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Cimifugin Relieves Histamine-Independent Itch in Atopic Dermatitis via Targeting the CQ Receptor MrgprA3

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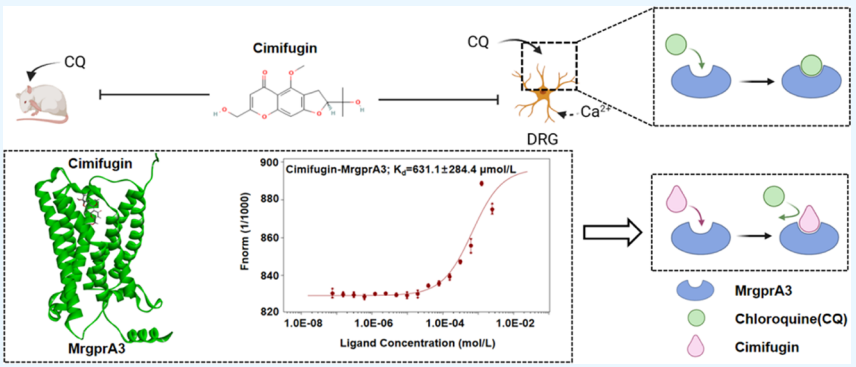

ABSTRACT: *Background*: We previously found that cimifugin has a potent antiallergic inflammatory effect in atopic dermatitis (AD). However, whether cimifugin has an antipruritic effect in AD was unknown. *Methods*: Mouse scratching behavior tests were performed to verify the proposed antipruritic effect of cimifugin on DNFB- or FITC-mediated AD. Chloroquine (CQ)- and compound 48/80-evoked acute itch models were employed to clarify the effect of cimifugin on histamine-dependent or -independent itch. Intracellular calcium changes were assessed in a primary culture of mouse dorsal root ganglia (DRG) in response to pruritogen exposure with or without cimifugin treatment, including CQ, histamine, allyl-isothiocyanate (AITC), and capsaicin. Molecular docking and microscale thermophoresis (MST) assays were performed to predict and verify the binding ability and modes between cimifugin and the CQ receptor MrgprA3, respectively. *Results*: We found that cimifugin attenuates itch behaviors effectively in FITC-induced AD. Notably, cimifugin significantly alleviated acute itching behaviors induced by CQ but not compound 48/80 in vivo. Moreover, cimifugin remarkably inhibited CQ-evoked calcium influx in DRG cells but had no obvious effect on histamine-induced calcium influx. Nevertheless, cimifugin did not interfere with either AITC-stimulated TRPA1 activation- or capsaicin-stimulated TRPV1 activation-mediated calcium influx in DRG cells. Molecular docking predicted that CQ and cimifugin might share similar binding abilities and binding modes with MrgprA3. MST assay confirmed cimifugin directly targeting MrgprA3. *Conclusion*: The present study demonstrates that cimifugin has a potent antipruritic effect in AD with a histamine-independent mechanism via targeting the CQ receptor MrgprA3. Thus, cimifugin is a promising candidate antipruritic agent for AD.

INTRODUCTION

Atopic dermatitis (AD) is a common inflammatory skin disorder that affects both children and adults worldwide and has a substantial psychosocial impact on patients and relatives.[1] Besides chronic skin inflammation, persistent itch is the most prominent yet most difficult symptom to control in patients with AD. It has been recognized that the allergic inflammatory mediators derived from immune cells and keratinocytes play a key role in the pathogenesis of AD and atopic itch.[2] Although the central nature of the inflammatory pathway in AD has been proved by the potent therapeutic effects of dupilumab (an IL-4Ra antagonist) and nemolizumab (an IL-31RA antagonist) application clinically,[3,4] there is an accumulating appreciation for the contribution of the nervous system in AD-associated itch.[5]

Itch often occurs when sensory nerves are exposed to exogenous and endogenous pruritogens. Pruritogens bind to a subset of primary afferent C-fiber somatosensory neurons (pruritoceptors) that innervate skin. After pruritogens activate pruritogen receptors on the cutaneous nerve endings of pruritoceptors, calcium influx and activation of intracellular signaling pathways result in the transmission of an electrical

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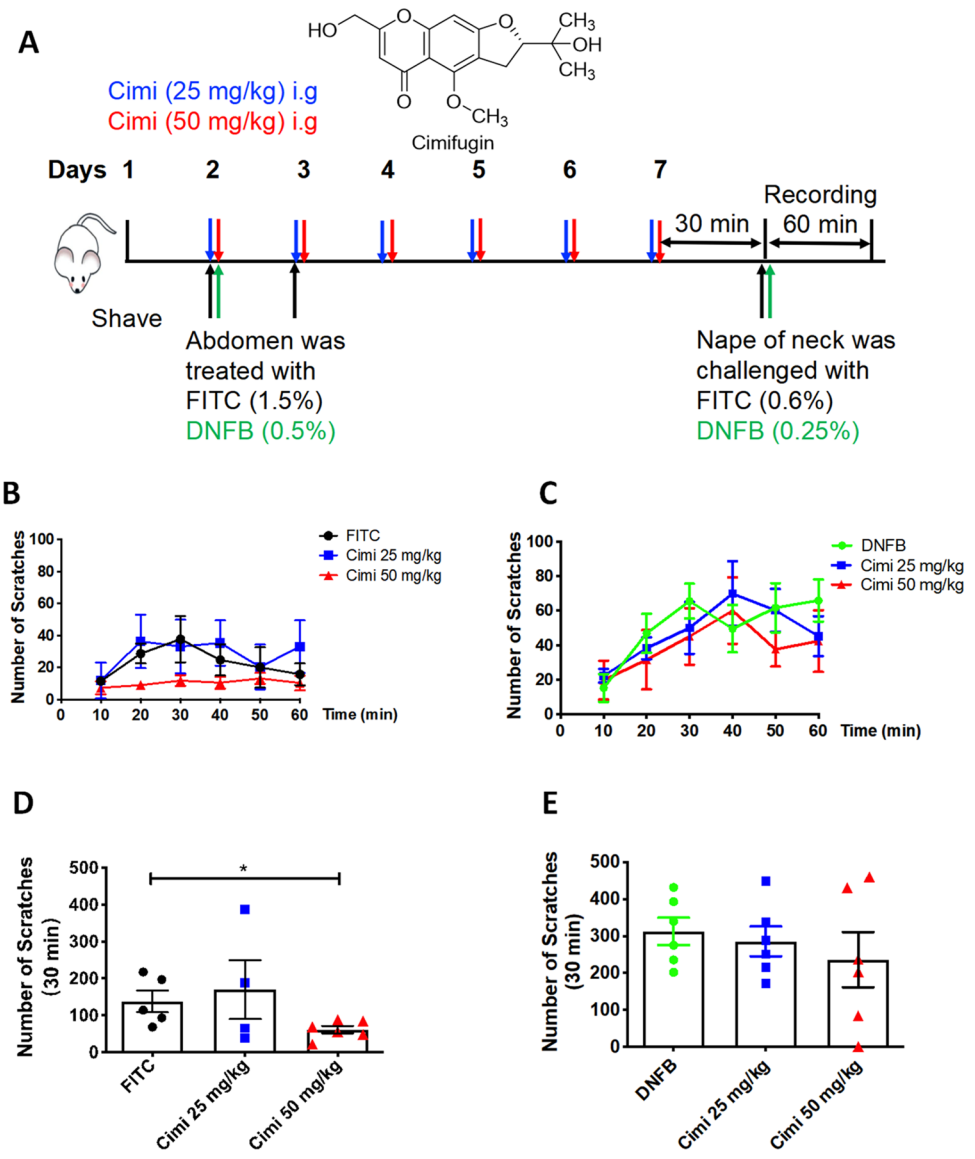

Figure 1. Effects of cimifugin on itch behaviors in atopic dermatitis mice. (A) Time schedule of FITC (1.5% for sensitization and 0.6% elicitation)- or DNFB (0.5% for sensitization and 0.25% for elicitation)-induced atopic dermatitis (AD) models and the cimifugin regimen. Cimifugin (25, 50 mg/kg) was administrated intragastrically for 6 consecutive days. (B) FITC (0.6%) challenge was performed 30 min after the last administration of cimifugin. Numbers of scratching induced by FITC were counted every 10 min in 60 min. (C) DNFB (0.25%) challenge was performed 30 min after the last administration of cimifugin. Numbers of scratching induced by DNFB were counted every 10 min in 60 min. (D) Total numbers of scratches induced by FITC counted in 30 min. (E) Total numbers of scratching induced by DNFB counted in 30 min. All data are presented as mean ± SEM, $n = 4-6$, $^*P < 0.05$ vs FITC group.

impulse from the skin to the dorsal root ganglia (DRG) and the spinal cord.[6] Subsequently, the itch signal is further processed in the brain, and scratching behavior is induced.[7] Itch can be conventionally classified into two subtypes, histamine-dependent and histamine-independent itches. Many forms of itch are mediated by the histaminergic pathway, and consequently, antihistamines are the first choice for the anti-itch purpose. However, antihistamines are only marginally effective in resolving most chronic itch in AD, indicating that histamine-dependent pathways do not contribute substantially to AD.[6] Therefore, further unraveling the pathologic mechanism of chronic itch in AD may help outline the possible therapeutic approach to target AD-associated itch.

Cimifugin is a natural component of chromones derived from the dry roots of *Saposhnikovia divaricata* for treating inflammatory diseases.[8] We previously reported the antiallergic inflammatory effects of cimifugin in the mouse model of AD and demonstrated that cimifugin could effectively inhibit epithelial-derived alarmin thymic stromal lymphopoietin (TSLP) production and repair the tight junction expression in the epithelial barrier.[9,10] In the present study, we sought to explore whether cimifugin has an antipruritic effect on AD-associated itch and its potential mechanism.

RESULTS

Cimifugin Attenuates Itch Behaviors in FITC-Induced Atopic Dermatitis Mice. Our previous studies have demonstrated that cimifugin can effectively inhibit allergic inflammatory response in atopic dermatitis (AD) mice.[9,10] Since pruritus is a primary symptom of AD, while cimifugin is a common compound in antipruritic Chinese medicine, we

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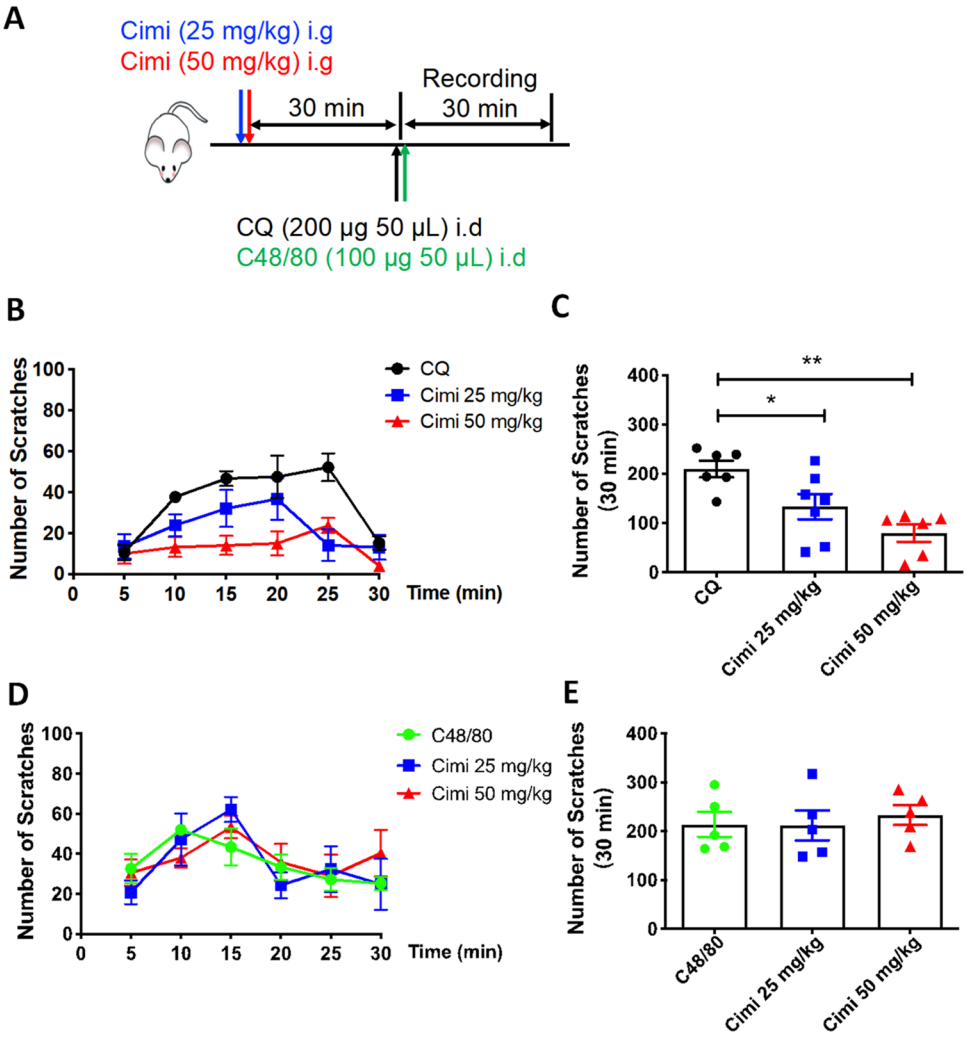

Figure 2. Effects of cimifugin on itch behaviors induced by histamine-dependent and histamine-independent pruritogens in mice. (A) Time schedule of CQ (4 mg/mL in 50 μL)- or compound 48/80 (2 mg/mL in 50 μL)-induced mouse itch behaviors and cimifugin (25, 50 mg/kg) regimen. (B) Numbers of scratching induced by CQ recorded every 5 min in 30 min. (C) Total numbers of scratches induced by CQ counted in 30 min. (D) Numbers of scratching induced by compound 48/80 (C48/80) counted every 5 min in 30 min. (E) Total numbers of scratching induced by compound 48/80 counted in 30 min. All data are presented as mean ± SEM, n = 5−7, $^{**}P < 0.01$ vs CQ group.

speculated that cimifugin might have a potential antipruritic effect on AD. Therefore, we evaluated the effect of cimifugin on itch behavior in DNFB (Th1-dominant inflammatory)- or FITC (Th2-dominant inflammatory)-mediated AD mice (Figure 1A). The dosages of cimifugin applied in this study are determined according to our previous study and other group's report.[9,11] The result showed that the topical application of DNFB (0.5% for sensitization and 0.25% for elicitation) or FITC (1.5% for sensitization and 0.6% for elicitation) induced robust itching behavior, which lasted for more than 30 min (Figure 1B,C). Preventive treatment of cimifugin (50 mg/kg) significantly reduced FITC-invoked scratching behavior in AD mice as shown in Figure 1B,D ($P < 0.05$). However, preventive treatment with cimifugin (50 mg/kg) had no significant effect on the DNFB-induced itching behavior (Figure 1C,E). These results indicate that cimifugin selectively inhibits Th2-dominant inflammation-mediated itch.

Cimifugin Alleviates Histamine-Independent Itch Behaviors in Mice. Itch is often divided into histamine-dependent and histamine-independent subtypes. Therefore, chloroquine (CQ), as a histamine-independent pruritogen, and compound 48/80, as a histamine-dependent pruritogen, were employed in this study to further verify the effect of cimifugin on the itch behavior in mice (Figure 2A). As seen in Figure 2B,C, i.d. injection of CQ evoked pronounced acute scratching behavior in mice and cimifugin pretreatment (25, 50 mg/kg, i.g.) significantly inhibited CQ-induced scratching behavior induced ($P < 0.05$, $P < 0.01$) with a dose-dependent manner. Similarity, i.d. injection of compound 48/80 induced immediate scratching behavior in mice as well; however, cimifugin pretreatment showed no significant effect on compound 48/80-induced scratches (Figure 2D,E). These results suggested that cimifugin could effectively alleviate histamine-independent pruritogen-evoked itch behavior.

Cimifugin Inhibits CQ-Induced Calcium Response in DRG Neurons. To explore the mechanism of cimifugin's antipruritic action, calcium imaging recordings were performed as both histamine and CQ induce calcium responses in DRG neurons.[12] We first evaluated the effect of cimifugin on CQ-evoked calcium influx in DRG neurons. As shown in Figure 3A, CQ (100 μM) treatment induced an intracellular calcium increase in 29 out of 131 small-size DRG neurons. When cells

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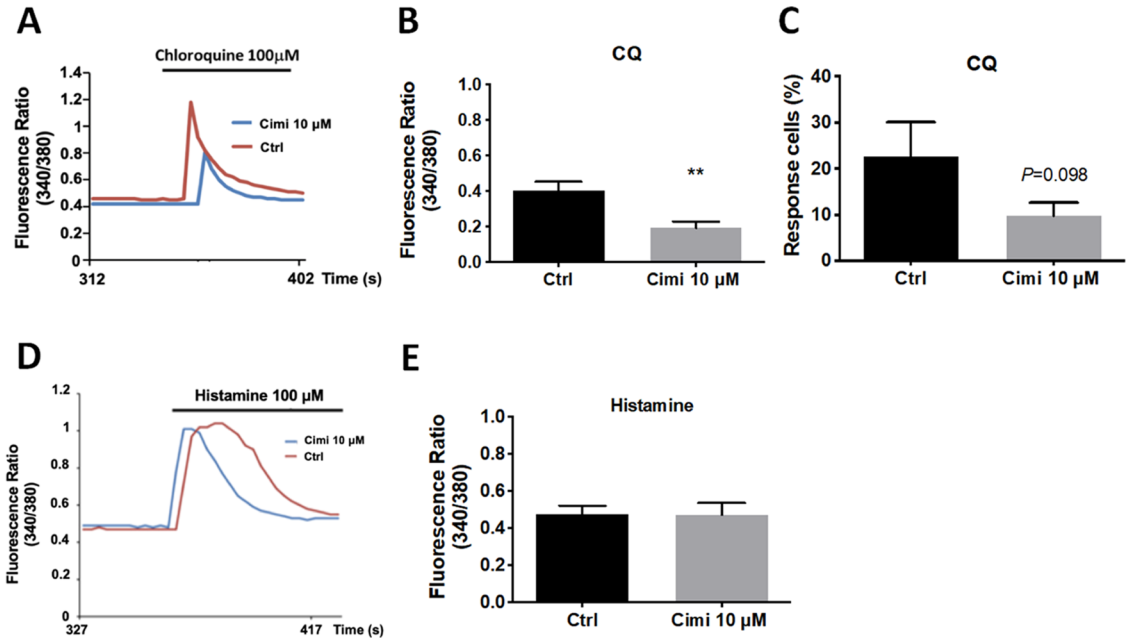

Figure 3. Cimifugin inhibits CQ-induced calcium influx in DRG cells. (A) Time course curve of CQ (100 μM)-induced calcium influx in DRG neurons. Each trace indicates the responses of representative DRG neurons without (Ctrl) or with cimifugin (Cimi, 10 μM) pretreatment in calcium imaging assay. (B) Effect of cimifugin on CQ-induced intracellular calcium flux to DRG neurons. (C) Histogram of the DRG cell response rate in response to CQ (100 μM) application. The percentages are given as the number of CQ-responding neurons of the total number of DRG neurons counted. Approximately 400−800 cells were studied in at least three separate experiments. (D) Time course curve of histamine (100 μM)-induced calcium influx in DRG neurons. Each trace indicates the responses of representative DRG neurons without (Ctrl) or with cimifugin (Cimi, 10 μM) pretreatment in calcium imaging assay. (E) Effect of cimifugin on histamine-induced intracellular calcium flux to DRG neurons. Approximately 400−800 cells were studied in at least three separate experiments. Data are presented as mean ± SEM, $^{**}P < 0.01$ vs Ctrl.

were pretreated with cimifugin (10 μM), however, the amplitude of the CQ-induced calcium response was greatly decreased (Figure 3A,B). In addition, the percentage of CQ-responding neurons also decreased in the cimifugin-treated group (Figure 3C). These data indicate that cimifugin inhibits the calcium response invoked by histamine-independent pruritogen in DRG neurons.

We then tested the effect of cimifugin on histamine-evoked calcium influx in cultured DRG neurons. As shown in Figure 3, histamine (100 μM) exposure induced an intracellular calcium increase in 56 out of 335 small DRG neurons. However, cimifugin (10 μM) pretreatment showed no significant effect on the peak of the calcium response in histamine-evoked DRG neurons (Figure 3D,E). These results were consistent with in vivo experiments that showed that cimifugin showed no effects on histamine-dependent itch behaviors.

Cimifugin Has No Effect on TRPA1- or TRPV1-Mediated Calcium Responses. It has been recognized that CQ binds to MrgprA3 (Mas-related G-protein-coupled receptor member A3) of DRG neurons and further relays itch signals via activation of TRPA1 (transient receptor potential ankyrin 1).[13] TRPA1 is required for nonhistaminergic itch.[14] We then assessed the effect of cimifugin on allyl-isothiocyanate (AITC, a TRPA1 agonist)-evoked calcium influx in DRG neurons. We found that stimulation of DRG cells with AITC (100 μM) increased intracellular calcium flux in DRG cells (Figure 4A). Cimifugin (10 μM) pretreatment or post-treatment showed no significant effects on AITC-induced intracellular calcium influx (Figure 4A−C). We also compared the slope of the time response curves of calcium imaging; there was no obvious difference between with or without cimifugin treatment in the AITC-evoked calcium influx. These results suggested that cimifugin has no effect on the TRPA1-mediated calcium influx.

Although transient receptor potential vanilloid 1 (TRPV1) channel activation is mainly involved in histamine-induced itch, it has been reported that binding of CQ to MrgprA3 causes activation of $G\alpha_q$ protein, leading to the sensitization of TRPV1 and subsequently enhancing itch sensation.[15] We then assessed the effect of cimifugin on the capsaicin (a TRPV1 agonist)-evoked calcium influx in DRG neurons. Capsaicin (1 μM) induced increased intracellular calcium in small- and medium-size DRG neurons (Figure 4D). Cimifugin (10 μM) pretreatment or post-treatment showed no significant effects on the capsaicin-induced calcium influx (Figure 4D−F). Therefore, these results suggested that cimifugin has no effect on the TRPV1 function.

Cimifugin Has the Potential to Bind to MrgprA3. CQ binds to its receptor MrgprA3 and further relays its signal to the TRPA1 ion channel, allowing calcium influx. Since we found that cimifugin inhibited the CQ-induced calcium influx without affecting TRPA1 activation, we then speculated whether cimifugin, like CQ, could directly bind to MrgprA3 to inhibit the histamine-independent itch signal. Based on predicting the predominant binding modes of a ligand with a protein of known three-dimensional structure, the molecular docking technique was used in this study to predict the potential binding ability and modes between cimifugin and MrgprA3 protein. Our molecular docking outcomes predicted that CQ and cimifugin were combined in the same pocket of MrgprA3 with similar binding abilities as the binding energy of cimifugin and MrgprA3 is identical to that of CQ and MrgprA3 (Table 1). In addition, CQ and cimifugin shared most of the amino acids when combined with MrgprA3, which were

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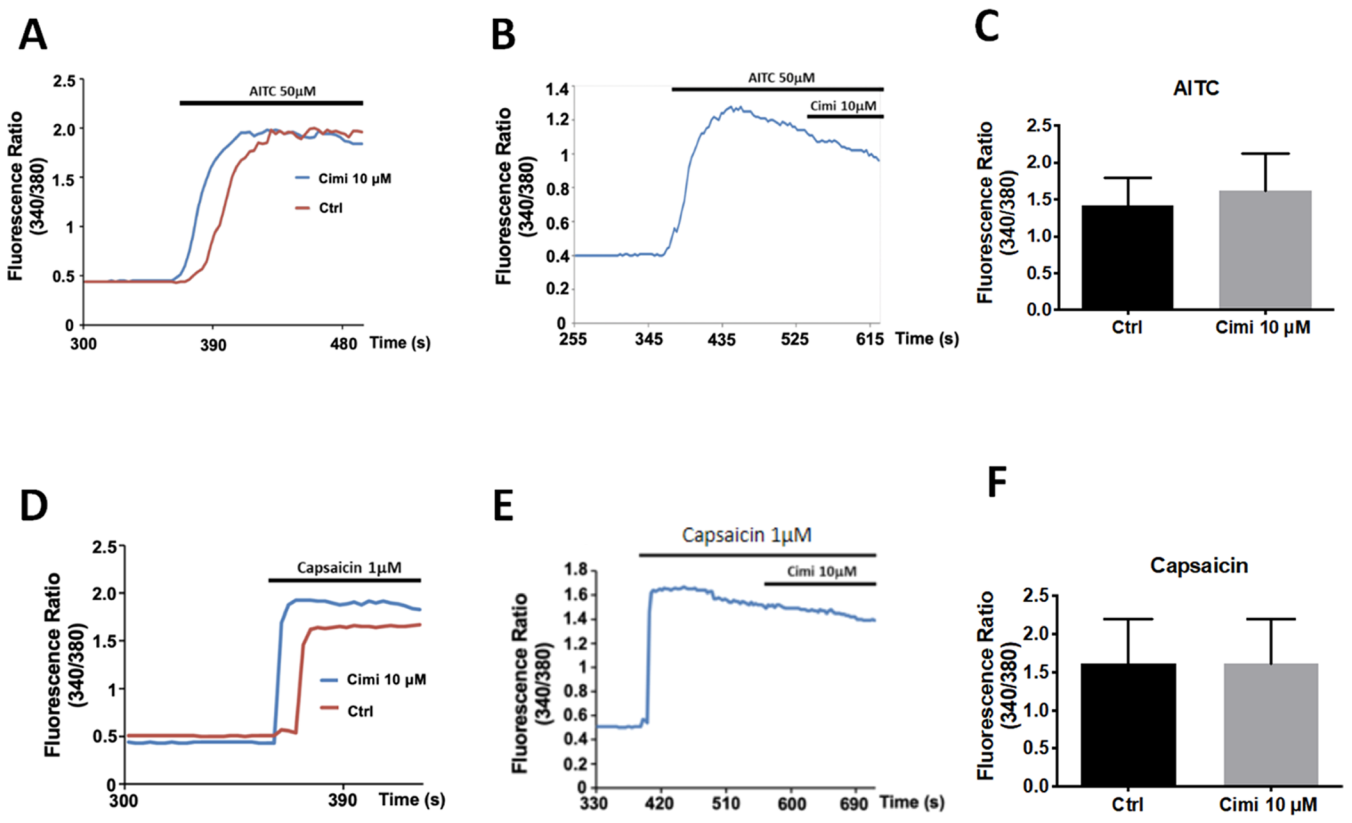

Figure 4. Cimifugin has no effect on TRPA1 or TRPV1 activation-mediated calcium influx. (A) Representative trace of calcium imaging shows that pretreatment of cimifugin (10 μM) has no effect on AITC (50 μM)-evoked calcium influx. (B) Representative trace of calcium imaging shows that post-treatment of cimifugin (10 μM) has no effect on AITC (50 μM)-evoked calcium influx. (C) Histogram of AITC-induced calcium influx with or without cimifugin (10 μM) treatment in DRG cells. (D) Representative trace of calcium imaging shows that pretreatment of cimifugin (10 μM) has no effect on capsaicin (1 μM)-evoked calcium influx. (E) Representative trace of calcium imaging shows that post-treatment of cimifugin (10 μM) has no effect on capsaicin-evoked calcium influx. (F) Histogram of capsaicin-induced calcium influx with or without cimifugin (10 μM) treatment in DRG cells. Approximately 400−800 cells were studied in at least three separate experiments. Data are presented as mean ± SEM.

Table 1. Binding Energies of Compounds Binding to the Mouse MrgprA3 Protein Crystal Structure

compounds	compounds CID	docking score (kcl/mol)
chloroquine	2719	−5.134
cimifugin	441960	−5.409

HID88, ARG92, TYR152, TRP222, THR225, and PHE230 (Figure 5). These results suggested that cimifugin has a potent ability to bind to the CQ receptor MrgprA3 and that it may potentially compete with CQ to bind to MrgprA3.

Cimifugin Directly Interacts with the CQ Receptor MrgprA3. Microscale thermophoresis (MST) is a biophysical assay to quantify the interaction between molecules such as proteins and small molecules. In recent years, the MST assay has been used to detect protein−protein and protein−drug interactions.[16,17] To further investigate the direct interaction between cimifugin and MrgprA3 protein, we performed MST assay by using the lysates of HEK293T cells with overexpression of MrgprA3 labeled with GFP (Figure 6A,B). MST results showed that both CQ and cimifugin directly interacted with MrgprA3 protein in a positive dose-dependent manner (Figure 6C,D), but no interaction was detected between the GFP empty vector, as the negative control, and MrgprA3 (Figure 6E,F). The determined equilibrium dissociation constant (K_d) for cimifugin binding to MrgprA3 protein was 631.1 μmol/L, which was much lower than the K_d value of CQ (1353.0 μmol/L) (Figure 6C,D). As the K_d value and affinity are inversely related, these results suggested that cimifugin had a stronger affinity with MrgprA3 compared with CQ and cimifugin may be able to competitively bind to the CQ receptor MrgprA3. Thus, cimifugin has a potent antipruritic effect in AD with a histamine-independent mechanism via competitively binding to the CQ receptor MrgprA3.

In summary, cimifugin alleviates histamine-independent itch in FITC-induced AD. Cimifugin inhibited histamine-independent itch likely through competitively binding to the CQ receptor MrgprA3 rather than directly affecting TRPA1 and TRPV1 ion channel activation. Therefore, cimifugin is a promising drug for the treatment of AD, as it not only effectively inhibits allergic inflammation of AD but also relieves AD-associated itch.

DISCUSSION

Cimifugin is an important active component of *Saposhnikovia divaricata* and *Cimicifuga racemosa*, both of which are Chinese medicine frequently used as an anti-inflammatory and analgesic remedy.[18,19] Many biological functions of cimifugin have been recently revealed, such as anti-inflammation,[20] antioxidative stress,[8] and preventing hepatocyte damage and osteoclastogenesis.[21,22] We previously demonstrated that cimifugin derived from *S. divaricata* is a natural compound with potent antiallergic activity.[9,10] In the present study, we found that cimifugin also has a strong anti-itch activity in FITC-induced

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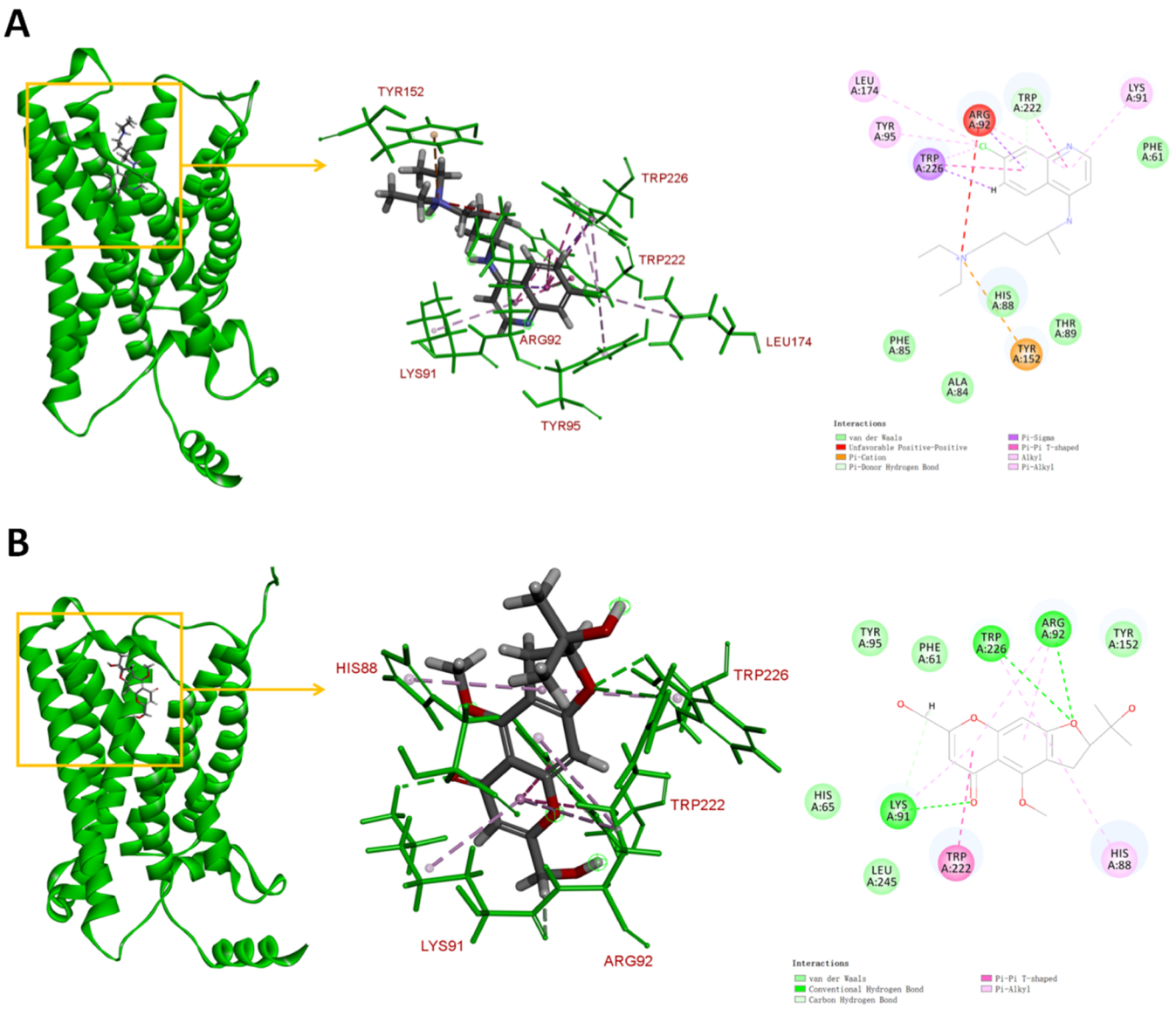

Figure 5. Cimifugin and CQ bind to MrgprA3 through common amino acids. (A) Combination sites of CQ and MrgprA3 and docking interaction mode. (B) Combination sites of cimifugin and MrgprA3 and docking interaction mode.

AD mice, suggesting that cimifugin may be developed as a promising treatment for AD, as it not only inhibits allergic inflammation but also possesses an antipruritic effect.

Notably, the antipruritic effects of cimifugin have recently been reported in psoriasis, primarily through inhibiting TRPV4.[11] TRPV4 is a member of the TRP ion channel. It is expressed in epidermal keratinocytes and functions as a pruriceptor in acute histaminergic itch but not in non-histaminergic itch evoked by CQ.[23] In the present study, however, we found that cimifugin alleviated acute scratching behavior induced by CQ but had no effect on histamine-dependent itch behavior induced by compound 48/80. In addition, cimifugin significantly inhibited the CQ-evoked calcium influx in DRG neurons in vitro but did not impact histamine-induced calcium influx. These results suggested that the mechanism of cimifugin relieving itch in AD may be different from that of inhibiting itch in psoriasis.

TRP channels are nonselective calcium-permeable cation channels, which have been best recognized for their contributions to sensory transduction including itch sensation.[24] Among the large TRP superfamily proteins, TRPV1 and TRPA1 are both expressed in DRG neurons. The non-histaminergic mechanism of itch has been identified, including CQ-induced calcium influx through the TRPA1 channel in DRG neurons.[25] We therefore investigated the effect of cimifugin on TRPA1 function using the TRPA1 agonist AITC. However, the results showed that cimifugin had no effect on AITC-evoked calcium influx, indicating that cimifugin might not directly interfere with TRPA1 function. Although TRPV1 is recognized as a key transducer in mediating histamine-dependent itch signaling, it also has been suggested to be involved in many chronic itch conditions like AD.[26] Besides, it has been reported that TRPA1 and TRPV1 are coexpressed in a subpopulation of nociceptive DRG neurons. We then assessed the effect of cimifugin on TRPV1-mediated calcium influx using the TRPV1 agonist capsaicin. We found that cimifugin did not affect capsaicin-evoked calcium influx, either. Therefore, our results indicated that cimifugin's antipruritic effect might not be mediated by TRPA1 and TRPV1.

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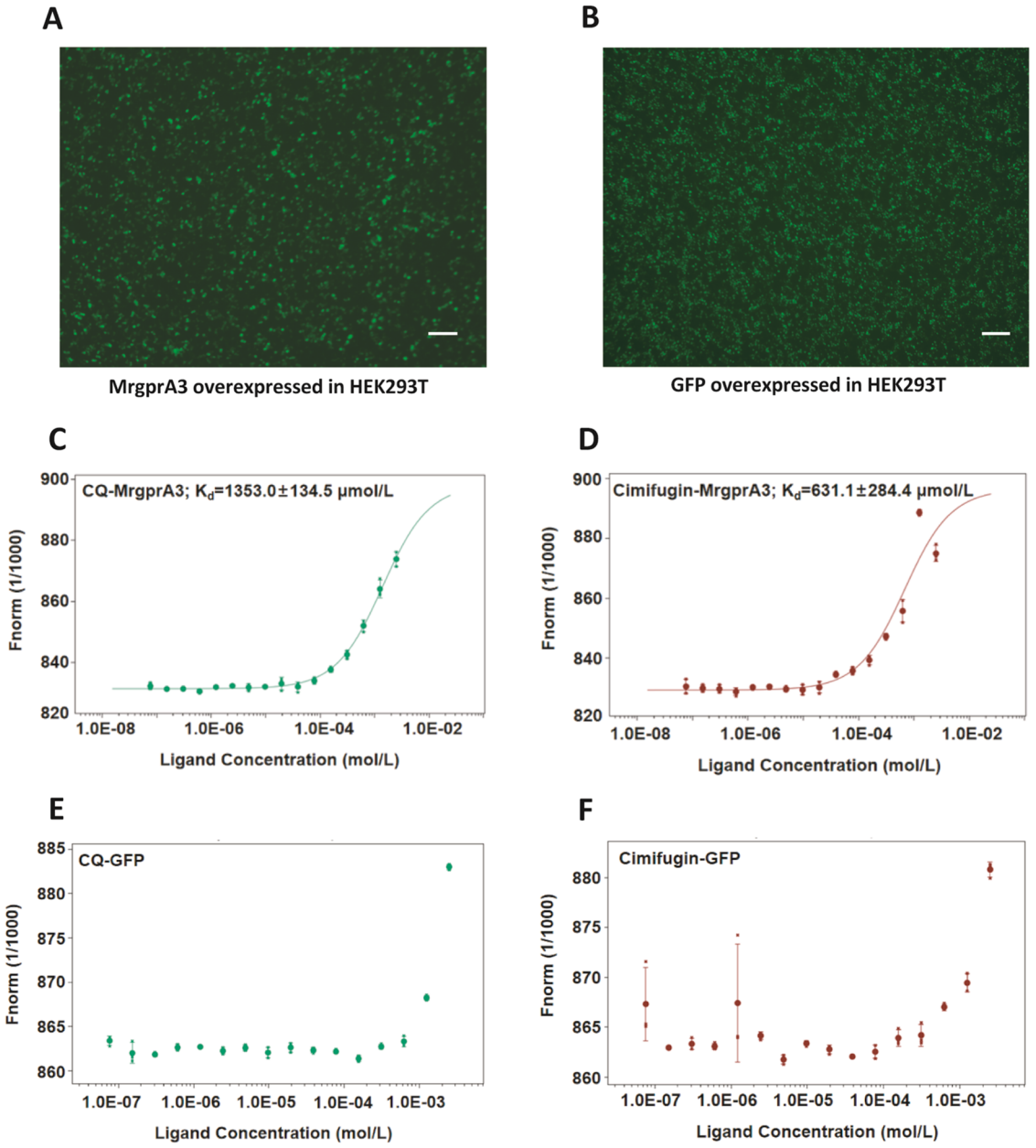

Figure 6. Cimifugin directly binds to the CQ receptor MrgprA3. (A) Transfection efficiency of the pEGFP-C1-MrgprA3 plasmid in HEK293T cells (bar = 200 μm). (B) Transfection efficiency of the pEGFP-C1 plasmid in HEK293T cells (bar = 200 μm). (C) Dose−response curve of the ligand fitted by MST analysis software of CQ and MrgprA3-GFP. (D) Dose−response curve of the ligand fitted by MST analysis software of cimifugin and MrgprA3-GFP. (E) Dose−response curve of the ligand fitted by MST analysis software of CQ and the GFP empty vector. (F) Dose−response curve of the ligand fitted by MST analysis software of cimifugin and the GFP empty vector.

MrgprA3 is the specific receptor for the antimalaria drug CQ and is important for nonhistaminergic and TRPV1-independent itch.[25] We then speculated that cimifugin's inhibition of nonhistaminergic itch in AD may be through direct binding to the CQ receptor MrpgrA3 to compete for the combination of CQ with it. We performed a molecular docking technique to predict the binding ability and binding modes between cimifugin and MrgprA3 by using CQ as a positive control. We found that cimifugin possessed a similar binding energy as CQ to MrgprA3 and shared several of the same amino acid binding sites. Moreover, MST assay is a novel technology based on detecting the electrophoretic mobility changes of biomolecules in a temperature gradient, which can detect the binding and dissociation processes of biomolecules and obtain the information on molecular interaction patterns and kinetic constants.[16,17] Therefore, it was employed in this study to further confirm the direct binding ability of cimifugin to MrgprA3. We found that cimifugin directly interacted with MrgprA3 in a positive dose-dependent manner. More importantly, the equilibrium dissociation constant of cimifugin binding to MrgprA3 protein was obviously lower than that of CQ binding to the MrgprA3 protein. This result suggested that cimifugin can competitively bind to the CQ receptor MrgprA3, which is an important mechanism by which cimifugin inhibits histamine-independent itch in AD.

Based on our present results, we proposed that cimifugin alleviates histamine-independent itch in AD by competitively binding to the CQ receptor MrgprA3 rather than directly

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inhibiting TRPA1 and TRPV1 ion channel activation. These findings not only merely enrich our knowledge of the potentially pharmaceutical mechanism of cimifugin in treating AD but also point toward new therapeutic strategies for itch by targeting the CQ receptor MrgprA3.

CONCLUSIONS

This study demonstrated that cimifugin not only inhibited the allergic inflammatory response in AD but also had potent antipruritic effects. Cimifugin has an antipruritic effect on AD mainly by targeting the CQ receptor MrgprA3 through a histamine-independent pathway without interfering with TRPA1 and TRPV1 ion channel activation. Therefore, this study suggests that targeting MrgprA3 may be an important strategy for the intervention of histamine-independent itch and that cimifugin is a promising candidate compound for the treatment of AD.

MATERIALS AND METHODS

Mice. All procedures were carried out in accordance with the guidelines for the care and use of laboratory animals and were approved by the Animal Care and Ethical Committee of Nanjing University of Chinese Medicine and Drexel University. CD1 and BALB/c mice (Charles River Laboratories and Beijing Vital River Laboratory Animal Technology Co., Ltd., China) aged 5−10 weeks were used in the present study. All experimental animals were housed in specific pathogen-free conditions with a 12 h light−dark cycle and ad libitum access to water and chow. Temperature and humidity were maintained at 18−25 °C and 40−70%, respectively.

Reagents. Cimifugin was purchased from Yongjian Pharmaceutical (China, Purity ≥98%). Fluorescein isothiocyanate (FITC), 2,4-dinitrofluorobenzene (DNFB), chloroquine (CQ), compound 48/80, histamine, allyl-isothiocyanate (AITC), and capsaicin were all purchased from Sigma-Aldrich. The stock solutions of drugs were prepared in DMSO and stored at −20 °C. The drugs were further diluted with saline, and the final concentration of DMSO does not exceed 1% when used in behavioral tests or does not exceed 0.1% when applied in the calcium imaging experiments.

DNFB- or FITC-Induced Atopic Dermatitis Model of Itch. The mouse models of DNFB- or FITC-induced AD were established based on the previous literature with minor modifications.[10,27] Briefly, mice were shaved with an area of approximately 3 × 3 cm^2 on the abdomens and 2 × 2 cm^2 on the nape of necks on day 1 and topically sensitized with 50 μL of 0.5% DNFB (4:1 dissolved in acetone and olive oil mixture) or 80 μL of 1.5% FITC (1:1 dissolved in acetone and dibutyl phthalate) solution on days 1 and 2. Mice were challenged with 30 μL of 0.25% DNFB or 20 μL of 0.6% FITC solution by topical application to the shaved area on the nape of neck on day 7. Mice were then put into plastic chambers (14 × 18 × 12 cm^3) on an elevated metal mesh floor and were allowed to acclimate for 30 min. Scratching was recorded for 60 min. A direct scratching event involved the mouse lifting its hind paw to the shaved region and returning the paw to its original position or to the mouth for licking.

Acute Scratching Behavior Test. Acute itch elicited by intradermal injection of pruritogens was established as previously reported with minor modifications.[28] Mice were shaved with an area of approximately 2 × 2 cm^2 on the nape of the necks and then were allowed to acclimate in plastic chambers (14 × 18 × 12 cm^3) on an elevated metal mesh floor for 30 min. To induce acute scratching behaviors, 50 μL of CQ (200 μg) or compound 48/80 (100 μg) was intradermally (i.d.) injected with a 28-gauge needle in the nape of the mice. Scratching behaviors were recorded for 30 min. One bout of scratching was defined as the mouse lifting its hind paw and scratching continuously for any length of time until the paw returned to the floor or to the mouth for licking.

Primary Dorsal Root Ganglia Neuron Culture. Mouse dorsal root ganglia (DRG) neurons were primarily cultured as described.[15] Briefly, DRGs were isolated from adult CD1 mice (5 and 7 weeks) and incubated in HBSS (Invitrogen) containing type IV collagenase/papain (2.5 mg/mL) at 37 °C for 30 min. After incubation, DRGs were washed with HBSS and cultured in neurobasal medium (Gibco) followed by mechanical dissociation with a pipette in the presence of 0.05% DNase I (Sigma-Aldrich). The dissociated neurons were plated onto coverslips precoated with poly-D-lysine (0.01%, Sigma) and collagen (0.125 mg/mL). DRG cells were then incubated in a 5% CO_2 atmosphere at 37 °C and were maintained for 24 h before use.

Calcium Imaging. Intracellular Ca^{2+} dynamics was measured by the calcium imaging technique as described in our previous study,[29] which was performed using Fura-2-based microfluorimetry and an imaging analysis system. Briefly, DRGs were cultured in Tyrode's solution (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 5.6 mM glucose) containing 4 μM Fura-2-AM (Life Technologies) for 30 min at room temperature. After incubation, the solution containing Fura-2-AM was washed out with Tyrode's solution, and DRGs were incubated in Tyrode's solution for 20 min. CQ (100 μM), histamine (100 μM), AITC (100 μM), and capsaicin (1 μM) were applied to DRG cells to elicit calcium influx. Cimifugin (10 μM) was preincubated for 5 min prior to the pruritogen application or given 2 min after each pruritogen exposure. The fluorescence intensity was measured at 340 and 380 nm with an interval of 3 s under an Olympus inverted microscope equipped with a CCD camera (Hamamatsu ORCA-03G, Japan). The fluorescence images were recorded and analyzed using software MetaFluor 7.7.9 (Molecular Devices). Intracellular Ca^{2+} changes were expressed as the F_{340}/F_{380} ratios.

Molecular Docking. The three-dimensional crystal structure of the mouse MrgprA3 protein (AlphaFoldDBID: Q91WW3) obtained from Uniprot was used for molecular docking. First, MrgprA3 protein and the ligands cimifugin (CID: 441960) and chloroquine (CID: 2719) were prepared by the Protein Preparation Wizard and LigPrep modules in the Schrödinger Maestro 11.9 system, respectively. Hydrogens were added and water molecules were removed, followed by energy minimization and optimization. Then, molecular docking analysis was performed with the Glide module. SiteMap was used to simulate the ligand-binding sites, and Receptor Gird Generation was applied to generate a docking pocket. Other parameters were set as default. The ability of compounds to directly bind to the MrgprA3 structure was predicted based on the docking score.

Microscale Thermophoresis (MST) Assay.[30] HEK-293T cells (CL-0005) were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). Cells were transfected with expression plasmids for pEGFP-C1 and pEGFP-C1-MrgprA3 (synthesized by Sangon Biotech Shanghai, China),

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ACS Omega 2024, 9, 7239−7248

and the lysates were collected as assay buffer for MST experiments. A NanoTemper Monolith instrument (NT.115) (NanoTemper Technologies, München, Germany) was employed for measuring thermophoresis. Samples were loaded into Monolith NT.115$_{Pico}$ MST standard-treated capillaries and measured using Monolith NT.115$_{Pico}$ and MO.Control software (NanoTemper Technologies) at 37 °C.

Statistical Analysis. Statistical analysis was performed by using GraphPad Prism 8.0. All data were presented as the mean ± SEM. Unpaired two-tailed Student's *t* test was used when comparing two groups. One-way ANOVA analysis of variance with Dunnett's test was applied to compare multiple groups. A *P* value <0.05 was considered statically significant for all experiments.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AD	atopic dermatitis
DRG	dorsal root ganglia
TSLP	thymic stromal lymphopoietin
FITC	fluorescein isothiocyanate
DNFB	2,4-dinitrofluorobenzene
CQ	chloroquine
AITC	allyl-isothiocyanate
MST	microscale thermophoresis
MrgprA3	Mas-related G-protein-coupled receptor member A3
TRPA1	transient receptor potential ankyrin 1
TRPV1	transient receptor potential vanilloid 1

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