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INVITED REVIEW

Anti-pruritic effect of isothiocyanates: Potential involvement of toll-like receptor 3 signaling

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Abstract

The innate immune system has an emerging role as a mediator of neuro-immune communication and a therapeutic target for itch. Toll-like receptor 3 (TLR3) plays an important role in itch, as shown in TLR3 knock-out mice. In this study, to evaluate effects of TLR3 inhibitors on histamine-independent itch, we used two kinds of isothiocyanate (ITC). Both phenethyl isothiocyanate (PEITC) and sulforaphane (SFN) inhibited Poly I:C (PIC)-induced signaling in the RAW264.7 cell line. We then investigated the anti-pruritic effect of these compounds on PIC- and chloroquine (CQ)-induced scratching behavior. PEITC and SFN both suppressed PIC-evoked scratching behavior in mice, and PEITC also inhibited CQ-induced acute itch. Finally, we examined the oxazolone-induced chronic itch model in mice. Surprisingly, oral dosing of both compounds suppressed scratching behaviors that were observed in mice. Our findings demonstrate that TLR3 is a critical mediator in acute and chronic itch transduction in mice and may be a promising therapeutic target for pruritus in human skin disorders. It is noteworthy that SFN has potential for use as an antipruritic as it is a phytochemical that is used as a supplement.

KEYWORDS
atopic dermatitis, chloroquine, isothiocyanate, itch, pruritus, sulforaphane, toll-like receptor 3

1 | INTRODUCTION

Chronic pruritus is a debilitating symptom that occurs in various conditions such as systemic and dermatological diseases, or as a side effect of medications; it is frequently refractory to treatment. According to the International Forum for the Study of Itch (IFSI), pruritus is defined as an uncomfortable sensation that elicits the desire to scratch; the chronic form lasts 6 weeks or longer.[1] The sensation of itch appears to be transmitted through the free nerve endings of C-fibers, which are mainly located in the epidermis and at the dermoepidermal junction. Atopic dermatitis (AD) is a common dermatologic disease that is accompanied by severe pruritus,[2,3] which is the most important symptom in this disease. Furthermore, in patients with AD, severe pruritus not only influences the patient's quality of life, but also elicits intense and persistent scratching, which aggravates the lesions. In treating AD, we thus need to develop an efficient strategy for controlling pruritus and scratching. However, the precise mechanisms of pruritus in AD remain unclear. It has been

Abbreviations: CQ, chloroquine; ITC, isothiocyanate; PBS, phosphate-buffered saline; PEITC, phenethyl isothiocyanate; PIC, poly I:C; SFN, sulforaphane; TLR, toll-like receptor.

reported that the number of the patients with AD is about 20 million in the United States alone, costing over 300 million USD annually, a figure that is still increasing year by year.[4] Worldwide, a number of pharmacological medications have been developed to treat AD, and certain biological agents and Janus kinase (JAK) inhibitors have recently become available for the treatment of AD, in addition to traditional drugs such as topical steroids and calcineurin inhibitors.[5–7] More recently, it was demonstrated that nemolizumab, an anti-IL31 receptor alpha subunit antibody, alleviated pruritus in AD patients[8] and this drug has now been released in Japan. The itch-scratch cycle has been considered as an exacerbating factor in dermatological diseases and termination of this cycle contributes to and effectively speeds treatment of these diseases; moreover, it is recognized that chronic pruritus may remain in some patients with AD whose skin lesions have been cured. For the above reasons, anti-pruritic drugs have been desperately sought in the treatment of AD.

In controlling pruritus, the role of the innate immune system is crucial, and mediators of neuro-immune communication[9,10] have thus become therapeutic targets for chronic pruritus. In particular, Toll-like receptor 3 (TLR3) plays an important role in itch, shown by the fact that its expression is enhanced in the non-lesional and/or lesional skin of patients with AD, psoriasis (PS) and prurigo nodularis (PN) and by the observation that TLR3 activators upregulate endothelin-1 (ET-1) and thymic stromal lymphopoietin (TSLP), a pruritogenic cytokine, in human keratinocytes.[11,12] Moreover, in areas of affected skin, the stratum corneum TLR3 expression level correlates with the severity score of the skin lesions and also tends to correlate with the degree of itching.[13] In addition, it has been reported that intradermal injection of a TLR3 activator elicits scratching behavior in mice by generating action potentials in dorsal root ganglia (DRG) neurons. The scratching behaviors evoked by both histaminergic and non-histaminergic pruritogens are prominently attenuated in TLR-3 knock-out or knock-down mice.[14] In addition, the level of TLR3 expression in the dry skin of mouse model is markedly upregulated and provokes robust scratching behaviors.[14]

From these observations, TLR3 is considered to be the key molecule for control of chronic pruritus in inflammatory skin diseases and modulation of this receptor could be a promising target for the treatment of chronic pruritus and skin diseases.

In the present study, we evaluated the anti-pruritic effect of the isothiocyanate-derived TLR3 inhibitors phenethyl isothiocyanate (PEITC) and sulforaphane (SFN) on both acute itch evoked by histamine-independent poly I:C (PIC) or chloroquine (CQ), and oxazolone-induced chronic itch, referred to as "an AD model in mice". It has been reported that PEITC and SFN inhibit the response mediated by TLR3 activation.[15,16] On the other hand, recently many literatures report more roles of ITCs (PEITC and SFN) for antimicrobial action, anti-cancer effects and neuroprotective actions in neurodegenerative diseases.[17–20] Although these ITCs certainly have a multitude of mechanisms, we assume anti-pruritic effects of PEITC and SFN are mainly exerted by inhibiting of TLR3 because of strong association between TLR3 and itch. We found that the oral administration of PEITC and SFN suppressed scratching behavior in both the acute and chronic itch models, without showing any sedative effect. These results suggest that TLR3 inhibitors might be promising drugs for the treatment of acute and chronic pruritus associated with human skin diseases.

2 | MATERIALS AND METHODS

2.1 | Cell culture and reagents

Cells from a mouse macrophage line, RAW264.7, were cultured in RPMI1640 containing 10% FBS (Fetal Bovine Serum) and penicillin/streptomycin. Synthetic dsRNA poly(I):(C) (PIC) was applied to the medium in order to evoke TLR3 signaling.

2.2 | Assessment of TLR3 antagonists in a RAW264.7 cell-based assay

RAW264.7 cells were seeded at 1×10^5 cells/well with RPMI1640 containing 10% FBS and penicillin/streptomycin in a 96-well culture plate and cultured for 24 h. The culture medium was then replaced with fresh medium that included PIC (a TLR3 agonist) and vehicle/ITCs. After 24 h, the culture medium was harvested. The content of nitrite in the culture medium was measured using a Nitrate/Nitrite Fluorometric Assay Kit procured from Cayman Chemical, and drug activity was assessed based on the nitrite content.

2.3 | Animals

Five-week-old CD1 male mice (Japan SLC, Inc.) and seven-week-old HR-1 female mice (Hoshino Laboratory Animals, Inc.) were purchased. These animals had free access to food and water in a specific pathogen-free animal room that was maintained at 24 ± 1°C with a 12-h light–dark cycle. This study was reviewed by the Animal Care and Use Committee and approved by the head of the test facility, and performed in accordance with the Guideline for the Animal Experiments, Research & Development Division, Toray Industries, Inc.

2.4 | Drugs

Poly I:C (PIC; Sigma-Aldrich and Yamasa), chloroquine diphosphate (CQ; Sigma-Aldrich) (an agonist of sensory neuron-specific GPCR MrgprA3), phenethyl isothiocyanate (PEITC; Sigma-Aldrich), D,L-sulforaphane (SFN; Santa Cruz Biotechnology) and 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone; Wako Pure Chemical Industries, Ltd.) were purchased. TRK-820 (nalfurafine hydrochloride) was synthesized at Toray Industries, Inc. PIC and CQ were dissolved in phosphate buffered saline (PBS) pH 7.4. PEITC and SFN were suspended in distilled water with 1% dimethyl sulfoxide and

1% Tween 80 (v/v). TRK-820 was dissolved in the same vehicle as PEITC and SFN. Oxazolone was dissolved in acetone: olive oil (4:1, v/v) as a 3% (w/v) solution.

2.5 | Measurement of scratching behavior

Scratching behavior in mice was automatically detected and objectively evaluated using MicroAct® (Neuroscience Inc.), as previously reported.[21,22] Briefly, under isoflurane anesthesia, a small parafilm®-coated magnet (1 mm in diameter, 3 mm in length) was implanted subcutaneously into the dorsa of both hind paws at least 5 days before scratching behavior was recorded. On the day of the experiment, mice were put into an observation chamber (11 cm in diameter, 18 cm high), which was surrounded by a round coil, for habituation for at least 30 min. Subsequently, the electric current induced in the coil by the movement of the magnet-implanted hind paws was recorded to measure the number of scratching behavior. The electric current data were analyzed using MicroAct® software ver. 2.15A.

2.6 | Acute itch induced by PIC- or CQ

On the day before behavioral recording for PIC- or CQ-induced scratching on CD1 mice, the fur on the rostral back was shaved. On the day of the experiment, mice were habituated to the observation chamber. PEITC, SFN or vehicle was administered orally 1 h before, and TRK-820 was administered orally 15 min before the intradermal injection of the pruritogen or vehicle. Immediately after the intradermal injection of PIC (500 μg/50 μl), CQ (100 μg/50 μl) or vehicle in the nape of the neck, each mouse was swiftly placed in an observation chamber and scratching behavior was measured.

2.7 | Chronic itch associated with dermatitis induced by oxazolone

All HR-1 mice were sensitized by an epicutaneous application of 100 μl/site of 3% oxazolone solution to the whole back. Seven days later, 60 μl/site of 3% oxazolone solution or vehicle was repeatedly applied to the rostral back area at one- or two-day intervals for at least 4 weeks or more. Two days before the evaluation of the effects of compounds, scratching behavior was measured according to the procedure described above for each group, and then the last application of oxazolone or vehicle was performed. Oxazolone-treated mice were divided into four groups based on the number of scratching behavior over 1 h. Two days after the last application of oxazolone or vehicle, mice were habituated to the observation chamber, and then measurement of scratching behavior was performed 1 h after the oral administration of PEITC, SFN or vehicle, or 15 min after administration of TRK-820. PEITC or SFN was evaluated in two independent experiments.

2.8 | Wheel-running test

Each CD1 mouse was individually placed into the wheel of an apparatus (KN-78; Natsume Seisakusho Co, Ltd.) 1 h after the oral administration of PEITC, SFN or vehicle, and then the number of wheel rotations was recorded for 30 min.

2.9 | Data analysis

The data were presented as means and SEM. Student's *t*-test was used to analyze differences between the two groups. Multiple comparisons among the test substance (PEITC, SFN or TRK-820)-administered groups and the vehicle-administered group were performed using Dunnett's test or Steel's test, based on the results of Bartlett's test for homogeneity of variances. Values of $p < .05$ were considered to be statistically significant. For statistical analysis, SAS ver. 9.1.3 (SAS Institute Japan) was used.

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,[23] and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.[24]

3 | RESULTS

3.1 | Inhibitory effects of ITCs on TLR3 signaling

To investigate inhibitory effects of PEITC and SFN on the cellular response mediated by activation of TLR3 signaling, we used the mouse macrophage RAW264.7 cell line. As expected, PIC-stimulated induction of nitrite was inhibited by both PEITC and SFN, with respective IC_{50}s of 10.3 and 7.2 μmol/L (Figure 1).

3.2 | Effects of ITCs on PIC-induced scratching behavior

The effects of PEITC and SFN on PIC-induced scratching behavior are shown in Figure 2. One hour after the administration of PEITC or SFN, PIC (500 μg/site) was injected intradermally in the nape of the neck and the number of scratching behavior was measured for 30 min. PEITC (100 or 300 mg/kg, p.o.) and SFN (100 mg/kg, p.o.) significantly inhibited PIC-induced scratching behavior. TRK-820 (0.1 mg/kg, p.o.) was administered 15 min before the intradermal injection of PIC as a positive control of the anti-pruritic agent, and also inhibited scratching behavior.

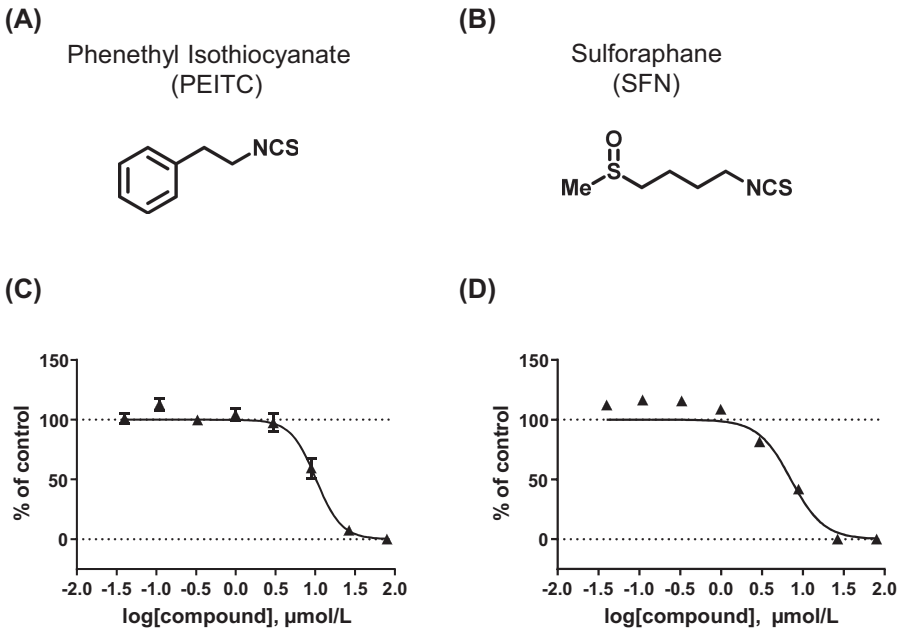

FIGURE 1 Isothiocyanate derivatives (ITCs) and their inhibitory effects on TLR3. Phenethyl isothiocyanate (PEITC) (A) and sulforaphane (SFN) (B) were evaluated in a RAW264.7 cell-based assay. RAW264.7 cells were seeded at 1×10^5 cells/well and cultured for 24 h. The culture medium was changed to fresh medium that include PIC (TLR3 agonist) and vehicle/ITCs. After 24 h, culture medium was harvested. The nitrite content in the culture medium was measured by Nitrate/Nitrite Fluorometric Assay Kit and drug activity was assessed with the nitrite contents for PIC (C) and SFN (D).

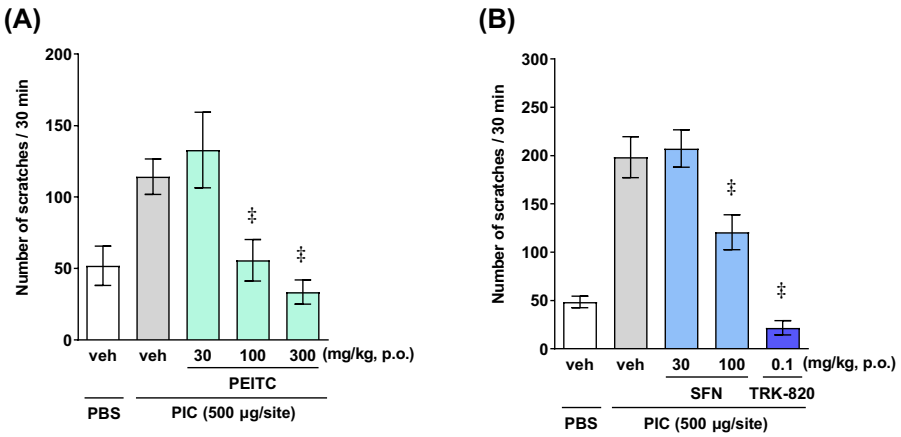

FIGURE 2 Effects of ITCs on PIC-induced scratching behavior. The ICR mice were administered oral PEITC (A) or SFN (B) and 1 h later, PIC (500 μg/site) or PBS was injected intradermally. TRK-820 (B) was administered p.o. 15 min before i.d. injection of PIC. Immediately after the i.d. injection, scratching behavior was recorded for 30 min. Ordinate represents the number of scratching events over 30 min (means and SEM). Vehicle/PBS group: $n = 8$ per group, SFN (30 mg/kg)/PIC group: $n = 9$ per group, the other groups: $n = 10$ per group. $^{‡}p < .05$, significantly different from vehicle/PIC-treated group (Steel's test).

3.3 | Effect of ITC on the CQ-induced scratching behavior

The effect of PEITC on CQ-induced scratching behavior is shown in Figure 3. One hour after the oral administration of PEITC, CQ (100 μg/site) was injected intradermally in the nape of the neck and the number of scratching behavior was measured for 30 min. PEITC (100 mg/kg, p.o.) significantly attenuated CQ-induced scratching behavior.

3.4 | Effects of ITCs on spontaneous motor activity

PEITC and SFN exerted an inhibitory effect on scratching behavior, but raised concerns that this effect might simply be associated with decreased motor activity, for example, due to sedation. To test this possibility, the effects of PEITC and SFN on spontaneous motor activity was evaluated with a wheel-running test in CD1 strain mice (Figure 4). PEITC (30, 100 or 300 mg/kg) and SFN (30 or 100 mg/kg) were administrated orally 1 h before measurement. Neither agent affected spontaneous motor activity at the effective dose for acute itch induced by PIC or CQ.

3.5 | Effects of ITCs on scratching behaviors associated with dermatitis induced by oxazolone

The effects of PEITC and SFN on scratching behavior associated with oxazolone-induced dermatitis are shown in Figure 5. This study

was performed 2 days after the last application of oxazolone to the skin. One hour after the oral administration of PEITC or SFN, the number of scratching behavior was measured for 1 h. PEITC and SFN (both 100 mg/kg, p.o.) significantly inhibited dermatitis-associated scratching behavior. TRK-820 (0.05 mg/kg, p.o.) was administered 15 min before the measurement, and had an inhibitory effect on dermatitis-associated scratching behavior.

4 | DISCUSSION

To date, it has been reported that several ITCs inhibit the response mediated by TLR3 activation, but it remains unclear whether those compounds have anti-pruritic effects.[15,16] It has been reported in the patent literature that an extract from cruciferous plants improved skin lesion and reduced itch in psoriasis patient (US8158161 B2). However, this test was conducted with a plant extract that included various ingredients in addition to certain ITCs. Thus, it was not clear whether specific ITCs had such effects because a purified single component extracted from the plants was not applied to the patient in the test. In this study, we demonstrate not only an inhibitory effect of ITCs on the cellular response induced by a TLR3 agonist with an in vitro, cell-based assay but also an anti-pruritic effect on scratching behavior in mice in a model of acute and chronic itch, using two structurally similar and naturally occurring ITCs, PEITC and SFN.

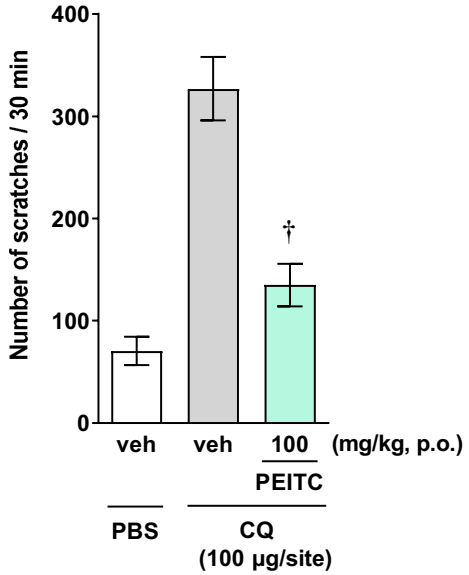

FIGURE 3 The ICR mice were administered oral PEITC and 1 h later, CQ (100 μg/site) or PBS was injected intradermally. Immediately after the i.d. injection, scratching behavior was recorded for 30 min. Ordinate represents the number of scratching events over 30 min (means and SEM). Vehicle/PBS group: $n = 8$ per group, vehicle/CQ group: $n = 9$ per group, PEITC/CQ group: $n = 10$ per group. $^{\dagger}p < .05$, significantly different from vehicle/CQ-treated group (Student's *t*-test).

It was revealed that TLR3 participated not only in acute itch induced by Compound 48/80 and chloroquine as well as PIC, but also in chronic itch in a dry-skin model that used TLR3 knock-out mice.[14] First, we developed a cell-based assay system using RAW264.7 cells to evaluate the TLR3-inhibitory effect of two ITCs, something previously observed in several reports.[15,16] We thus confirmed that PEITC and SFN inhibit TLR3 activation induced by PIC. Although nitric oxide was detected as an output of cell activation by PIC in this system and the IC_{50} was estimated to be 10.3 and 7.2 μmol/L, respectively, there was no marked difference in the potency of the inhibitory effects of ITCs based on the IC_{50} value in comparison to findings in previous reports.[15,16] Conversely, it was not clear whether a specific compound such as a TLR3 inhibitor will show similar in vivo anti-pruritic effects as well, despite showing inhibitory effect against TLR3 in vitro. Since we confirmed that both PEITC and SFN have inhibitory activity against TLR3 activation in vitro, their in vivo efficacies were tested based on scratching behavior elicited by experimental models of itch. We evaluated the anti-pruritic effect of PEITC and SFN against acute itch evoked by PIC and chloroquine in vivo. Both compounds clearly inhibited scratching behaviors evoked by PIC; moreover, PEITC decreased chloroquine-induced acute itch as well. These results revealed for the first time that a single dose of an orally administered compound was able to control acute, histamine-independent itch via TLR3 and MrgprA3 activation. Further evaluation will be needed to confirm the capability of SFN to reduce chloroquine-induced itch.

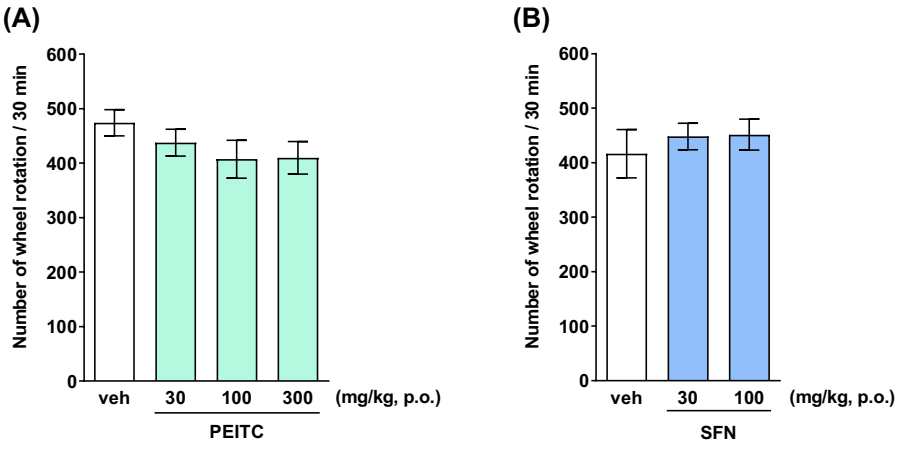

FIGURE 4 The ICR mice were given an oral administration of PEITC (A) or SFN (B). One hour later, the mice were placed in a wheel cage and spontaneous motor activity was measured for 30 min. Ordinate represents the number of wheel rotations in 30 min (means and SEM). $n = 10$ per group.

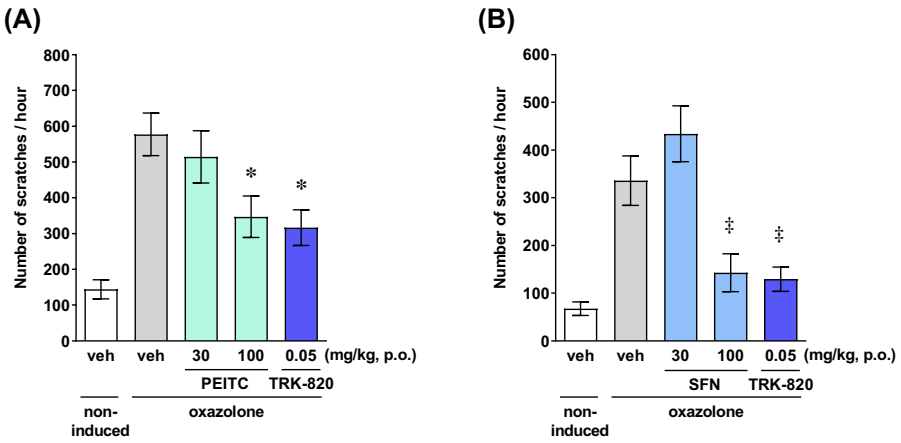

FIGURE 5 Effects of ITCs on scratching behavior associated with dermatitis induced by oxazolone. All HR-1 mice were sensitized over the whole back with oxazolone. To induce dermatitis, oxazolone was applied to the rostral back area repeatedly, three times per week for at least 4 weeks or more starting from 7 days after sensitization. Two days after the last application of oxazolone or vehicle, mice were given an oral administration of PEITC (A), SFN (B) or TRK-820. Measurement of scratching behavior was performed 1 h after p.o. of PEITC, SFN or vehicle, or 15 min after p.o. of TRK-820. Ordinate represents the number of scratching events in 1 h (means and SEM). Non-induced (vehicle-application) group, $n = 6$ (A) and $n = 8$ (B) per group. Oxazolone-application group, $n = 18$ per group. $^{*}p < .05$ (Dunnett's test) or $^{‡}p < .05$ (Steel's test), significantly different from vehicle/oxazolone-application group.

A prior report indicates that a Japanese horseradish extract that includes 6-MSITC, an ITC, improved skin condition and suppressed scratching behavior in a model of atopic dermatitis in HR-1 mice that were given a special diet lacking in magnesium.[25] However, we could find no references to itch suppression using either PEITC or SFN in the literature. Moreover, this experiment was conducted with a Japanese horseradish extract that included various ingredients in addition of 6-MSITC. Thus, it was not clear whether 6-MSITC had such effects because a purified single component extracted from Japanese horseradish was not administered to mice in this experiment. There is also a report in the patent literature that describes improvement of skin symptom in patients with atopic dermatitis who took Japanese horseradish extract in tablet form for 8 weeks. They refer to SFN in the patent specification but provide no details of data concerning an anti-pruritic effect of either PEITC or SFN prior to our study, or indeed any evidence of efficacy of these ITCs for atopic dermatitis-related itch. We thus developed an in-house atopic dermatitis model in mice, using repeated application of oxazolone to the back of the nape to evaluate the efficacy of PEITC and SFN against chronic itch. Surprisingly, both PEITC and SFN suppressed scratching behavior in mice with atopic dermatitis, with reductions of 53% and 72%, respectively, compared with vehicle. These results indicate that PEITC and SFN can be a medicine for atopic dermatitis. It is suggested that TLR3 pathway is activated in the chronic itch condition in the peripheral nerve and its activation results in the increase of the scratching behaviors, and then ITCs attenuate the responses through at least the blockade of the TLR3 pathway. However, it is not sure how oxazolone activates the TLR3 pathway in the peripheral nerve and if there are other pathways which the ITCs can involve. We will keep investigating the detailed mechanism of the ITCs to control itch.

While we adopted oral administration of two ITCs in the in vivo evaluation, it was also important to assess efficacy when they were applied to peripheral tissue, in order to determine whether ITCs can modulate scratching behavior at the level of the skin. We then evaluated the effect of topical application of ITC on scratching behavior in a mouse model, showing that topical application of ITCs to the skin did not suppress the scratching behavior evoked by intradermal chloroquine (the results are not shown).

Although TLRs are typically expressed by immune cells and glial cells, increasing evidence indicates that primary sensory neurons also express TLRs (e.g., TLR3, TLR4 and TLR9).[14,26–29] In cultured sensory neurons from E14 DRGs, TLR3 is localized to the soma and neurites, and is concentrated in filopodial structures along the leading edge of the growth cone.[26] It is also reported that functional TLR3 is expressed by primary sensory neurons that co-express this with TRPV1 and GRP, and it is known that this subset of nociceptors is indispensable for itch sensation.[14] Therefore, intradermal injection of PIC might evoke itch through TLR3 activation at the end of the primary sensory neuron in the skin. On the other hand, MrgprA3, which is the receptor for CQ, is expressed on itch-related DRG neurons (the NP2 sub-population) that co-express with TRPV1; CQ also causes itch through MrgprA3 expressed at the ends of this sub-population of neurons in skin.[30–33] Oral administration of ITCs could attenuate scratching behavior induced by CQ, but its topical application could not. These results imply that ITCs modulate scratching behavior induced by CQ through TLR3 at more proximal parts of the primary afferent, possibly in the cell soma of the DRG neuron, but not at the level of nerve endings in the skin. Our findings demonstrate that TLR3 is a critical mediator involved in acute and chronic itch transduction in mice, and may be a promising therapeutic target for pruritic disorders. It is noteworthy that SFN can potentially be

used as an antipruritic medicine since it is a phytochemical used as a supplement.

AUTHOR CONTRIBUTIONS

Participated in research and study design: Moriyama, Konno, Hayashi, Suzuki, Kainoh. Conducted experiments and the study: Moriyama, Konno, Serizawa, Yuzawa, Majima, Hayashi. Performed data analysis: Moriyama, Konno, Serizawa, Yuzawa, Majima. Wrote or contributed to the writing of the manuscript: Moriyama, Konno, Serizawa.

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FUNDING INFORMATION

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CONFLICT OF INTEREST

All authors are current or former employees of Toray Industries, Inc. M.K. and I.H. are listed as inventors on a patent application covering the use of sulforaphane described in this manuscript (Publication number: WO2017111069).

ETHICS STATEMENT

This study was reviewed by the Animal Care and Use Committee and approved by the head of the test facility, and performed in accordance with the Guideline for the Animal Experiments, Research & Development Division, Toray Industries, Inc.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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