic scissors, and the spine was separated. The cleaned spine is placed on ice for trimming. Under the stereoscopic dissecting microscope, remove the spinal cord, expose the DRG, carefully remove the body of the DRG with ophthalmic forceps, and cut the connected fibers (all the above operations are done on ice).

2.7. Real-Time PCR

The mice after 4 times treatment were dissected. Skin and DRG samples were isolated from mice and stored in TRIzol reagent (R401–01, Vazyme), then RNA was extracted from the tissue samples immediately. Reverse transcription was performed using HiScript II Q T SuperMix (R222–01, Vazyme). For qPCR, AceQ qPCR SYBR Green Master Mix (Without ROX) (Q711–02, Vazyme) was used. The reaction was run in a Light Cycler 480 II Real-Time PCR instrument (Roche,Basel, Switzerland) using 1 μl of the cDNA in a 20μl reaction according to the instructions of manufacture. The sequence of the TRPV4 primers were as follows: forward primer, TCCACCCTATATGAGTCCTCGG and reverse primer, TAGGTGCCGTAGTCAAACAGT. The sequences of GAPDH primers were as follows: forward primer: GGAGCGAGATCCCTCCAAAAT and reverse primer: GGCTGTTGTCATACTTCTCATGG. Calibrations and normalizations were performed using the following $2^{-\Delta\Delta CT}$ method, where ΔΔCT = (CT (target gene) – CT (reference gene)) - (CT (calibrator) – CT (reference gene)). GAPDH was used as the reference gene for qPCR experiments [25].

Table 1
The histopathological Baker grading of psoriasis patients.

Patient	1	2	3
Munro microabscess	0	2	2
Hyperkeratosis	0	0.5	0.5
Parakeratosis	0	0	1
Thickening of spinous layer	0.5	1	1.5
Skin extension and undulation	0.5	1	1.5
Inflammatory cellinfiltration	0.5	1	1.5
Telangiectasia	0	0.5	0.5
Grading	2	6	8

2.8. Immunostaining of skin and DRG

The isolated DRGs were fixed with 4% PFA at 4 °C for 30 min, and the skin was fixed with 4% PFA at 4 °C for 2 h, and then all tissues were placed in 20% sucrose solution for 24 h. Use OCT (optimal cutting temperature compound) embedding agent at −20 °C. Cryoembedded tissues were cut into 10 μm thick slices on a sliding microtome (CM1950; Leica). For immunostaining of transient receptor potential cation channel V4 (TRPV4), sections were incubated in blocking solution (containing 5% FBS, 0.1% Triton X-100, and 0.02% sodium azidein PBS) for 2 h at room temperature and then incubated with rabbit anti-TRPV4 (1:200, novusbio, NB110–74,960) at 4 °C overnight. Next, the sections were incubated in Alexa Fluor-conjugated goat anti-rabbit IgG secondary antibody (1:200, Beyotime, A0453) at room temperature for 1 h. Three mice from each group were analyzed. Image J software was applied to measure the positive signals of immunofluorescence.

2.9. Immunostaining of skin section of psoriasis patients

Psoriasis patients' skin was provided by the Department of Dermatology, the First Affiliated Hospital of Nanjing Medical University. Roasted slices, 60 °C, roasted slices for 1 h, and ewaxing in xylene I (1330–20–7) for 5 min, dewaxing in xylene II for 5 min, dewaxing in xylene III for 5 min; absolute ethanol I for 10 min, absolute ethanol II (10,009,257) for 10 min, 95% ethanol for 10 min, 95% Ethanol for 10 min, distilled water for 5 min, and distilled water for 5 min. Then put the slices into the citric acid repair solution to antigen repair, keep the temperature above 95 °C for 20 min (low heat in microwave oven, boiling water bath), take out, and naturally cool to room temperature. Removal of endogenous enzymes, to take the slides into a wet box, add 3% H_2O_2 (7722–84–1), leave it for 10 min, wash with PBS 3 times, 5 min each time. The next steps are the same as the above. Image J software was applied to measure the positive signals of immunofluorescence.

2.10. Western blot

Skin and DRG tissues of mice were taken out and ground into homogenates with 100 mg/ml protein lysis solution (Beyotime, P0013). The samples were collected in microcentrifuge tubes, and lysed for 30 min. Then the homogenates were centrifuged at 12,000 rpm at 4 °C for 15 min. Finally, the protein concentration of the tissue or cell sample is determined. Total protein extracts were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Germany). PVDF membranes were then blocked in TBST with 5% non-fat milk powder and 0.5% Albumin Bovine V at room temperature for 1 h. After blocking, the membranes were washed with TBST 3 times for 10 min. Then incubated with antibodies against TRPV4 (1:1000 dilution; AT24724, SCIBEN, China) at room temperature for 60 min and at 4 °C overnight. On the other day, the membranes were washed 3 times with TBST for 10 min. After washing, membranes were incubated at

Table 2
$TRPV4^+$ fluorescence intensity in skin of the patients with psoriasis.

Patient	1	2	3
Fluorescence intensity in total epidermis	13.357	28.599	69.470
Fluorescence intensity in per unit area	2.785	8.366	16.885

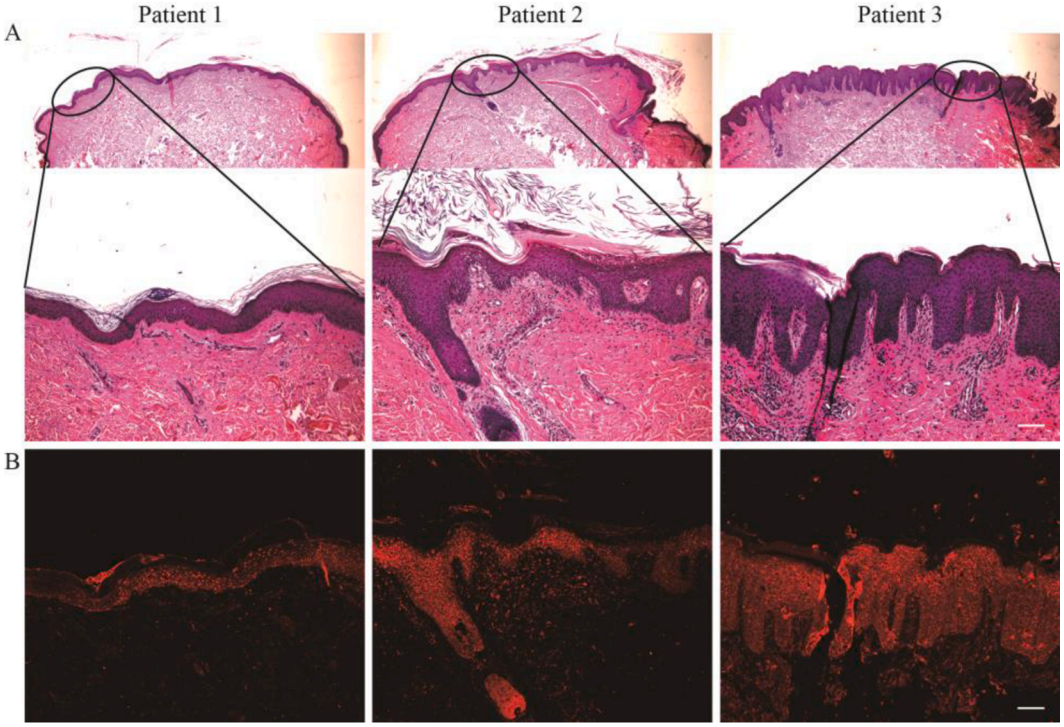

Fig. 1. HE staining and TRPV4 fluorescence detection of skin biopsies from patients with psoriasis. (A) HE chromatogram of skin biopsies from three patients with psoriasis. The picture in the below line is the enlargement of the above line. (B) TRPV4 fluorescence staining of patient skin tissues. The expression of TRPV4 increased with the severity of the psoriasis from left to right. Scale bar is 100 μm.

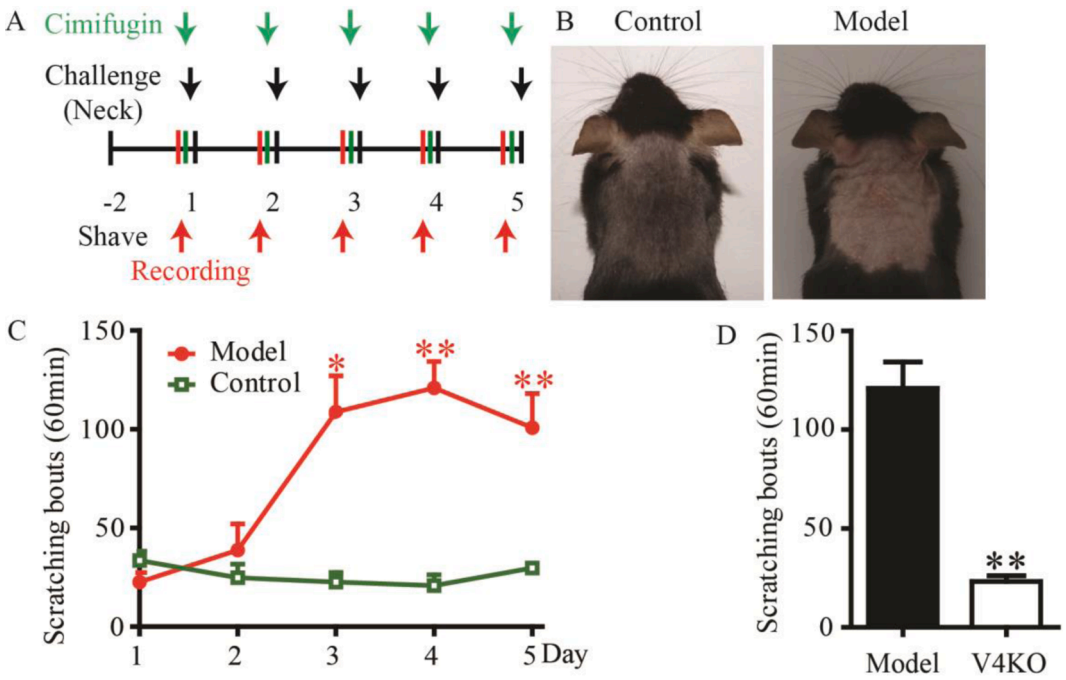

Fig. 2. TRPV4 is required for psoriasis-induced chronic itch. (A) Time schedule of imiquimod-induced mouse psoriasis model and cimifugin treatment. (B) Mouse skin performance before imiquimod treatment and treated with imiquimod 5 days later. (C) Compare to control group (mice treated with vehicle), the scratching behavior was significantly increased in psoriasis model mice (21 ± 5 (Control group) VS 121 ± 13 (Model group), day 4, $n = 5$). (D) *TRPV4*$^{-/-}$ mice decreased scratching behavior induced by psoriasis. * $P<0.05$, ** $P<0.01$.

room temperature with secondary peroxidase-linked goat anti rabbit IgG (1:2000 dilution; AB6721, Abcam, USA) for 1 h. After washing, Protein bands were visualized and the protein expressions were quantified [12].

2.11. Cell culture

HaCaT cells (immortalized human keratinocyte) and HEK293 cells were purchased from the Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China). HaCaT cells were cultured in MEM (A1049001, Hyclone, Thermo scientific, Waltham, USA) supplemented with 10% fetal bovine serum in an incubator (95% O_2 and 5% CO_2) at 37 °C. HEK293T cells were cultured in DMEM (AF29431640, Hyclone, Thermo scientific, Waltham, USA) supplemented with 10% fetal bovine serum in an incubator (95% O_2 and 5% CO_2) at 37 °C [12]. DRG neurons were cultured in DMEM F-12 (31,331,093, Hyclone, Thermo scientific, Waltham, USA) supplemented with 10% fetal bovine serum in an incubator (95% O_2 and 5% CO_2) at 37 °C [12].

2.12. TRPV4 plasmid transfection

HEK293 cells were transfected with cDNA for rat TRPV4 (rTRPV4) using Lipofectamine 2000 (2,004,958, Life Technologies, Carlsbad, USA) [25]. Media was changed 5 h after transfection, and after 24 h of culture, calcium imaging and whole cell patch clamp studies were performed.

2.13. Calcium imaging

HaCaT cells, HEK293 cells and DRG neurons were incubated with fura-2 AM (344,911, Sigma, USA) for 30 min at 37 °C in the dark. After washing twice with Ca^{2+}buffer (containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose (pH 7.3)), the glass coverslip was placed in the chamber and perfused with Ca^{2+}buffer [26]. A high-speed continuous scanning monochromatic light source was used to excite at 340 and 380 nm to detect changes in intracellular free calcium concentration.

2.14. Whole cell patch clamp

Whole-cell currents were record using Multiclamp 700 B and Digidata 1440 A (Molecular Devices, Inc., San Jose, USA), capacitance transients and series resistance were minimized by using the capacitance neutralization circuits on the amplifier. The currents were evoked by holding the membrane potential at −60 mV. The currents were digitized (sampled at a frequency of 10 kHz and filtered at 0.1 kHz for analysis), stored and subsequently analyzed by using Clampex 10.3 (Molecular Devices, Inc., San Jose, USA). Experiments were performed with a perfusion system, drugs were added with pipette directly to the recording chamber. The volume of chamber is fixed (100 μL). When a certain volume of drug is added, the terminal concentration of the drug can be calculated. All the experiments were performed at room temperature. The external solution contained 140 mM NaCl; 4 mM KCl; 2 mM $CaCl_2$; 2 mM $MgCl_2$•$6H_2O$; 10 mM HEPES; 5 mM glucose; pH 7.4 (adjusted by NaOH). Patch pipettes resistance of typically 4–6 MΩ. The pipette solution contained 135 mM KCl; 1.1 mM $CaCl_2$; 2 mM EGTA; 3 mM Mg^{2+}-ATP; 0.5 mM Na^{2+}-ATP; pH 7.4 (adjusted by KOH); Osmolality was adjusted to 300–310 mOsM [26]. Non-paired t-tests (two tails) were used for data analysis.

2.15. Homology modeling and molecular docking

The three dimensional structure of rTRPV4 was constructed by homology modeling in the software of Discovery Studio 4.1 using the template of *Xenopus Tropicalis* TRPV4 (PDB ID: 6BBJ) [27]. The sequence identity of rTRPV4 with xtTRPV4 is 77.4%. The best constructed model was optimized with a restrained minimization using OPLS3 force field in Maestro 10.3. The H-bonds was assigned using PROPKA at pH 7.0. Then the optimized structure was used for molecular docking.

The structures of cimifugin and the reported TRPV4 agonist GSK101 were built and prepared using OPLS3 force field in Maestro 10.3. Epik was used to generate possible states at target pH 7.0 ± 2.0. The ligand binding site of rTRPV4 was defined referring to the site of capsazepine

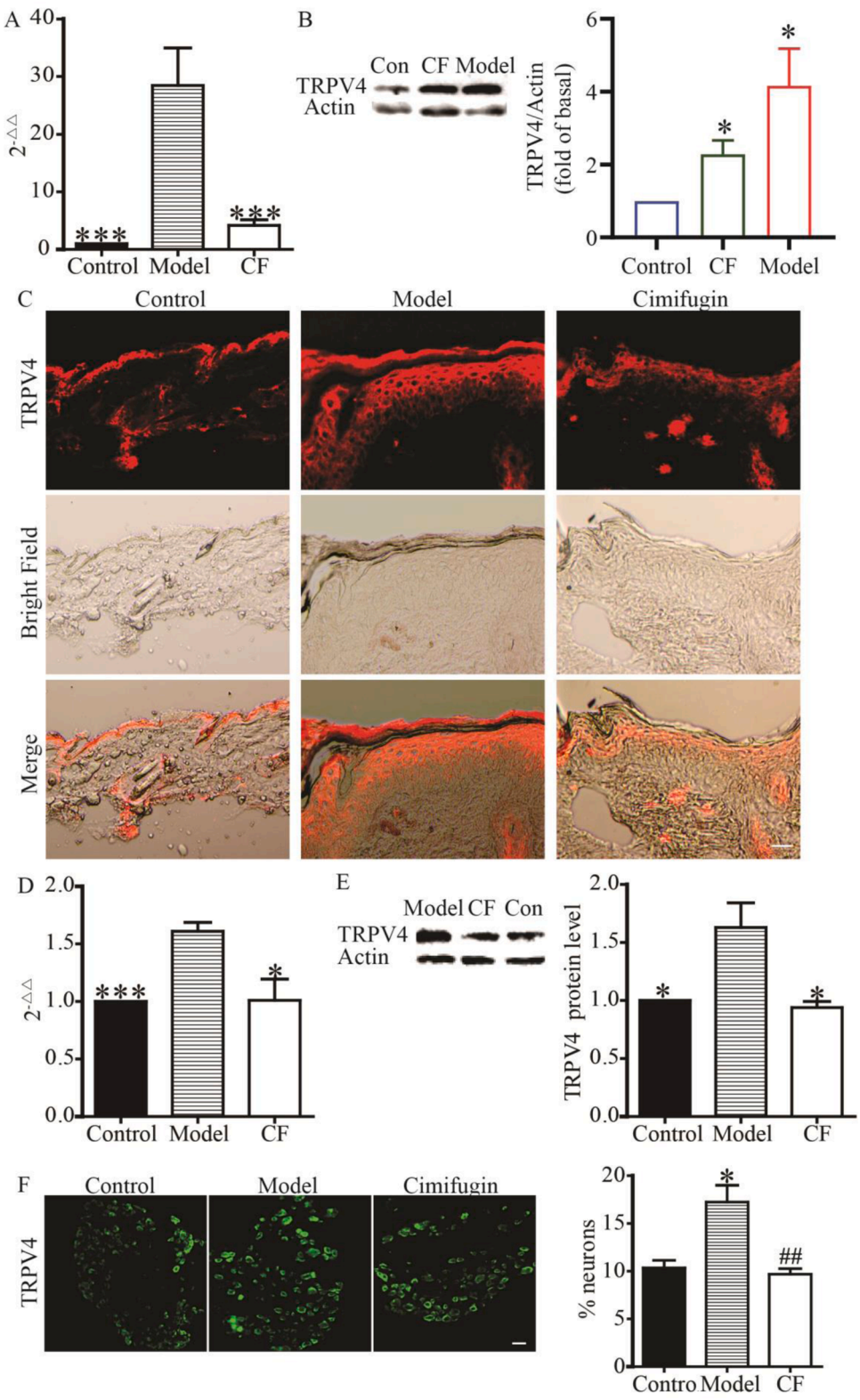

Fig. 3. Psoriasis increased TRPV4 expression in epidermis and DRG, cimifugin can inhibit psoriasis-induced TRPV4 expression increase. (A) *TRPV4* mRNA was up-regulated in the skin of psoriasis mice model and cimifugin decreased it. (B) Western blot assay reveled TRPV4 increased in psoriasis mice skin and cimifugin could down regulate the expression of TRPV4. (C) TRPV4^{+} cells in the skin of psoriasis model mice were increased and cimifigin treatment reversed it. (D) *TRPV4* mRNA was up-regulated in the DRG of psoriasis mice model and cimifugin decreased it. (E) Western blot assay reveled TRPV4 increased in psoriasis mice DRG and cimifugin could down regulate the expression of TRPV4 in psoriasis mice DRG. (F) TRPV4^{+} DRG neurons in psoriasis mice model was increased and cimifigin treatment reversed it (*, *** compared to Control, $^{\#\#}$ compared to Model). * $P<0.05$, *** $P<0.001$, $^{\#\#}$ $P<0.01$. All scale bars represent 50 μm.

Table 3
TRPV4^{+} fluorescence intensity in skin of the mice with psoriasis.

	Control	Model	Cimifugin
Fluorescence intensity in total epidermis	13.357	54.374	18.440
Fluorescence intensity in per unit area	6.259	18.538	6.347

binding with TRPV1 [28]. Then molecular docking was conducted in the Glide module of Maestro 10.3. The docking score of SP (standard precision) was used.

2.16. Data analyses

Statistical analyses were performed using GraphPad Prism 8.0

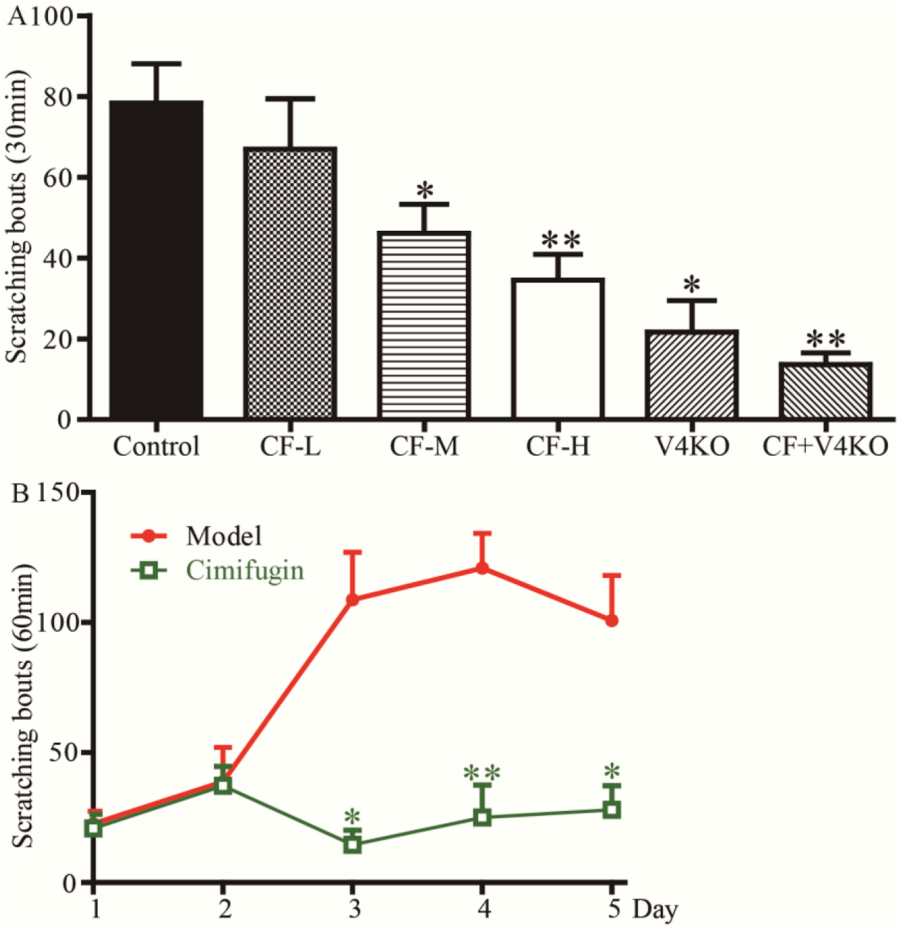

Fig. 4. Cimifugin alleviated TRPV4 mediated acute and chronic itch. (A) Cimifugin decreased scratching behavior induced by GSK101 in dose dependent (CF-L: 1.625 mM cimifugin pretreatment, CF-M: 4.875 mM cimifugin pretreatment, CF-H: 8.125 mM cimifugin pretreatment; $n = 6$, * compare with control group). (B) Cimifugin treatment alleviated the scratching behavior of psoriasis model mice ($n = 5$,* compare with model group). * $p<0.05$, ** $p<0.01$.

software. All data are presented as mean ± SEM for n independent observations. Student's *t*-test was used to analyze statistical significance between two groups. ANOVA and repeated measures tests were used to test hypotheses about effects in multiple groups occurring over time. $P < 0.05$ was considered significantly different.

3. Results

3.1. The expression level of TRPV4 in human skin is closely related to the severity of psoriasis

TRPV4 is an important molecule in the production of itch sensation and expressed in the skin, but the knowledge of TRPV4 in psoriasis pruritus is very limited. To prove that TRPV4 in the skin of psoriasis patients is an important factor of pruritus, we cooperated with the Dermatology Department of Jiangsu Provincial People's Hospital to obtain skin samples of patients with different degrees of psoriasis, and detected the expression of TRPV4 in the skin of these patients. Under the condition of complying with the hospital policy, the severity of the skin of patients with psoriasis was determined according to the method of histopathological classification (Baker grading) [29]. The skin condition of three patients with psoriasis was tested. The skin injury grades of the patients were 2, 6 and 8, respectively. They belong to the patients with mild, moderate and severe psoriasis (Table 1). Then, HE staining and immunofluorescence were used to detect the skin hyperplasia and the expression of TRPV4 in three patients. The results showed that the degree of epidermis hyperplasia was closely related to the severity of the disease, and the epidermis hyperplasia was the largest in the patients with the most serious psoriasis (Fig. 1A). The expression level of TRPV4 in the skin of three patients is also positively correlated with the severity of the psoriasis (Fig. 1B), the intensity of immunofluorescence was measures by Image J (Table 2).

3.2. Psoriasis induced itch was TRPV4 dependent

TRPV4 has been determined to be closely related to psoriasis. To further study the function of TRPV4 in psoriasis model, imiquimod was applied to the shaved back skin of the mice for 5 consecutive days to induce psoriasis model (Fig. 2A), the model caused obviously skin damage (Fig. 2B). As human psoriasis, mice psoriasis model also could induce pruritus. After 3 times imiquimod treatment, the scratching behavior of mice was significantly higher than that of the control group (Fig. 2C). Importantly, the scratching behavior was abolished in TRPV4 KO mice when compared to WT mice in our psoriasis model (23 ± 3, day 4, $n = 5$) (Fig. 2D).

3.3. Psoriasis increased TRPV4 expression in epidermis and DRG

The expression of TRPV4 in the skin of patients with psoriasis is closely related to the severity of the disease, we speculated that the expression of TRPV4 should also change in mice psoriasis model. Next, the expression of TRPV4 in the DRG from the model mice and the skin from the injured site was detected. The results indicated that TRPV4 increase was the most significant in skin (Fig. 3A) in model group. In addition, we also detected the expression of TRPV4 protein in the skin by immunohistochemistry and western blot. Correlating well with mRNA

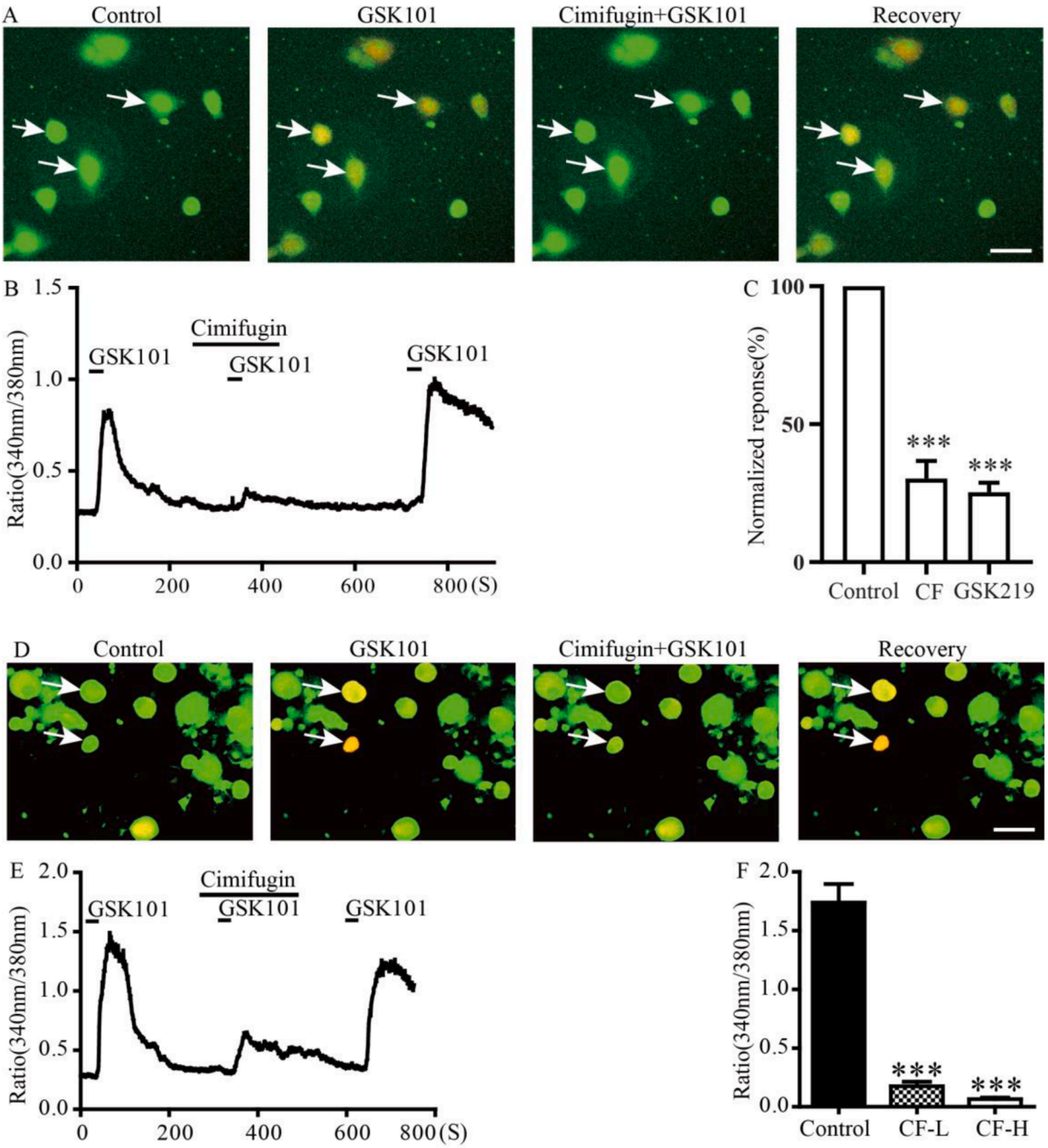

Fig. 5. Cimifugin inhibited TRPV4 mediated calcium influx in HaCaT cells and DRG neurons. (A) Cimifugin inhibits the GSK101-evoked calcium influx in HaCaT cells (cell number, $n > 1000$) (White arrow represents positive cells). (B) Time-response curve showed GSK101 induced obviously calcium influx in HaCaT cells and this calcium response can be inhibited by cimifugin pre-treatment. (C) Both cimifugin and GSK219 inhibited calcium influx caused by GSK101 in HaCaT cells (cell number, $n > 1000$; ** compare with control group). (D) Cimifugin inhibited the GSK101-evoked calcium influx in DRG (White arrow represents positive cells). (E) Time-response curve showed GSK101 induced calcium influx in DRG neurons and this calcium response can be inhibited by cimifugin pre-treatment. (F) Cimifugin inhibited calcium influx of DRG neurons caused by GSK101 in a concentration dependent manner (CF-L: 1.625 μM, CF-H: 4.874 μM; mice number, $n = 3$; *** compare with control group). ***$p<0.001$. All scale bars represent 50 μm.

expression result, western blot result confirmed that imiquimod treatment increased TRPV4 protein expression in the skin (Fig. 3B), and immunofluorescence response also revealed a significant increase of $TRPV4^+$ cells in skin (Fig. 3C, Table 3, Fig. S1). TRPV4 also expressed in DRG and involved in itch sensation transmission. Our results indicated that imiquimod treatment also increased TRPV4 expression in mRNA and protein level of DRG (Fig. 3D-F).

3.4. Cimifugin relieved TRPV4-mediated acute and chronic psoriasis itching

Cimifugin is an active ketone ingredient from many traditional antipruritic herbs, such as Saposhnikovia divaricate, Rhizoma cimicifugae. Cimifugin has been reported to have bacteriostatic and antiviral effects. Studies have showed that cimifugin inhibits allergic inflammation by reducing the levels of epithelial-derived TSLP and IL-33 by modulating tight junction in the initial stage of AD, and improves the

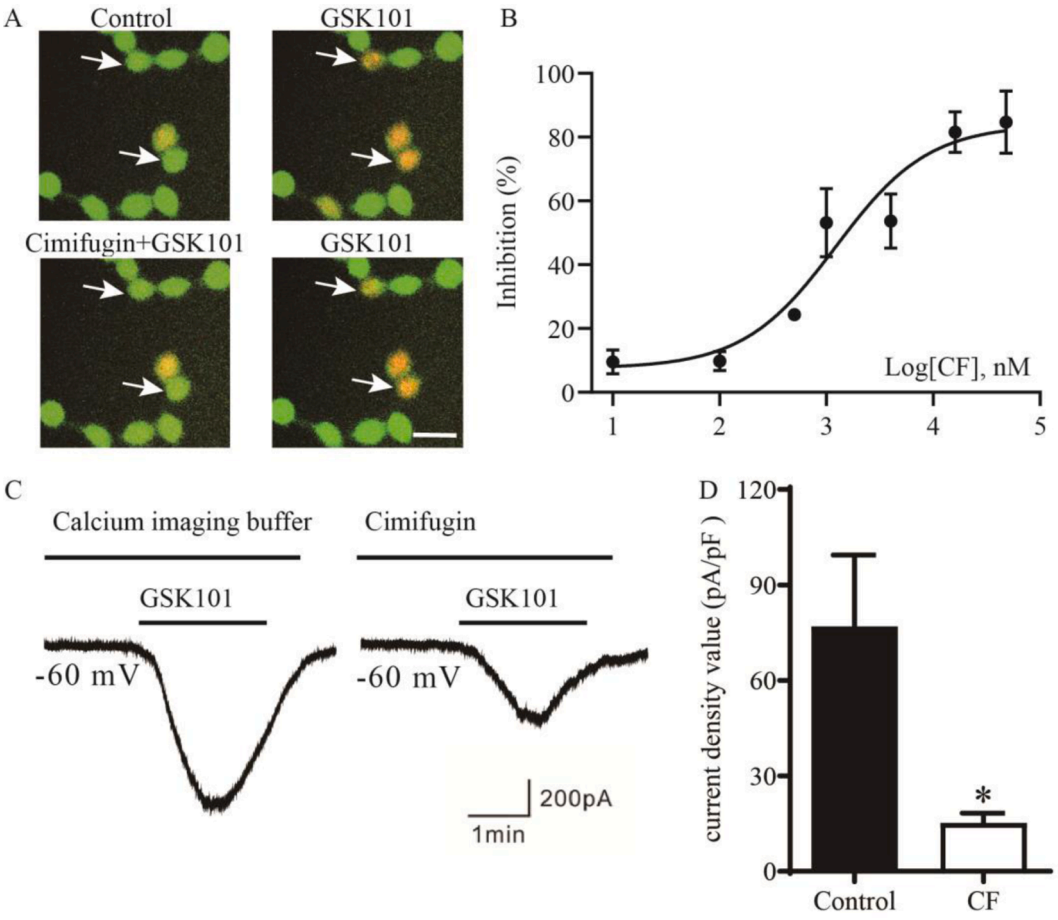

Fig. 6. Cimifugin inhibited TRPV4 function in transfected HEK293 cells. (A) The responses of TRPV4 transfected HEK293 cells to GSK101 and Cimifugin + GSK101 (cell number, $n > 1000$) (White arrow represents positive cells). (B) The dose-inhibition curve of cimifugin to GSK101 was measured by calcium imaging and the IC_{50} is about 1.585 μM ($R^2 = 0.8965$, the X axis represents concentration of cimifugin). (C) The inward current traces of GSK101 and cimifugin + GSK101 to transfected HEK293 cells. (D) The inward current of transfected HEK293 cells treated with cimifugin ($n = 10$) was significantly lower than that of the control group ($n = 13$). * $p<0.05$. All scale bars represent 50 μm.

recurrent inflammation in the mice AD recurrence model by maintaining skin barrier integrity and recovery of tight junction expression [30]. Thus, we propose the hypothesis whether cimifugin relieve TRPV4-mediated acute and chronic itching. Pretreatment with subcutaneous injection of cimifugin (1.625 mM, 4.875 mM, 8.125 mM, 25μL) in the mouse nape 30 min before GSK101 (60 μM, 25μL, i.d in the same site) stimulation, scratching behavior induced by GSK101 was obviously inhibited by cimifugin in a dose-dependent manner (45 ± 4 for 4.875 mM; 35 ± 1 for 8.125 mM; $n = 6$) (Fig. 4A). Moreover, the pruritus of psoriasis model mice could also be inhibited by cimifugin intragastric administration (25 ± 13 (Cimifugin group) VS 121 ± 13 (Model group), day 4) (Fig. 4B). Psoriasis increased TRPV4 expression, while cimifugin treatment could reverse the increase of TRPV4 expression both in skin and DRG (Fig. 3).

3.5. Cimifugin inhibited TRPV4 induced calcium influx in HaCaT cells and mice DRG neurons

It is obvious that cimifugin could inhibit the scratching behavior of psoriasis model mice through TRPV4. To further confirm whether cimifugin can directly inhibit the functional activity of TRPV4 channel, HaCaT cells were used to detect the interaction between cimifugin and TRPV4. HaCaT cells are the immortalized human keratinocytes and have been extensively used to study the epidermal homeostasis and its pathophysiology. TRPV4 has high expression in keratinocyte, therefore, calcium imaging was applied to study the effect of cimifugin on TRPV4 channels in HaCaT cells. The results indicated that cimifugin could inhibit the calcium influx of TRPV4 channel induced by GSK101 (Fig. 5A-C). GSK101 (100 nM) elicited intracellular calcium elevation in HaCaT cells, however pre-incubation of cimifugin (9.75 μM) could inhibit this calcium response induced by GSK101 (Fig. 5C). Then, we detected the effect of cimifugin to TRP channels on DRG neurons in mice. Similarly, cimifugin (1.625 μM, 4.874 μM) had a significant inhibitory effect on GSK101-induced (1 μM) intracellular calcium flux to DRG neurons (Fig. 5D-F). But, Cimifugin did not inhibit the activity of TRPV1 or TRPA1 in DRG neurons (Fig. S2).

3.6. Cimifugin inhibited calcium influx and inward current of TRPV4 channel in HEK293 transfected cells

To further confirm the inhibitory effect of cimifugin on TRPV4 channel, we selected another cell line (HEK293), and used calcium imaging technique to detect the effect of cimifugin on the calcium influx of HEK293 cells transfected with TRPV4 plasmid. The results showed that the inhibitory effect of cimifugin on GSK101 (250 nM) induced calcium influx was similar to that of HaCaT cells and DRG neurons in TRPV4-transfected HEK293 cells (Fig. 6A) and this inhibition is concentration dependent (EC_{50} = 1.585 μM) (Fig. 6B). Electrophysiological experiments also showed that cimifugin could inhibit GSK101 induced inward current on TRPV4-transfected HEK293 cells. GSK101 of 100 nM could induce inward current obviously. However, GSK101 induced inward current was significantly reduced by pretreatment of these cells with

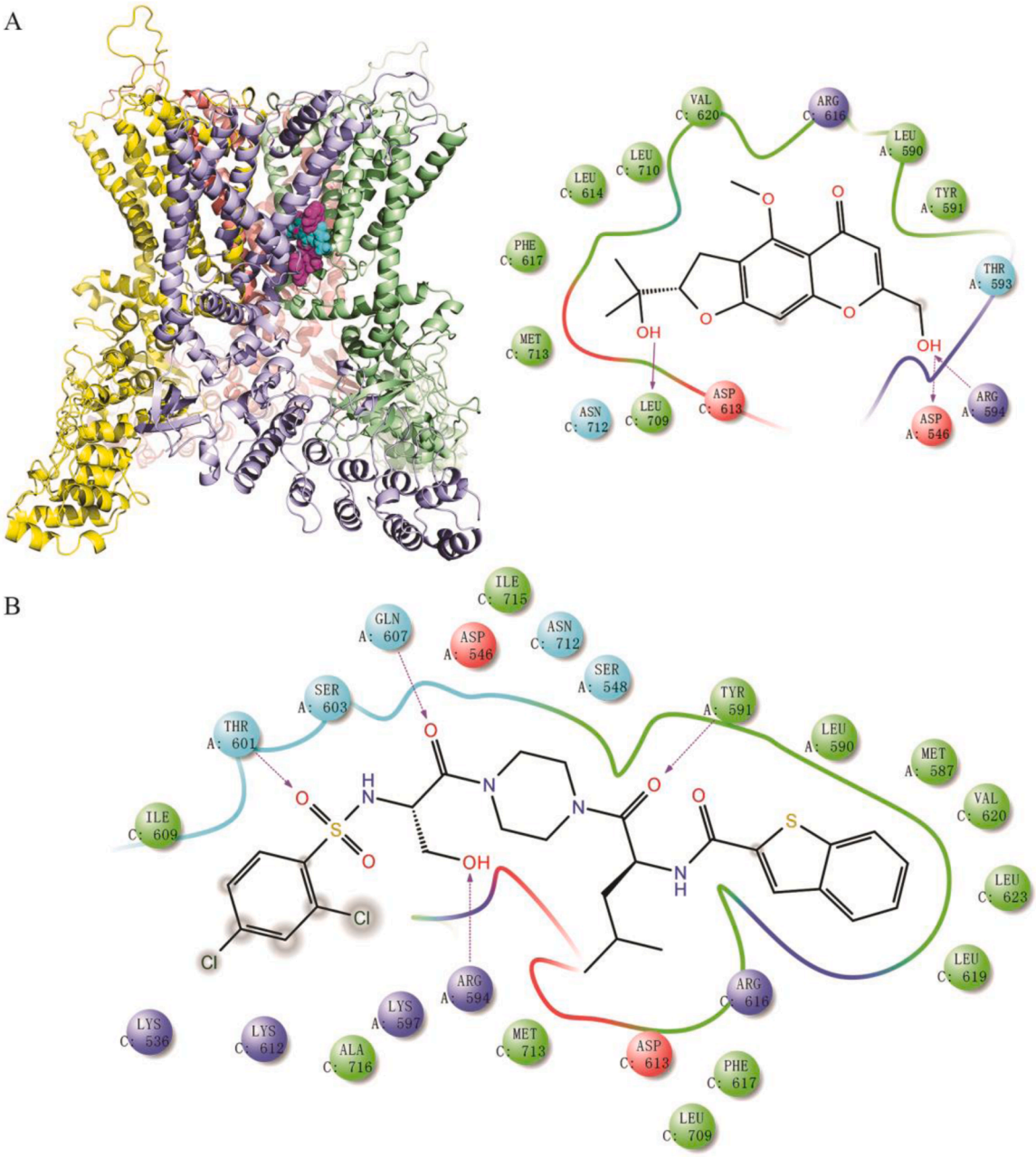

Fig. 7. GSK1016790A and Cimifugin bind ratTRPV4 through some common amino acids. (A) Combination sites of Cimifugin and ratTRPV4 and specific interaction mode. (B) Combination sites of GSK1016790A and ratTRPV4 and specific interaction mode.

9.75 μM cimifugin for 3 min. The current density values was 76.98 ± 22.39 pA/pF (GSK101 group) and 15.29 ± 3.09 pA/pF (GSK101+Cimifugin) group, respectively (Fig. 6C and D).

4. Discussion

TRPV4 was expressed in skin, peripheral nervous system (DRG and TG) and other tissues [17]. Our study found that there was high expression of TRPV4 in skin cells from psoriatic patients, and the expression level of TRPV4 was closely related to the severity of the patients, that TRPV4 was involved in the development of psoriasis. Then, the expression of TRPV4 in skin and DRG was checked, these results confirmed that TRPV4 was involved in the formation of psoriasis pruritus in model mice. In addition, behavioral experiments of TRPV4 gene knockout mice further defined that psoriasis pruritus is partly depend on the expression of TRPV4. These findings not only explain the cause of psoriasis pruritus, but also provide an important drug target for the treatment of patients with psoriasis pruritus.

Cimifugin, an active ketone ingredient from many traditional antipruritic Chinese medicines, has also been reported to have antibacterial and antiviral effects [30, 31]. However, in this study, we found that cimifugin has a significant effect on reducing the itching of psoriasis, which is the first evidence that cimifugin has such an effect. In DRG neurons, HaCaT cells and HEK293 cells transfected with TRPV4 plasmid, cimifugin can effectively inhibit GSK101 activated cell response. It is clear that the antipruritic mechanism of cimifugin is achieved by inhibiting the activity of TRPV4 channel. TRPV4 has been identified as the target of cimifugin. This not only expands the types of TRPV4 channel ligands, deepens the understanding of the functional mechanism of cimifugin, and provides the experimental basis for the future application of cimifugin, but also supplies a choice for the treatment of psoriasis patients.

Molecular docking is an important tool in structural molecular biology and computer-assisted drug design [32]. The goal of ligand-protein docking is to predict the predominant binding mode of a ligand with a protein of known three-dimensional structure. By using the molecular docking technology, the possible way of combining cimifugin with TRPV4 was simulated. Our molecular docking experiments predicted that GSK1016790A and Cimifugin are combined in the same pocket of ratTRPV4, and GSK1016790A and Cimifugin shared most of amino acids when combined with TRPV4, which were Arg594, Leu590 or Phe617 (Fig 7). These showed that cimifugin was a potential and effective TRPV4 inhibitor. We will explore the interaction between cimifugin and TRPV4 in future research. We will perform point mutations on these binding sites, and then observe the inhibitory effect of cimifugin on the function of TRPV4 after mutation, so as to determine the exact target of cimifugin binding to TRPV4. This helps to understand how cimifugin exert an antipruritic effect and reveals that cimifugin may be a potential drug for the treatment of chronic psoriasis pruritus in humans.

Although we have some new findings in this manuscript, there are still have a few interesting results need to be further studied. TRPV4 is expressed in both skin and DRG, in terms of the current data (acute and chronic itch behavior), we can defined that cimifugin is involved in the inhibition of pruritus in mice by inhibiting TRPV4 channel on DRG neurons. Keratinocyte can release several kinds of itching substances (such as, TSLP and ILs [33]), it's reasonable to speculate that the anti-pruritic effect of cimifugin is related to TRPV4 expression in keratinocyte. However, according to current study, it is still difficult to defined which kind of tissue expressed TRPV4 is more important in the anti-pruritic effect of cimifugin. Hyperknesis (stronger itch responses to pruritogens than normal) is a common symptom of patients with chronic itch. To determine whether this hyperknesis is associated with up-regulation of TRPV4, the expression of TRPV4 in the DRGs and skin of model mice also been checked in our experiemnt. Both immunostaining and RT-qPCR revealed that TRPV4 expression is significantly up-regulated in both DRG and skin. At the same time, cimifugin was found that can inhibit the expression of TRPV4, but the mechanism of how cimifugin regulated the expression of TRPV4 is still elusive.

In conclusion, our data indicated that cimifugin displays its anti-pruritic effect on psoriasis by inhibiting the activation of TRPV4 channel.

Authors contribution

Jinjin Yan: Methodology, Investigation, Formal analysis, Writing - original draft. **Fan Ye**: Methodology, Investigation, Formal analysis. **Ying Ju**: Methodology, Investigation. **Dijun Wang**: Methodology, Investigation. **Jiao Chen**: Methodology, Investigation. **Xinyu Zhang**: Methodology, Investigation. **Zhi Yin**: Methodology, Investigation. **Changming Wang**: Methodology, Investigation. **Yan Yang**: Methodology, Investigation. **Chan Zhu**: Methodology, Investigation. **Yuan Zhou**: Methodology, Investigation. **Peng Cao**: Investigation, Resources. **Yang Xu**: Investigation, Resources. **Guang Yu**: Methodology, Formal analysis, Conceptualization, Supervision, Funding acquisition, Writing - review & editing. **Zongxiang Tang**: Conceptualization, Supervision, Funding acquisition, Writing review & editing.

Ethics approval and consent to participate

Animal experiments were conducted in accordance with the relevant guidelines and regulations of the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine (Ethics licence ACU190601, 20,190,605). All protocols were approved by the International Association for the Study of Pain, and every effort was made to minimize the number and suffering of the included animals, including providing standard living condition and no additional damage appearing during the study. Psoriasis patients' skin was provided by the Department of Dermatology, the First Affiliated Hospital of Nanjing Medical University, and this study was approved by the hospital ethics committee. The study was not pre-registered.

Declaration of Competing Interest

The authors declare that they have no relevant conflicts of interest.

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Supplementary materials

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