(E6005 group, 7.7%; vehicle group, 23.1%). The strength of the most commonly used topical corticosteroid was "very strong" in both groups. The demographics and clinical characteristics of the vehicle group were not significantly different from those of the E6005 group.

Adverse events

Treatment-emergent adverse event (TEAE) is defined as an event occurring on or after the first dose of study drugs. Neither death nor serious TEAEs were encountered in the entire study period. In the randomization phase, the incidence of TEAEs was 50.0% (26/52 subjects) in the E6005 group and 38.5% (10/26 subjects) in the vehicle group. The incidence of TEAEs leading to study withdrawal was 9.6% (5/52 subjects) in the E6005 group and 15.4% (4/26 subjects) in the vehicle group. The incidence of treatment-related TEAEs that are possibly or probably related to the study drug or vehicle was 36.5% (19/52 subjects) in the E6005 group and 26.9% (7/26 subjects) in the vehicle group. The TEAEs that occurred in ≥5% of subjects in either treatment group were application site reactions, exacerbation of atopic dermatitis, nasopharyngitis, Kaposi's varicelliform eruption and pruritus (Table 2). The incidence of skin infections including cellulitis, furuncle, herpes simplex, impetigo, tinea pedis, Kaposi's varicelliform eruption, and acne was 9.6% (5/52 subjects) in the E6005 group and 15.4% (4/26 subjects) in the vehicle group. In the randomization phase, all TEAEs were CTCAE grade 1 or grade 2, except for one grade 3 hypertriglyceridemia in the vehicle group. Changes in laboratory values and body weight related to E6005 treatment were not observed.

Treatment-emergent adverse events were reported in 69.2% (36/52 subjects) in the group that applied E6005 for 12 weeks. TEAEs that occurred in ≥5% of subjects were application site reactions, nasopharyngitis, exacerbation of atopic dermatitis, urticaria, and acne. The overall TEAE profile of 0.2% E6005 treatment for up to 12 weeks was similar to that for the first 4 weeks.

Pharmacokinetic profiles

The plasma E6005 concentration was below the detection limit at all sampling points for all subjects. The plasma M11 concentration was detected in 34 of 72 subjects who were treated with at least one dose of E6005 (maximum value was 13.6 ng/mL). No major difference in TEAE profile was found between subjects with detectable plasma M11 and those without detectable plasma M11 (data not shown).

Clinical efficacy

Table 3 shows scores of EASI, SCORAD-objective, SCORAD-C, itch Behavioral Rating Scale, and severity of the targeted lesion in the randomization phase (LOCF). Although not statistically significant, the percent changes of EASI, SCORAD-objective and severity of targeted lesions at week 4 in the E6005 group were improved compared with those in the vehicle group. Figure 2 shows representative photographs achieving marked improvement in targeted lesions from two E6005-treated subjects.

Since several subjects in the vehicle group experienced rapid deterioration in their SCORAD-C score, the difference in percent change from baseline at week 4 between the E6005 and vehicle groups was large in the FAS analysis. However, the itch Behavioral Rating Scale did not show such a large difference (Table 3).

Also in the PPS analysis, the percent changes from baseline at week 4 (LOCF) of these efficacy parameters showed improvements in the E6005 group compared with those in the vehicle group. The differences in the percent changes in the PPS analysis were greater than those in the FAS analysis, possibly owing to the fact that the PPS analysis excluded subjects who applied <10% of the expected volume of ointment (E6005 or vehicle) during the study period and those who applied ointment during <70% of the study period.

Figure 3 shows the box plots of the scores for EASI, SCORAD-objective, SCORAD-C, and itch Behavioral Rating Scale at weeks 0 (baseline), 4, and 12 for subjects who were treated with E6005 in both the randomization and extension phases. Except for the itch Behavioral Rating Scale, these scores at week 12 were significantly reduced from those at baseline (EASI, $P = 0.030$; SCORAD-objective, $P < 0.001$; SCORAD-C, $P = 0.038$; and itch Behavioral Rating Scale, $P = 0.462$).

DISCUSSION

In the present study, the safety and efficacy of 0.2% E6005 ointment were evaluated in the initial 4-week randomization phase that included the use of a vehicle control. Subjects

Table 2. Treatment-emergent adverse events (TEAEs) occurring in ≥5% of subjects in either treatment group in the randomization phase

	E6005 ($n = 52$)		Vehicle ($n = 26$)	
	TEAE*	Treatment-related TEAE†	TEAE*	Treatment-related TEAE†
Application site reactions‡, *n* (%)	8 (15.4)	8 (15.4)	4 (15.4)	4 (15.4)
Exacerbation of atopic dermatitis, *n* (%)	7 (13.5)	7 (13.5)	3 (11.5)	2 (7.7)
Nasopharyngitis, *n* (%)	3 (5.8)	1 (1.9)	0 (0.0)	0 (0.0)
Kaposi's varicelliform eruption, *n* (%)	1 (1.9)	1 (1.9)	2 (7.7)	0 (0.0)
Pruritus, *n* (%)	0 (0.0)	0 (0.0)	2 (7.7)	1 (3.8)

*TEAE is defined as an event occurred on or after the first dose of study drug.
†Treatment-related TEAEs are TEAEs that are considered by investigators to be possibly or probably related to the study drug or vehicle.
‡Application site reactions include application site irritation, application site pruritus, application site warmth, and application site paraesthesia.

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Table 3. Summary of efficacy parameters in FAS and PPS populations at week 4 (LOCF)

	FAS			PPS		
	Baseline	Week 4	% Change	Baseline	Week 4	% Change
EASI						
E6005, Mean ± SD	9.2 ± 4.6	8.8 ± 8.0	−2.2 ± 105.1	8.8 ± 4.4	7.7 ± 6.8	−7.9 ± 108.8
Vehicle, Mean ± SD	9.4 ± 5.3	9.9 ± 9.1	6.7 ± 82.9	9.5 ± 5.4	10.5 ± 9.1	13.2 ± 83.7
Mean difference (95% CI)			−9.0 (−56.4, 38.4)			−22.4 (−74.5, 29.8)
SCORAD-objective						
E6005, Mean ± SD	31.1 ± 7.5	27.8 ± 11.6	−8.9 ± 36.1	30.7 ± 7.3	25.8 ± 10.3	−13.6 ± 35.2
Vehicle, Mean ± SD	29.1 ± 6.7	28.2 ± 13.4	−3.7 ± 39.0	29.1 ± 6.3	28.9 ± 13.5	−1.8 ± 39.0
Mean difference (95% CI)			−4.1 (−22.0, 13.8)			−10.5 (−29.2, 8.3)
SCORAD-C						
E6005, Mean ± SD	7.8 ± 4.3	7.6 ± 5.2	3.5 ± 58.0*	7.4 ± 4.0	6.8 ± 4.6	−1.8 ± 56.3†
Vehicle, Mean ± SD	8.8 ± 5.4	9.1 ± 5.0	46.9 ± 126.3	8.5 ± 5.3	9.6 ± 4.9	59.9 ± 128.4
Mean difference (95% CI)			−50.0 (−88.4, −11.7)			−69.5 (−110.2, −28.7)
Behavioral rating scale						
E6005, Mean ± SD	4.2 ± 1.4	4.3 ± 1.7	4.9 ± 42.3*	4.0 ± 1.3	4.0 ± 1.5	2.4 ± 43.8*
Vehicle, Mean ± SD	4.4 ± 1.4	4.4 ± 1.7	3.9 ± 39.4	4.4 ± 1.4	4.7 ± 1.5	11.3 ± 34.2
Mean difference (95% CI)			−0.9 (−19.9, 18.2)			−12.3 (−32.3, 7.7)
Severity of the targeted lesion						
E6005, Mean ± SD	6.1 ± 1.7	4.5 ± 2.2	−25.1 ± 36.9	6.0 ± 1.7	4.2 ± 2.1	−27.7 ± 36.2
Vehicle, Mean ± SD	5.7 ± 2.2	4.8 ± 3.0	−10.0 ± 53.3	5.7 ± 2.1	5.1 ± 3.0	−5.1 ± 54.1
Mean difference (95% CI)			−13.1 (−33.6, 7.4)			−20.8 (−42.6, 1.0)

CI, confidence interval; FAS, full analysis set; LOCF, last observation carried forward; PPS, per protocol set.
The % change represents the% change in the measure of efficacy at week 4 from baseline. The mean difference shows the least square mean difference of% changes in the measure of efficacy between the E6005 and vehicle groups (ANCOVA model). n = 52 for the E6005 group and n = 26 for the vehicle group in the FAS analysis, and n = 45 for the E6005 group and n = 23 for the vehicle group in the PPS analysis.
*n = 51.
†n = 44.

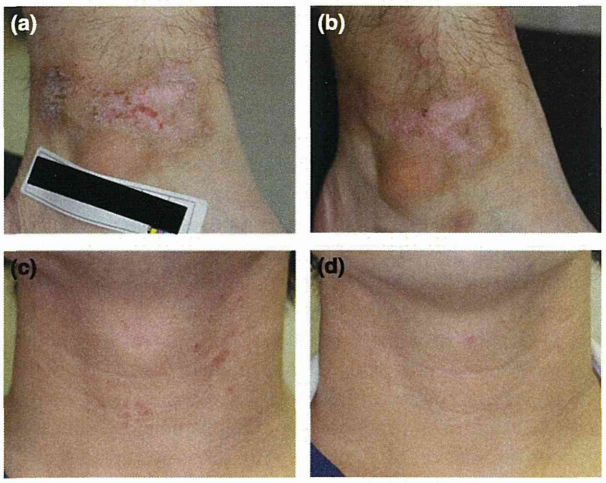

Figure 2. Clinical photographs of two subjects treated with E6005 ointment showing symptomatic improvement. In one subject, the total severity score of targeted lesion decreased from (a) 9 at baseline to (b) 3 at week 4 with improvement in the symptom of oozing/crust, excoriation and lichenification. In another subject, the score decreased from (c) 9 at baseline to (d) 4 at week 4 with improvement of erythema and excoriation.

who completed the randomization phase entered the 8-week extension phase in which all subjects were treated with E6005.

The present study showed that the overall AE profile for up to 12 weeks was similar to that for the first 4 weeks, indicating that 0.2% E6005 ointment was well tolerated. The most frequent TEAEs in both groups were application site reactions including burning, pain and warmth, and the incidence of application site reactions in the E6005 group (15.4%) was similar to that in the vehicle group (15.4%) in the randomization phase. Topical application of immunosuppressants such as pimecrolimus and tacrolimus which inhibit calcineurin-mediated T-cell activation is efficacious for AD treatment. However, these agents are associated with a high incidence of application site reactions such as skin burning and irritation.[17,18] Two phase III studies of 0.1% tacrolimus ointment in Japanese adults patients with AD showed high incidence of application site reactions compared with those in the corticosteroid groups (80.0% vs 6.6%, 59.1% vs 8.9%).[19,20] Furthermore, the application of 0.2% E6005 ointment did not significantly increase the incidence of skin infections at the application site. Although a direct comparison of the results of tacrolimus studies with E6005 cannot be performed, E6005 may be safer than other current topical treatments for AD.

The plasma E6005 concentration was below the detection limit in all subjects who participated in the E6005 analysis, suggesting that systemic absorption of E6005 was negligible or very low. The absorbed E6005 may be quickly metabolized into M11 by carboxylesterase in the liver.[21] In fact, *in vitro* human and animal liver microsomes rapidly hydrolyze E6005 into M11

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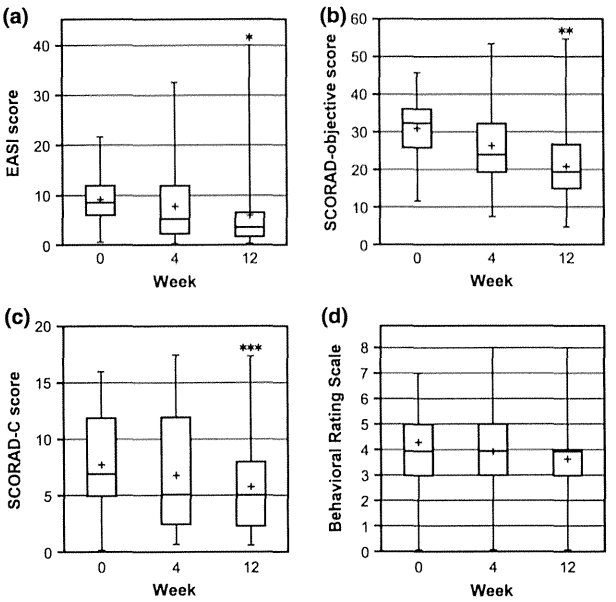

Figure 3. Box plots of efficacy parameters at baseline, week 4, and week 12. (a) Eczema Area and Severity Index (EASI), (b) Severity Scoring Atopic Dermatitis (SCORAD)-objective, (c) SCORAD-C (visual analog scales for pruritus and sleep loss), and (d) Behavioral Rating Scale. The box represents the interquartile range. Upper and lower bars include all outliers. Plus sign (+) and horizontal line (–) in boxes indicate the mean and median, respectively. The number of subjects who were treated with 0.2% E6005 ointment in both the randomization and extension phases was 52 at baseline and 47 at weeks 4 and 12. *P = 0.030, **P < 0.001, ***P = 0.038.

(S. Kawaguchi and K. Kusano 2014, unpublished data). In the present study, the plasma M11 concentration was quantifiable in 47% of subjects and the maximum concentration was found to be 13.6 ng/mL. When E6005 (4 mg/kg per day) at a no observed adverse effect level (NOAEL) was administered intravenously for 4 weeks to rats, the plasma concentration of M11 was in the range of 1093–1458 ng/mL (N. Katsutani, 2008, unpublished data), which was about 100-fold the level observed in the present study (13.6 ng/mL). Furthermore, after intravenous administration of ^{14}C labeled E6005 to rats, the radioactivity was found to be low in the central nervous system.[13] The potency of M11 in inhibiting isolated PDE4 enzyme (IC_{50} = 7.9 nmol/L) was one third of that of E6005 (IC_{50} = 2.8 nmol/L)[13] (also M. Shirato, 2009, unpublished data). However, the potency of M11 in suppressing the production of cytokines such as IL-2, IL-4, IL-12, TNFα, and IFN-γ in human monocytes (IC_{50} = 100–360 nmol/L) was about 1/100 that of E6005 (IC_{50} = 0.49–3.1 nmol/L), possibly due to the inferior membrane permeability of M11 compared with that of E6005 (N. Ishii, 2008, unpublished data). From these findings, we speculate that M11 resulting from the systemic absorption of E6005 is of little or no physiological consequence and has negligible toxicity.

The use of an oral PDE 4 inhibitor, roflumilast, as a COPD drug is limited because of systemic AEs such as diarrhea, emesis, weight loss, and headache, probably due to its agonistic effect on the α2 adrenoceptor.[22,23] In the present study, we found that treatment with 0.2% E6005 ointment up to 12 weeks did not induce systemic AEs. This suggests that there is low systemic absorption of E6005, low level of tissue distribution, and rapid metabolization of absorbed E6005 into M11.

At the end of week 4, EASI, SCORAD-objective, SCORAD-C and severity of the targeted lesions in the E6005 group tended to improve compared with those in the vehicle group. In the FAS analysis, the EASI percent change from baseline at week 4 was −2.2% in the E6005 group and 6.7% in the vehicle group, and the difference between the two groups was −9.0% (Table 3). In the PPS analysis, the corresponding difference of percent change in EASI between the two groups was −22.4%. Similarly, the magnitude of the changes in other parameters of efficacy including SCORAD-objective, SCORAD-C, itch Behavioral Rating Scale, and severity of targeted lesions were greater in the PPS analysis than in the FAS analysis. In the PPS analysis, subjects who were non-compliant with the application volume and/or period of the study drug were excluded before all analyses were initiated. The difference in efficacy results between FAS and PPS analyses suggests that adequate subject instruction on topical drug application including application amount, and adherence to the study protocol might improve the efficacy of E6005 treatment.

The group that received 0.2% E6005 ointment for 12 weeks showed a significant reduction in EASI (P = 0.030), SCORAD-objective (P < 0.001) and SCORAD-C (P = 0.038) from baseline. Before screening, more than half of these subjects had been treated with topical corticosteroids of strongest, very strong and/or medium potency. A washout phase was included and concomitant use of topical corticosteroids was not allowed during the entire study period. Taking these facts into consideration, our study suggests that treatment with 0.2% E6005 ointment for 12 weeks is efficacious; and E6005 ointment might be a desirable option especially for long-term use through prevention of flares in AD with its excellent safety profiles, although this will need further study.

In vitro studies showed that E6005 selectively inhibited human PDE4 activity with IC_{50} of 2.8 nmol/L, and suppressed the production of various cytokines from human lymphocytes and monocytes with IC_{50} values ranging 0.49–3.1 nmol/L.[13] The topical application of E6005 in oxazolone-induced dermatitis in NC/Nga mice produced an anti-inflammatory effect with reduced expression of cytokines and adhesion molecules.[13] In addition to the anti-inflammatory effect, E6005 produced an immediate antipruritic effects even after a single application. The immediate antipruritic action in mice may suggest that E6005 directly inhibits C-fiber nerve activation, which is responsible for the perception of itch and its transmission to the central nervous system,[13] based on the finding that a PDE4 inhibitor, rolipram, reduced the release of sensory neuropeptides from C-fiber endings.[24] It was found that isolated primary dorsal root ganglion (DRG) neurons from Wistar rats expressed PDE4B subtype protein. Furthermore, the intracellular cAMP level in DRG neurons was elevated by E6005, and

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the capsaicin-induced C-fiber nerve depolarization in DRG neurons was inhibited by E6005 (H. Wakita, M. Ohkuro, N. Ishii, I. Hishinuma and M. Shirato, unpublished data). On the other hand, protease activated protein 2 (PAR2) is implicated in the pathogenesis of AD[25–27] and pruritus.[28–30] Recently, E6005 was shown to inhibit PAR2-mediated pruritus in mice models (T. Andoh and Y. Kuraishi 2014, unpublished data).

In conclusion, treatment of AD with 0.2% E6005 ointment up to 12 weeks had an adequate safety profile, was well-tolerated, and the systemic distribution of E6005 was very limited. E6005 treatment for 12 weeks significantly improved efficacy scores from baseline for the affected area and intensity of eczema (EASI and SCORAD-objective) and pruritus (SCORAD-C). These findings supported that topical E6005 might be useful especially for long-term management in the treatment of AD. It is considered that a further study using a higher concentration (>0.2%) of E6005 ointment for adult AD patients would be valuable. Furthermore, the good safety profile of E6005 should prompt a clinical study to evaluate the safety and efficacy in pediatric AD patients.

ACKNOWLEDGMENTS: This study was funded by Eisai Co., Ltd., Tokyo, Japan. The authors thank all the collaborating members of the JAPANESE E6005 Study Investigators. Principal investigators and medical institutions are described in Appendix 1. We also thank the study team at Eisai Co., Ltd. for their support of the development of the study report and study operations including data management and monitoring.

CONFLICT OF INTEREST: This study was supported by Eisai Co., Ltd. Y.K., H.A. and S.H. are employees of Eisai Co., Ltd. H.N. received grants and research support from Eisai Co., Ltd. M.F., N.H. and H.N. served as consultants to Eisai Co., Ltd.

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APPENDIX 1

THE JAPANESE E6005 STUDY INVESTIGATORS

Osamu Nemoto (Sapporo Skin Clinic), Masaru Homma (Asahikawa Medical College Hospital), Takafumi Etoh (Tokyo Teishin Hospital), Atsuyuki Igarashi (NTT Medical Center Tokyo), Takaoki Ishiji (The Jikei University Hospital), Hiroyuki Yamao (Kitaayase Skin Clinic), Yasushi Suga (Juntendo University Urayasu Hospital), Tokuya Omi (Queen's Square Medical Center), Akimichi Morita (Nagoya City University Hospital), Juichiro Nakayama (Fukuoka University Hospital), and Satoshi Takeuchi (Kyushu University Hospital).

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proliferation and an enhancement of stimuli responses. Metabolic process genes are highly enriched in both hyper- and hypomethylated groups indicates that the shifting of metabolic processes might be important in skin ageing and may represent a fruitful area for further inquiry.

We also observed a locus of a keratin gene cluster is hypermethylated in ageing skin, and this has been partially cross-validated by a human counterpart study that both Krt1 and Krt16 are hypermethylated in skin ageing (20). However, omics-level study including MeDIP-chip is easily biased, the careful confirmation work is essential if any findings are interested to pursue further. Skin fibroblasts can be induced towards pluripotent stem cells by a combination of transcription factors, and it is essentially an epigenetic reprogramming process (30). Therefore, there is a likelihood that modulation of epigenetics could be an innovative anti-skin-ageing strategy in the future.

Acknowledgements

XX conceived this project, HQ performed the research, and XX wrote the paper. This work was supported by Yale University. We appreciate LEN for reviewing/editing the manuscript.

Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Genome-wide distribution of skin age-induced DNA methylation patterns.

Figure S2. Gene clusters change their methylation status with skin ageing.

Table S1. Age-induced hypermethylated promoters.

Table S2. Age-induced hypomethylated promoters.

Data S1. Materials and methods.

DOI: 10.1111/exd.12377
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Letter to the Editor

Topical E6005, a novel phosphodiesterase 4 inhibitor, attenuates spontaneous itch-related responses in mice with chronic atopy-like dermatitis

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Abstract: Atopic dermatitis is a chronic inflammatory cutaneous disease with difficult-to-treat pruritus. Although phosphodiesterase (PDE) 4 inhibitors have an anti-inflammatory effect on inflammatory skin diseases, such as atopic dermatitis, their acute antipruritic activities remain unclear. Therefore, in this study, we examined whether E6005, a novel PDE4 inhibitor, has antipruritic activity in dermatitis, using a mouse model of atopic dermatitis (NC/Nga mice). A single topical application of E6005 inhibited spontaneous hind-paw scratching, an itch-associated response and spontaneous activity of the cutaneous nerve in mice with chronic dermatitis. The cutaneous concentration of cAMP was significantly decreased in mice with chronic dermatitis, and this decrease was reversed by topical E6005 application. These results suggest that E6005 increases the cutaneous concentration of cAMP to relieve dermatitis-associated itching. Thus, E6005 may be a useful therapy for pruritic dermatitis such as atopic dermatitis.

Key words: atopic dermatitis – cAMP – itch – mice – phosphodiesterase

Accepted for publication 12 March 2014

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Background

Atopic dermatitis is a chronic inflammatory skin disease characterized by severe difficult-to-treat pruritus (1). Itching in atopic dermatitis may be mediated by manifold mediators, including interleukin-31 (2), thymic stromal lymphopoietin (3), tryptase (4), leukotriene B_4 (5) and sphingosylphosphorylcholine (6) and may not be fully relieved by selective receptor antagonists. Therefore, effective antipruritic medicines with novel mechanisms of action are required for the treatment of atopic dermatitis. Phosphodiesterase 4 (PDE4), which catalyses the conversion of cAMP to 5′-AMP, plays a critical role in the pathogenesis of inflammatory disorders. PDE4 inhibitors exert anti-inflammatory influences in inflammatory diseases (7–11), and topical application of PDE4 inhibitors exerts anti-inflammatory effects in atopic dermatitis (12). However, it is unclear whether PDE4 inhibitors have acute antipruritic activity in atopic dermatitis.

Questions addressed

E6005 was recently developed as a topically available selective PDE4 inhibitor and has anti-inflammatory and antipruritic activities in allergic contact dermatitis in mice (13). However, it remains unclear whether E6005 has antipruritic activity in chronic dermatitis. Therefore, we examined the acute effects of topical E6005 application on spontaneous itching behaviour, spontaneous activity of the cutaneous nerve and the cutaneous concentration of cAMP in mice with chronic dermatitis.

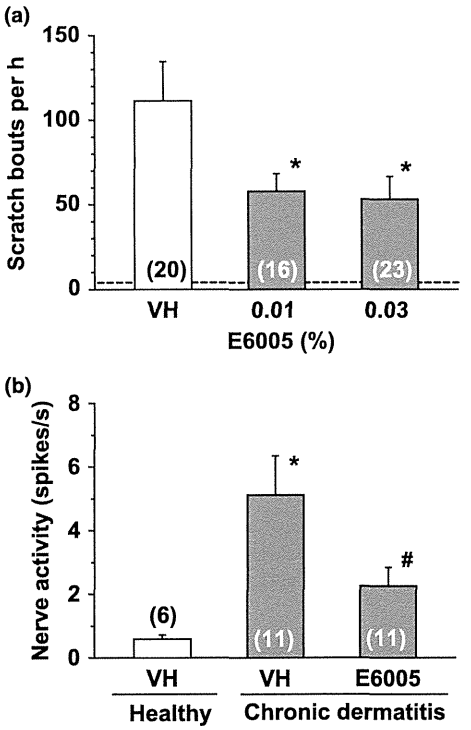

Figure 1. Effects of the PDE4 inhibitor E6005 on spontaneous scratching and cutaneous nerve firing in mice with dermatitis. (a) Effects of E6005 on spontaneous scratching. E6005 and vehicle (VH) were applied topically to the hair-clipped rostra back 1 h before the start of observation. The number of scratching episodes was counted for 1 h. A dashed line indicates the number of spontaneous scratching in healthy controls (n = 8). *P < 0.05 vs VH (Holm-Šidák test). (b) Effect of E6005 on spontaneous activity of the cutaneous nerve. The firing of the cutaneous nerve innervating the rostral back including the hair-clipped region was electrophysiologically recorded for 30 min. E6005 (0.03%) and VH were applied to the hair-clipped skin 1 h before recording. *P < 0.05 vs VH in healthy mice, #P < 0.05 vs VH in mice with dermatitis (Holm–Šidák test). Values represent mean ± SEM. The number in parentheses indicates the number of animals used.

Experimental design

Experimental procedures are detailed in Data S1. Briefly, Male NC/Nga mice with chronic dermatitis (14) and healthy NC/Nga mice were used. E6005, dissolved in an acetone–ethanol mixture, was applied to the hair-clipped rostral back. Observation of hind-paw scratching and electrophysiological recording of the cutaneous nerve branch were conducted according to our previous study (15). The effects of topical application of E6005 were evaluated 1 h after application, because topical application of the vehicle elicited hind-paw scratching for 40 min (Figure S1). The cutaneous concentration of cAMP was measured by enzyme immunoassay.

Results

Although mice with chronic dermatitis scratched throughout the rostral part of the body and head with the hind paws, in this study, we counted scratching of the hair-clipped skin. Healthy mice scratched only several times per hour, while mice with chronic dermatitis frequently scratched the hair-clipped skin (Fig. 1a). A single topical application of E6005 significantly inhibited spontaneous scratching during 1–2 h after application in mice with chronic dermatitis; the inhibition was partial and similar between 0.01% and 0.03% (Fig. 1a).

Spontaneous activity of the cutaneous nerve innervating the rostral back was recorded from 1 to 1.5 h after topical application. The spontaneous activity was as low as 0.6 spikes/s in healthy mice and markedly increased in mice with chronic dermatitis (Fig. 1b). Topical application of 0.03% E6005 to the rostral back significantly inhibited the increased activity of the cutaneous nerve in mice with chronic dermatitis (Fig. 1b). E6005 (0.03%) did not affect nerve activity evoked by punctuate stimulation with a von Frey filament (Figure S2).

The cutaneous concentration of cAMP was significantly decreased in mice with chronic dermatitis (Fig. 2). The decreased concentration of cAMP was significantly reversed 1 h after topical application of 0.03% E6005 in these mice (Fig. 2).

Conclusions

We found that a single topical application of the novel PDE4 inhibitor E6005 inhibited spontaneous hind-paw scratching in mice with chronic dermatitis, suggesting acute antipruritic activity. A single topical application of E6005 reduced the increased activity of the cutaneous branch of the nerve innervating the lesioned skin. These results suggest that E6005 acts locally on the lesioned skin to inhibit the generation of itch

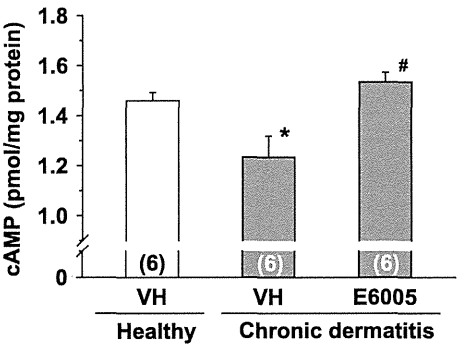

Figure 2. Effect of E6005 on cAMP concentration in the healthy and chronic lesioned skin of mice. E6005 (0.03%) and vehicle (VH) were applied topically to the rostral part of the back 1 h before skin sampling. The concentration of cAMP was measured using a cAMP enzyme immunoassay kit. Values represent mean ± SEM (n = 6). *P < 0.05 vs VH in healthy skin, #P < 0.05 vs VH in dermatitis skin (Holm-Šidák test). The number in parentheses indicates the number of animals used.

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signals. Topical application of E6005 did not affect mechanical stimulation-evoked nerve activity, suggesting that the inhibitory effects of E6005 are not due to nervous damage or local anaesthetic action.

The cutaneous concentration of cAMP was decreased in mice with dermatitis, and topical E6005 application reversed the decreased concentration. PDE4 is expressed in the keratinocytes (16). Thus, it is possible that the activation of PDE4 (a decrease in the cAMP concentration) in the epidermal keratinocytes is responsible for chronic dermatitis-associated itching and that the inhibition of activated PDE4 relieves the itching. The mechanisms of the cAMP-mediated antipruritic effect remain unclear. Protein kinase A inactivates 5-lipoxygenase through the phosphorylation of its Ser^{523} residue, leading to a decrease in leukotriene B_4 production (17). Leukotriene B_4 is a potent itch mediator (18) acting on the BLT1 leukotriene B_4 receptor in primary afferents (19). Leukotriene B_4 produced in keratinocytes is involved in spontaneous scratching in mice with dermatitis (5) and proteinase-activated receptor 2-mediated scratching (20). Thus, it is conceivable that an increase in cAMP concentration by E6005 results in the inhibition of increased production of leukotriene B_4 in keratinocytes in dermatitis.

Tryptase release from mast cells is increased in the lesioned skin of patients with atopic dermatitis (21) and NC mice with chronic dermatitis (4). PDE4 inhibitors suppress the degranulation of mast cells through the increase in intra-cellular cAMP (22). Inflammatory cells including T cells are increased in the skin of NC mice with dermatitis (23), T-cell cytokines including interleukin-31 are associated with scratching (2,24), and PDE4 inhibitors suppress the activation and cytokine release of these cells (25). Thus, the anti-inflammatory actions may also contribute to the antipruritic effects of E6005. Sensory C-fibres play an important role in spontaneous scratching in NC mice with dermatitis (26). The sensitivity of C-fibres to heat and chemical stimuli are increased or not affected by cAMP (27), and protein kinase A activation reduces TRPV1 channel desensitization (28). Thus, the direct action of E6005 on primary afferents may not play a role in its antipruritic effect.

The present study showed that E6005, a novel PDE4 inhibitor, has acute antipruritic activity in chronic dermatitis. Thus, E6005 is expected to be useful for the treatment of pruritic chronic dermatitis including atopic dermatitis.

Acknowledgements

This study was funded by Eisai Co. Ltd. Tokyo, Japan.

Author contributions

TA and YK designed experiments, and TA and TY performed experiments. TA and YK wrote the manuscript.

Conflict of interests

The authors do not report any conflict of interest relevant to this study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Figure S1. Hind-paw scratching following topical application of vehicle for E6005 in mice.

Figure S2. E6005 does not inhibit cutaneous nerve activity evoked by mechanical stimulation in mice.

DOI: 10.1111/exd.12382
www.wileyonlinelibrary.com/journal/EXD

Letter to the Editor

Genotype analysis in Hungarian patients with multiple primary melanoma

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Abstract: Multiple primary melanoma patients (MPMps) have better prognosis and are more prone to genetic predisposition than single melanoma patients. We aimed to compare genetic background (*CDKN2A, CDK4, MITF, MC1R*) of 43 Hungarian

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Pharmacological Reports 66 (2014) 699–703

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Short communication

Involvement of leukotriene B_4 in dermatophyte-related itch in mice

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ARTICLE INFO

Article history:
Received 20 June 2013
Received in revised form 27 December 2013
Accepted 3 January 2014
Available online 26 April 2014

Keywords:
Dermatophyte
Itch
Leukotriene B_4
5-Lipoxygenase
Proteinase-activated receptor 2

ABSTRACT

Background: Proteinase-activated receptor-2 (PAR_2) is involved in dermatophyte-induced scratching and leukotriene B_4 (LTB_4) release from keratinocytes. We investigated whether PAR_2-mediated LTB_4 production is involved in dermatophyte-induced scratching.
Methods: Dermatophyte extract was injected intradermally and scratching was observed in mice. LTB_4 was determined by enzyme immunoassay.
Results: Dermatophyte extract-induced scratching was inhibited by zileuton (5-lipoxygenase inhibitor), ONO-4057 (LTB_4 antagonist), FSLLRY-NH_2 (PAR_2 antagonist), and anti-PAR_2 antibody. Dermatophyte extract injection increased the cutaneous content of LTB_4, which was inhibited by zileuton and FSLLRY-NH_2.
Conclusion: These results suggest the involvement of LTB_4 in dermatophyte-associated itch. LTB_4 production might be due to PAR_2 stimulation in the skin.
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Introduction

Dermatophytosis (or tinea) is caused by a fungal infection, typically by dermatophytes. The most common functional symptom of tinea pedis is pruritus, which is reported by about 60% of patients with moderate or severe tinea pedis [9]. Details of the underlying mechanisms of dermatophytosis-associated pruritus remain poorly understood. During the infection process, dermatophytes secrete various enzymes, including proteases [15]. Of these secreted enzymes, proteases, and especially endopeptidases (proteinases), cause itching in humans and itch-related behavior in mice when administered to the skin [8,18]. Proteinase-activated receptor 2 (PAR_2) has been implicated as having an important role in proteinase-mediated itching [18].

Trichophyton mentagrophytes, including *Arthroderma vanbreuseghemii* (ADV), is known to infect both animals and humans [10]. Intradermal injection of ADV extract into the rostral back of mice elicits an itch-related response (hind-paw scratching), which has been shown to be suppressed by both a serine protease inhibitor and a PAR_2 antagonist [7]. Taken together, these findings suggest the possibility that proteinases secreted by dermatophytes cause itching through the stimulation of PAR_2 receptors in the skin.

An injection of SLIGKV-NH_2 (tethered ligand peptide of human PAR_2 receptor) into the human skin provokes itch and pain [17]. Injections of pruritogenic and algogenic substances (such as histamine and capsaicin, respectively) into the murine cheek elicit hind-paw scratching and pain-related behavior (forelimb wiping) of the injection site, respectively [16], and an injection of SLIGRL-NH_2 (tethered ligand peptide of murine PAR_2 receptor) into the murine cheek provokes marked hind-paw scratching and little forelimb wiping, suggesting that stimulation of PAR_2 receptor in the skin mainly induces itching in mice [1]. Similarly, an injection of ADV extract into the murine cheek increased markedly hind-paw scratching and only slightly forelimb wiping, suggesting that ADV extract is pruritogenic with weak algogenic activity [7]. ADV extract-induced scratching is inhibited by a PAR_2 antagonist, which suggests that ADV extract-induced scratching is mediated by PAR_2 receptors in the skin [7].

PAR_2 receptors are expressed in epidermal keratinocytes [17] and nerve fibers in the skin [17]. Because dermatophytes infect the superficial skin and require keratin for growth [12], the epidermis may be a key site of pruritic dermatophyte infection. Epidermal keratinocytes produce itch mediators [5,6]. Of these, leukotriene (LT) B_4, a potent itch mediator [2], is produced by keratinocytes in response to pruritogenic stimuli [5,6] and is involved in dermatitis-associated pruritus [4]. The activation of PAR_2 receptors on keratinocytes has been shown to induce LTB_4 production [20]. Therefore, the purpose of this study was to determine whether

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http://dx.doi.org/10.1016/j.pharep.2014.01.003
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LTB_4 produced by epidermal keratinocytes is involved in itching induced by ADV extract.

Materials and methods

Animals

Male Slc:ICR mice (5–8 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). They were housed in a room under controlled temperature (21–23 °C), humidity (45–65%), and light (7:00 AM to 7:00 PM) conditions. Food and water were available ad libitum. The study protocol was approved by the Committee for Animal Experiments at the University of Toyama.

Materials

Zileuton (Sigma, St. Louis, MO, USA), indomethacin (Sigma), and 5-[2-(2-carboxyethyl)-3-[6-(4-methoxyphenyl)-5E-hexenyl] oxyphenoxy] valeric acid (ONO-4057; Ono Pharmaceutical Co., Ltd., Osaka, Japan) were dissolved in 0.5% carboxymethyl cellulose (Wako Pure Chemical Ind., Osaka, Japan). Diclofenac sodium (Research Biochemical Inc., Natick, MA, USA) was dissolved in tap water. Zileuton and ONO-4057 were administered orally 1 h before ADV extract injection, and indomethacin and diclofenac were administered orally 30 min before ADV extract injection. FSLLRY-NH_2 (selective PAR_2 antagonist peptide) was dissolved in saline and was injected intradermally together with ADV extract. Anti-PAR_2 monoclonal antibody (SAM-11, which has epitope of amino acid 37–50 of human PAR_2 containing the tethered ligand sequence and binds to human and murine PAR_2, according to the manufacturer's specifications; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and non-specific mouse IgG were dissolved in phosphate-buffered saline and injected intradermally 10 min before ADV extract injection. The doses of zileuton, ONO-4057, indomethacin, diclofenac, FSLLRY-NH_2, and antibody SAM-11 were selected from the published literature [5,6,18].

Dermatophytes and extract preparation

The dermatophyte ADV (ID #58445) was obtained from the National BioResource Project (http://www.nbrp.jp/). It was cultured in Sabouraud's dextrose liquid culture medium at 37 °C for more than 3 days. ADV extract was prepared as described previously [7].

Behavior experiments

The day before behavior experiments, the animals' hair was clipped over the rostral part of the back. For acclimation, mice were placed individually in an acrylic cage composed of four cells (13 cm × 9 cm × 35 cm) for at least 1 h prior to testing. Immediately after intradermal injection, mice were returned to the same cells, and their behaviors were videotaped for 1 h. No personnel were present in the observation room during this time. Playback of the video allowed for counting of injection site scratching by the hind paw. The series of movements in which the mice stretched either hind paw toward the injection site, leaned the head toward it, and rapidly scratched several times for about 1 s were considered as one bout of scratching [7].

LTB_4 enzyme immunoassay

The skin (8 mm in diameter) at the injection site was removed 5 min after injection, immediately weighed, shredded using scissors, and put into 0.2 mL of ice-cold ethanol containing 10 μM indomethacin and 10 μM zileuton. Lipid extraction and LTB_4 enzyme immunoassay were performed as previously described [4].

Data processing

Data are presented as means ± standard error of the mean (SEM). Data were analyzed with a Student's *t*-test, two-way repeated measures analysis of variance (ANOVA) and *post hoc* Tukey's test, or one-way ANOVA and *post hoc* Dunnett's test or Holm–Šidák test; $p < 0.05$ was considered significant. The statistical analyses were performed using Sigmaplot graphing and statistical software (version 11; Systat Software, Inc., Chicago, IL, USA).

Results

Scratching behavior induced by ADV extract

An intradermal injection of ADV extract (20 μg/site) into the rostral back elicited hind-paw scratching toward the injection site, inducing no other apparent behavioral responses. This effect peaked during the first 10-min period and almost completely subsided after 30 min (Fig. 1A). When scratching during the 1-h duration was compared between treatment groups, we found that ADV extract increased scratching bouts 2.2-fold compared to control (Fig. 1B).

Effects of inhibitors of 5-lipoxygenase, cyclooxygenase, and the LTB_4 receptor on ADV extract-induced scratching

Scratching induced by ADV extract (20 μg/site) was markedly inhibited by oral pretreatment with the 5-lipoxygenase inhibitor zileuton (10–100 mg/kg) and the LTB_4 receptor antagonist ONO-4057 (10–100 mg/kg). Nearly complete inhibition was observed after treatment with 30 and 100 mg/kg of zileuton or treatment

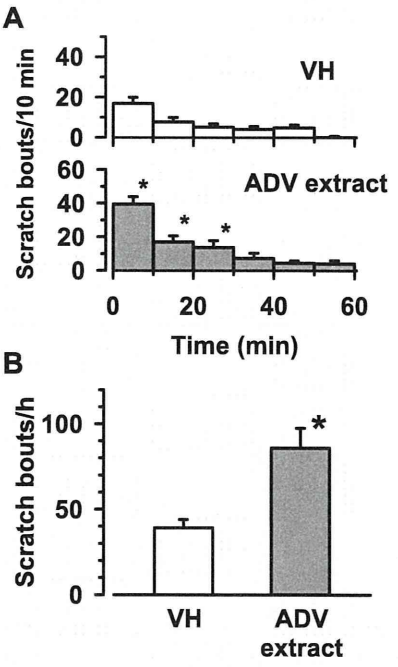

Fig. 1. Scratching response to an intradermal injection of dermatophyte extract into the rostral back in mice. Hind-paw scratching of the injection site was counted for 60 min after an intradermal injection of an extract of the dermatophyte *Arthroderma vanbreuseghemii* (ADV) or vehicle (VH). (A) Time-course of scratching after VH and ADV extract (20 μg/site) injections. *$p < 0.05$ vs. VH (Tukey's test). (B) Total number of scratching bouts for 1 h. *$p < 0.05$ (Student's *t*-test). The values represent the mean ± SEM ($n = 16$).

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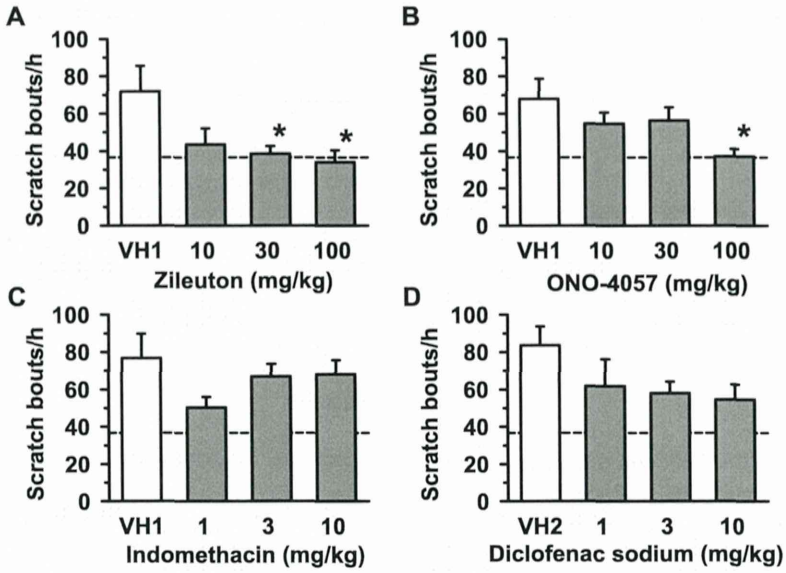

Fig. 2. Effects of inhibition of eicosanoid systems on dermatophyte extract-induced scratching in mice. Hind-paw scratching of the injection site was counted for 60 min after an intradermal injection of *Arthroderma vanbreuseghemii* (ADV) extract (20 μg/site). (A) Zileuton or vehicle (VH1, 0.5% carboxymethyl cellulose) was administered orally 60 min before ADV extract injection. $n = 8$ in each group. (B) ONO-4057 or VH1was administered orally 60 min before ADV extract injection. $n = 16$ each group. (C) Indomethacin or VH1 was administered orally 30 min before ADV extract injection. $n = 8$ in each group. (D) Diclofenac sodium or VH2 (tap water) was administered orally 30 min before ADV extract injection. $n = 8$ in each group. Dashed lines represent the average value of scratch bouts in mice given intradermal injection of saline. The values represent the mean ± SEM. $^*p < 0.05$ vs. VH1 or VH2 (Dunnett's test). These experiments were performed twice and similar results were obtained.

with 100 mg/kg of ONO-4057 (Fig. 2A and B). Oral pretreatment with the cyclooxygenase inhibitors indomethacin (1–10 mg/kg) or diclofenac sodium (1–10 mg/kg) had a tendency to decrease the ADV extract-induced scratching, but the effects were not statistically significant (Fig. 2C and D).

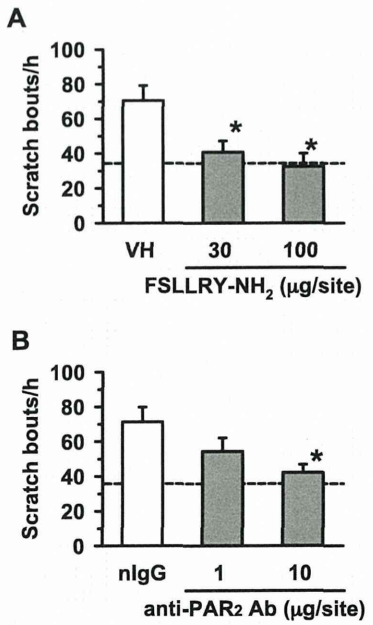

Fig. 3. Effects of PAR_2 antagonist and anti-PAR_2 antibody (Ab) on dermatophyte extract-induced scratching in mice. Hind-paw scratching of the injection site was counted for 60 min after an intradermal injection of *Arthroderma vanbreuseghemii* (ADV) extract (20 μg/site). (A) ADV extract was injected intradermally with or without the PAR_2 antagonist FSLLRY-NH_2 (30 and 100 μg/site). (B) Anti-PAR_2 monoclonal Ab (1 and 10 μg/site) and non-specific IgG (nIgG, 10 μg/site) were injected intradermally 10 min before ADV extract injection. The dashed line represents the average value of scratch bouts in mice given an intradermal injection of saline. The values represent the mean ± SEM ($n = 8$). $^*p < 0.05$ vs. nIgG (Dunnett's test). These experiments were performed twice and similar results were obtained.

Effects of PAR_2 antagonist and anti-PAR_2 antibody on ADV extract-induced scratching

Scratching induced by ADV extract (20 μg/site) was markedly inhibited by intradermal co-injection with FSLLRY-NH_2 (30 and 100 μg/site) (Fig. 3A). In addition, ADV extract-induced scratching was dose-dependently inhibited by intradermal pretreatment with a PAR_2 neutralizing antibody (1 and 10 μg/site) dose-dependently inhibited scratching induced by ADV extract (20 μg/site), as compared with a non-specific IgG (Fig. 3B).

LTB_4 production

Cutaneous LTB_4 content was significantly increased 5 min after intradermal injection of ADV extract (20 μg/site) (Fig. 4). This increase was significantly and almost completely prevented by oral pretreatment with zileuton (100 mg/kg) and intradermal co-injection with FSLLRY-NH_2 (100 μg/site) (Fig. 4).

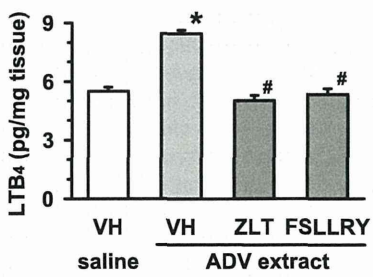

Fig. 4. Increased production of leukotriene (LT) B_4 after dermatophyte extract injection in the mouse skin. *Arthroderma vanbreuseghemii* (ADV) extract (20 μg/site) and saline were injected intradermally, and 5 min later LTB_4 content was measured in the treated skin. Zileuton (ZLT, 100 mg/kg; $n = 9$) and the vehicle (VH; $n = 8$ in each group) were administered orally 1 h before ADV extract injection. FSLLRY-NH_2 (FSLLRY, 100 μg/site; $n = 8$) was injected intradermally together with ADV extract. The values represent the mean ± SEM. $^*p < 0.05$ vs. VH + saline, $^\#p < 0.05$ vs. VH + ADV extract (Holm–Šidák test). These experiments were performed twice and similar results were obtained.

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Discussion

An intradermal injection of ADV extract (20 μg/site) elicited hind-paw scratching, confirming our previous report, which showed that intradermal ADV extract (1–20 μg/site) causes a dose-dependent increase in hind-paw scratching [7].

We have shown that ADV extract-induced scratching is inhibited by both the PAR_2 antagonist FSLLRY-NH_2 and the serine protease inhibitor nafamostat [7]. In the present study, we reconfirmed the inhibitory activity of FSLLRY-NH_2 and observed that a PAR_2-neutralizing antibody also inhibited ADV extract-induced scratching. The PAR_2 receptor is activated via the proteolytic cleavage of its N-terminal sequence by serine proteases. ADV extract cleaves a synthetic N-terminal peptide of PAR_2 similar to both trypsin and tryptase [7]. Therefore, serine protease(s) present in the ADV extract may elicit scratching through PAR_2 activation, although pruritogenic serine proteases have not yet been shown to be a component of ADV extract. In the skin, PAR_2 has been shown to be expressed mainly in nerve fibers [17] and epidermal keratinocytes [17]. Since ADV extract is equally effective at inducing scratching behavior in both mast cell-deficient mice and normal littermates [7], mast cells may not be essential for the pruritogenic action of ADV extract. Thus, an intradermal injection of ADV extract may primarily act on nerve fibers and epidermal keratinocytes to induce scratching.

ADV extract-induced scratching was inhibited by the LTB_4 receptor antagonist ONO-4057, suggesting that dermatophyte-associated itching involves LTB_4, a potent pruritogen in mice [2]. A key enzyme for LTB_4 production is 5-lipoxygenase [14]. Thus, the observation that the 5-lipoxygenase inhibitor zileuton suppressed scratching and LTB_4 production induced by the ADV extract supports the idea that LTB_4 is involved in dermatophyte-associated itching. Since ADV extract-induced scratching was fast in onset and short in duration, component and/or resident cells in the skin may be responsible. 5-Lipoxygenase is present in epidermal keratinocytes [4], and cultured keratinocytes produce LTB_4 upon stimulation with PAR_2 agonist [20]. Thus, it is plausible that PAR_2-mediated LTB_4 produced by epidermal keratinocytes is involved in ADV extract-induced scratching. Mast cells also produce LTB_4 following activation [13]. However, mast-cell deficiency does not reduce scratching induced by ADV extract or PAR_2 agonist [7], suggesting that mast cells do not play a key role in ADV extract-induced scratching. We do not rule out the possibility that LTB_4 produced by infiltrating leukocytes is involved under dermatitis conditions.

There are two LTB_4 receptor subtypes, BLT1 and BLT2, which have high and low binding affinities for LTB_4, respectively [19]. Since ONO-4057 has a similar affinity for BLT1 and BLT2 receptors [19], the results of the present study did not detect the relative contributions of BLT1 and BLT2 receptors in ADV extract-induced scratching. In the dorsal root ganglia and skin in normal mice, BLT1 receptor mRNA is expressed, but BLT2 receptor mRNA cannot be detected [3]. LTB_4 administration increases Ca^{2+} influx in cultured dorsal root ganglion neurons [3]. Most (73%) of the BLT1 immunoreactive neurons are small or medium in size and TRPV1 channel immunoreactivity (a marker of unmyelinated C-fiber neurons) has been shown to be present in BLT1-positive neurons [3]. Therefore, it is suggested that BLT1 receptors are responsible for an initiation phase of ADV extract-induced scratching. On the other hand, LTB_4 promotes the chemotaxis of leukocytes that express BLT1 and BLT2 receptors [19]. Therefore, it is possible that BLT2 receptors are involved in a late phase of ADV extract-induced scratching [11].

In this study, the cyclooxygenase inhibitors indomethacin and diclofenac did not significantly affect the ADV extract-induced scratching. Indomethacin does not inhibit scratching induced by PAR_2 agonist, substance P, and sphingosylphosphorylcholine in mice [5,6,20], although it inhibits prostaglandin E_2 production induced by PAR_2 agonist and substance P in cultured keratinocytes [5,20]. Intradermal injection of prostaglandin E_2 does not elicit scratching in mice [2]. With these findings taken into account, the present results suggest that although its production is increased by ADV extract, prostaglandin E_2 does not play a key role in ADV extract-induced scratching.

In summary, data shown here suggest that dermatophyte-contained serine protease(s) act on PAR_2 in the epidermis to produce LTB_4, which causes itching. This may be a causal mechanism of dermatophytosis-associated pruritus.

Conflict of interest

The authors state no conflict of interest.

Funding

This study was supported in part by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (19790051; 22790063), Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology, Japan (23390153), and Grants for Health Science from the Health, Labour and Welfare Ministry, Japan. This work was also supported in part by the National BioResource Project of Japan (http://www.nbrp.jp/).

Acknowledgement

We thank Dr. Jung-Bum Lee in the Department of Pharmacognosy, University of Toyama, for kindly providing FSLLRY-NH_2.

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Original Paper

Pharmacology

Pharmacology 2015;95:95–103
DOI: 10.1159/000371890

Received: September 1, 2014
Accepted after revision: January 6, 2015
Published online: February 19, 2015

S-777469, a Novel Cannabinoid Type 2 Receptor Agonist, Suppresses Itch-Associated Scratching Behavior in Rodents through Inhibition of Itch Signal Transmission

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Key Words
S-777469 · Cannabinoid type 2 receptor · Itch · Antipruritic agent

Abstract
We have previously reported that S-777469 [1-([6-ethyl-1-(4-fluorobenzyl)-5-methyl-2-oxo-1,2-dihydropyridine-3-carbonyl]amino)-cyclohexanecarboxylic acid], a novel cannabinoid type 2 receptor (CB_2) agonist, significantly suppressed compound 48/80-induced scratching behavior in mice in a dose-dependent manner when it was administered orally. Here, we demonstrated that the inhibitory effects of S-777469 on compound 48/80-induced scratching behavior are reversed by pretreatment with SR144528, a CB_2-selective antagonist. In addition, we investigated the effects of S-777469 on itch-associated scratching behavior induced by several pruritogenic agents in mice and rats. S-777469 significantly suppressed scratching behavior induced by histamine or substance P in mice or by serotonin in rats. In contrast, the H_1-antihistamine fexofenadine clearly inhibited histamine-induced scratching behavior but did not affect scratching behavior induced by substance P or serotonin. Moreover, S-777469 significantly inhibited histamine-induced peripheral nerve firing in mice. In conclusion, these results suggest that S-777469 produces its antipruritic effects by inhibiting itch signal transmission through CB_2 agonism.

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Introduction

Pruritus is an unpleasant cutaneous sensation that provokes the desire to scratch [1] and can profoundly affect a person's quality of life. It is a major symptom of many dermatological, systemic, neurological, and psychogenic diseases [2]. Because the development of the itching sensation, particularly in chronic skin disorders (e.g., atopic dermatitis), is a complex process with many underlying causes, common treatments such as with H_1-antihistamines are not always effective. It has been reported that itch is probably mediated in the periphery by a subpopulation of unmyelinated afferent C fibers [3–5]. This nociceptor population is also responsive to painful

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0031–7012/15/0952–0095$39.50/0

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stimuli. Therefore, it is plausible that the suppression of itch and pain transmission via C fibers could control these symptoms.

The cannabinoid receptor (CB) was discovered as a receptor for Δ^9-tetrahydrocannabinol, an active ingredient of marijuana manufactured from *Cannabis sativa*. There are at least two types of CB, cannabinoid type 1 receptor (CB_1) and cannabinoid type 2 receptor (CB_2), both of which couple to G proteins. CB_1 is abundantly found in the central nervous system (CNS) and may be involved in the regulation of higher brain functions such as memory, motion, and pain sensation [6]. By contrast, CB_2 – which is primarily found in peripheral nerves and organs as well as in cells related to the immune system – may not be significantly involved in mental functions [6–9]. In human skin, CB_2 is expressed in bundles of sensory nerve fibers, mast cells, macrophages, and keratinocytes [6, 10], suggesting the possibility that this receptor is involved in the regulation of skin inflammation, itching, and pain. From these findings, CB_2-selective agonists are potential therapeutics for itch and pain without undesirable effects on the CNS. Indeed, several CB_2-selective agonists with analgesic effects have been reported on [11–16].

Regarding the role of CB in itch, recent reports have suggested that CB is involved in the regulation of pruritus. For example, a nonselective CB agonist, HU-210, has been reported to attenuate histamine-induced itch in human subjects [17]. In addition, topical agonists of CB were shown to have an antipruritic effect in atopic eczema and uremic pruritus [18]. Therefore, we tried to find novel CB_2-selective agonists that were antipruritic agents without side effects in the CNS, and developed S-777469 [1-([6-ethyl-1-(4-fluorobenzyl)-5-methyl-2-oxo-1,2-dihydropyridine-3-carbonyl]amino)-cyclohexanecarboxylic acid]. In a previous paper, we demonstrated that S-777469 possessed selective binding as well as agonistic activities for CB_2 compared to CB_1 and significantly suppressed compound 48/80-induced scratching behavior in mice [19].

In the present study, to characterize the antipruritic effect of S-777469, we evaluated its effects on itch-associated scratching behaviors in mice or rats induced by various pruritogens such as histamine, substance P, and serotonin. In addition, the effects of S-777469 on histamine-induced nerve firing and the expression of CB_2 on mouse dorsal root ganglion (DRG) neurons were investigated. Our results support the use of S-777469 to control itching associated with a variety of pathophysiologic conditions via the inhibition of itch signal transmission.

Materials and Methods

Animals

Female ICR mice and F344 rats were purchased from Charles River Japan (Yokohama, Japan). Female JW/CSK rabbits were purchased from Japan SLC (Hamamatsu, Japan). These animals were all housed under controlled temperature (20–26°C), humidity (30–70%), and lighting (lights on from 8:00 to 20:00) conditions. Food and tap water were provided ad libitum. The animal experiments in this study were approved by the Animal Care and Use Committee of Shionogi Research Laboratories.

Drugs and Chemicals

S-777469 and SR144528 were synthesized in our laboratory. Fexofenadine hydrochloride was extracted from Allegra® tablets (Sanofi-Aventis, Paris, France) in our laboratory. The following reagents were used in this study: histamine (Nacalai Tesque, Kyoto, Japan); compound 48/80, substance P, serotonin, and methylcellulose (Wako Pure Chemicals, Osaka, Japan), and (3-aminopropyl)triethoxysilane (APTES; Dako, Glostrup, Denmark).

Scratching Behavior

The rostral part of the skin on the backs of mice and rats was clipped using an electric clipper 2–5 days before the induction of scratching behavior. To induce scratching behavior, 40 μl of compound 48/80 (3 μg/site), histamine (100 nmol/site), or substance P (100 nmol/site) solution was injected intradermally into the back of mice. In rats, 100 μl of serotonin solution (200 μg/site) was injected. Negative control mice and rats received a saline injection. Immediately after the injection, the mice and rats were placed in an observation chamber (width × depth × height: 10.8 × 20.1 × 14.5 cm), and their scratching behavior was observed for 30 min. Mice and rats generally scratch several times for about 1 s, and a series of these behaviors was counted as 1 incidence. S-777469 and fexofenadine were administered orally 1 h before the injection of pruritogens. SR144528 was administered orally just before the administration of S-777469. These agents were suspended in 0.5% methylcellulose.

Repeated Drug Administration

The antipruritic effects of repeated administration of S-777469 were evaluated using compound 48/80-induced scratching behavior in mice. In the repeated-administration group, S-777469 (10 mg/kg) was administered twice a day from days 0–5. On day 6, 1 h after the last administration of S-777469, 40 μl of compound 48/80 (3 μg/site) was injected. In the single-administration group, S-777469 was administered 1 h before the injection of compound 48/80.

Nerve Firing

The animals were anesthetized with urethane (1.5 g/kg, intraperitoneally) 50 min after drug administration, and the skin of the back was dissected and turned inside out. The mice were placed on a warming manipulation platform, and the inverted skin was laid on the platform to expose the cutaneous nerve branches. One of the exposed nerve branches was then dissected free from the surrounding tissues and connected to bipolar electrodes of silver wire to detect nerve potentials. After the operation, the dissected site of a nerve branch was maintained in a mineral oil pool to prevent drying. The nerve potentials detected at the bipolar electrodes were

amplified, and the noise was cut off by a bioelectric amplifier (MEG-2100; Nihon Kohden, Tokyo, Japan). The amplified analog signals were then converted to digital signals by a PowerLab system (PowerLab/8SP; ADInstruments Pty Ltd., Bella Vista, N.S.W., Australia), and the digital signals were recorded using the software Chart5 for Windows (AD Instruments). After stable nerve activity was obtained, 50 µl of histamine solution (12.5 µmol/site) was injected intradermally into a nerve-innervating region. Nerve potentials were recorded for 30 min. The raw chart data were processed for abatement of low frequency noises, and the number of spikes with potentials over a calibrated gain value was counted (using Chart5) to determine the magnitude of nerve firing.

Production of Rabbit Anti-CB_2-Specific Antibody for Immunohistochemistry

Glutathione S-transferase (GST) and maltose-binding protein (MBP)-tagged fusion proteins of the C-terminal region polypeptide 302–347 (46 amino acids) of CB_2 were expressed and purified using an *Escherichia coli* expression system. GST-CB_2 and MBP-CB_2 fusion proteins were used for the production of rabbit anti-CB_2 serum and the purification of CB_2-specific antibody from antiserum, respectively.

Three JW/CSK rabbits were intradermally immunized 7 times at 2-week intervals with GST-CB_2 fusion protein (32 kDa). At 10 days following the final immunization, anti-GST-CB_2 serum was collected from the immunized rabbits, and the antibody titer was assessed by competitive ELISA using biotin-labeled GST-CB_2. Antiserum that showed the highest titer in 3 antisera was used for the isolation of CB_2-specific antibody. The isolation was carried out by affinity chromatography using MBP-CB_2 coupled to AminoLink Coupling Resin (Thermo Fisher Scientific Inc., Rockford, Ill., USA). The CB_2-specific antibody obtained was confirmed to show the immunoreaction with MBP-CB_2 by ELISA and Western blot, but no cross-reaction with GST. The specific antibody was used for studies on immunohistochemistry after dialyzing against phosphate-buffered saline (PBS).

Immunohistochemistry

Under pentobarbital anesthesia, ICR mice were flushed with PBS through the abdominal aorta, followed by perfusion with 4% paraformaldehyde in PBS. After perfusion, the DRG were carefully removed and thoroughly rinsed with PBS. The fixed samples were dehydrated through a graded alcohol series, cleared in xylene, and embedded in paraffin. Vertical tissue sections (5 mm thickness) were cut from the embedded blocks and mounted on slides freshly coated with APTES. After dewaxing, the sections were briefly washed with PBS, preincubated in normal goat serum at a dilution of 1:500 in PBS for 30 min, and subsequently incubated in the rabbit anti-CB_2 antibody mentioned above at a concentration of 1.0 µg/ml in PBS for 30 min under a humidified condition at room temperature. These samples were then thoroughly rinsed with PBS and incubated in biotinylated anti-rabbit polyclonal goat antibodies (Vector Laboratories, Burlingame, Calif., USA) at a dilution of 1:200 in PBS for 30 min at room temperature and rinsed again. The samples were next treated with avidin-biotinylated horseradish peroxidase complex solution (Vector Laboratories) at room temperature for 60 min. After immunological incubation, the samples were extensively washed with PBS; then, the exogenous peroxidase activity was visualized as dark brownish color deposits with 10 min of drenching in 50 mmol/l Tris-HCl (pH 7.4) containing 200 µg/ml 3,3′-diaminobenzidine hydrochloride substrate and dissolved 0.006% H_2O_2 for 5 min at room temperature in the dark. After a brownish color had developed, the reaction was stopped by rinsing in TE buffer (10 mmol/l Tris-HCl, pH 7.6, containing 1 mmol/l EDTA), and the target CB_2 localizations were visualized. Then, the sections were nuclear counterstained with hematoxylin, dehydrated, and finally permanently mounted in Entellan new (Merck Co., Rahway, N.J., USA). The slides were examined under the bright-field microscope Eclipse-800 (Nikon, Tokyo, Japan) with particular attention paid to evaluation of the dark brownish signals in the DRG upon transverse view of the specimens.

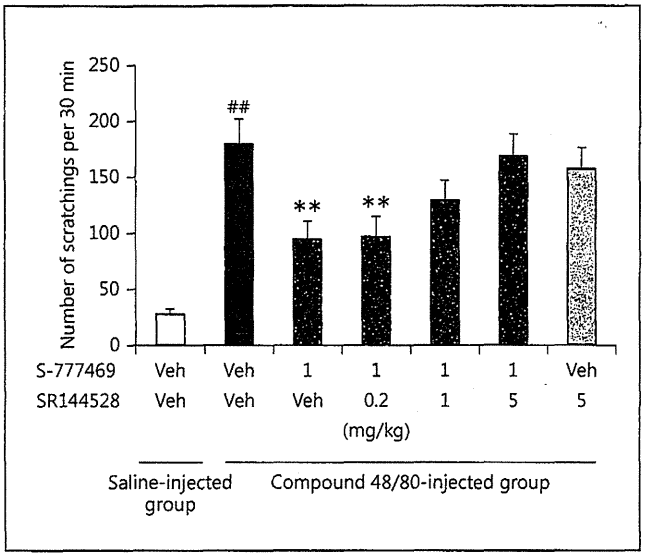


Fig. 1. Effects of S-777469 on compound 48/80-induced scratching behavior in mice with or without SR144528 treatment. SR144528 was administered orally just before S-777469 administration 1 h before the intradermal injection of compound 48/80. n = 14. All data represent means ± SE. Veh: vehicle group, which was administered 0.5% methylcellulose. ## $p < 0.01$ vs. Veh + Veh in the saline-injected group (Welch's t test); ** $p < 0.01$ vs. Veh + Veh in the compound 48/80-injected group (Dunnett's test).

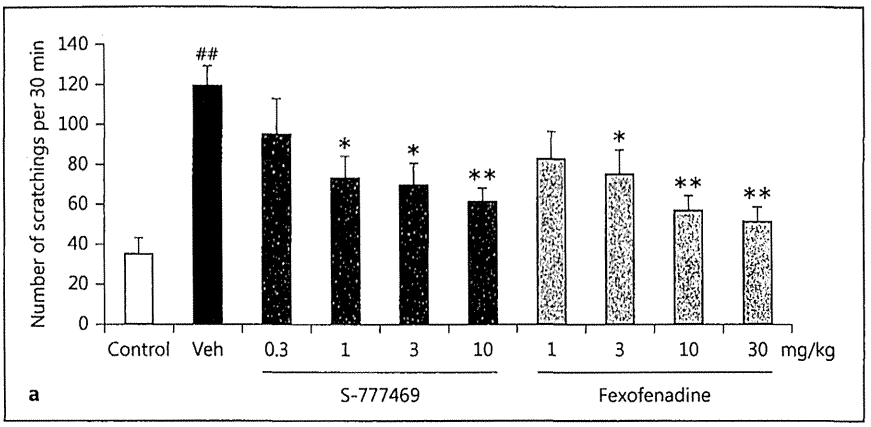
Statistical Analysis

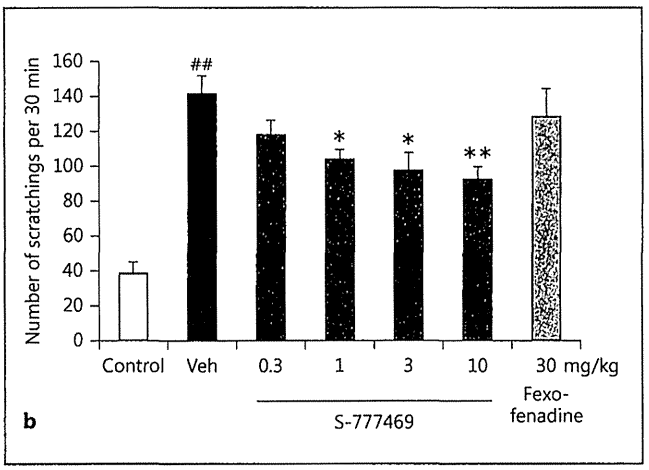
Data are expressed as means ± SE. Statistical significance was assessed by Welch's t test or Dunnett's test. In all tests, the significant level was at two-sided $p < 0.05$.

Results

Antagonistic Effects of SR144528 on Antipruritic Effects of S-777469

We previously showed that S-777469 dose-dependently inhibited compound 48/80-induced scratching behavior [19]. Here, we investigated the effects of pretreat-





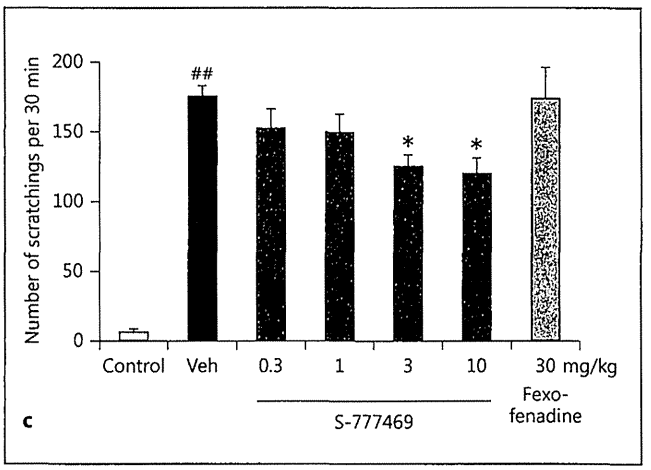


Fig. 2. Effects of S-777469 and fexofenadine on scratching behavior induced by histamine (**a**), substance P (**b**), and serotonin (**c**) in mice (**a**, **b**) and rats (**c**). S-777469 and fexofenadine were administered orally 1 h before the intradermal injection of pruritogens. n = 20. All data represent means ± SE. The control group was injected with physiological saline. Veh: vehicle group, which was administered 0.5% methylcellulose. ## $p < 0.01$ vs. control (Welch's t test); * $p < 0.05$, ** $p < 0.01$ vs. Veh (Dunnett's test).

ment with SR144528, a CB_2 antagonist, to clarify whether the inhibitory effects of S-777469 on compound 48/80-induced scratching behavior in mice are mediated by CB_2. Intradermal injection of compound 48/80, a mast cell stimulator, induced marked increases in itch-associated scratching behavior. S-777469 (1 mg/kg) was administered immediately after oral administration of SR144528 (0.2, 1, and 5 mg/kg) to evaluate its antagonistic action against S-777469. SR144528 alone (5 mg/kg) had no effect on compound 48/80-induced scratching behavior. S-777469 significantly inhibited scratching behavior in the absence of SR144528. However, in the presence of SR144528 (1 and 5 mg/kg), the inhibitory effects of S-777469 on scratching behavior were reduced to a level at which there was no statistically significant difference from that of the vehicle-treated group (fig. 1), indicating that the antipruritic effects of S-777469 are mediated through CB_2.

Scratching Behavior Induced by Various Pruritogens in Mice and Rats

To characterize the antipruritic effects of S-777469, we examined its effects on scratching behavior induced by several pruritogens in rodents in comparison to the effects of the H_1-antihistamine fexofenadine. Histamine (100 nmol/site) intradermally injected in mice provoked pronounced scratching behavior. This response was significantly suppressed by pretreatment with S-777469 in a dose-dependent manner, and the inhibition rates at doses of 0.3, 1, 3, and 10 mg/kg were 28, 55, 59, and 69%, respectively (fig. 2a). Fexofenadine also dose-dependently reduced histamine-induced scratching behavior, and the inhibition rates at doses of 1, 3, 10, and 30 mg/kg were 43, 52, 74, and 80%, respectively (fig. 2a).

Next, we examined the effects of S-777469 on substance P-induced scratching behavior in mice. As shown in figure 3b, substance P (100 nmol/site) induced obvious scratching behavior, and S-777469 inhibited this response in a

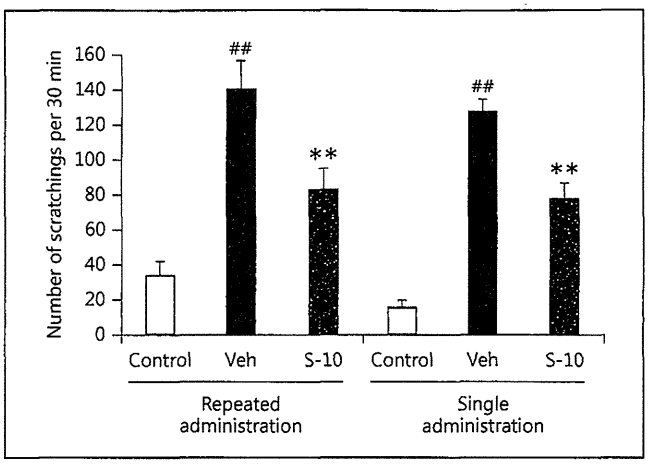


Fig. 3. Effects of S-777469 on compound 48/80-induced scratching behavior in mice after single or repeated administration. In the repeated-administration group, S-777469 was administered twice a day for 5 days and 1 h before the intradermal injection of compound 48/80. In the single-administration group, S-777469 was administered 1 h before the compound 48/80 injection. n = 15. All data represent means ± SE. Control: negative control. Veh: vehicle. S-10: S-777469 at 10 mg/kg. ## $p < 0.01$ vs. control (Welch's t test); ** $p < 0.01$ vs. Veh (Welch's t test).

dose-dependent manner. The efficacy was statistically significant at doses of 1 mg/kg or more. The inhibition rates for substance P-induced scratching behavior were 23, 37, 43, and 48%, respectively. On the other hand, fexofenadine at a dose of 30 mg/kg had no significant effects on scratching behavior induced by substance P. In a preliminary experiment, we also examined the effect of S-777469 on serotonin-induced scratching behavior in mice. S-777469 (10 mg/kg) significantly inhibited the response (the inhibition rate was 43%), and fexofenadine (30 mg/kg) had no significant effects on scratching behavior (data not shown).

Moreover, we tried to confirm the antipruritic effect of S-777469 in rats, since we used rats in S-777469 safety studies. It has been reported that intradermal injection of serotonin elicits scratching behavior in rats [20]; thus, we examined the effect of S-777469 on serotonin-induced scratching behavior. In F344 rats, intradermal injection of serotonin (200 μg/site) induced obvious scratching behavior, and S-777469 significantly inhibited the response in a dose-dependent manner. The inhibition rates at doses of 0.3, 1, 3, and 10 mg/kg were 14, 15, 30, and 33%, respectively. In contrast, fexofenadine at a dose of 30 mg/kg had no significant effects on scratching behavior (fig. 2c). These results suggest that S-777469 has an antipruritic effect regardless of the type of pruritogen.

Effects of S-777469 by Repeated Administration

For the treatment of chronic itch, a medication that can be given over a long time is required. Therefore, we next investigated whether the antipruritic effects of S-777469 persisted after repeated administration. As shown in figure 3, repeatedly administered S-777469 significantly inhibited compound 48/80-induced scratching behavior, and the degree of suppression was comparable to that obtained by single administration.

CB_2 Expression in DRG

To determine whether CB_2 is expressed by DRG neurons, we performed immunohistochemistry using a specific antibody. The dark brownish-colored positive immunoperoxidase signals for CB_2 were observed in DRG neurons, and they were especially apparent within smaller DRG neurons (<20 μm in diameter), whereas they were not detected in larger ones (>20 μm in diameter) (fig. 4). By qualitative observation, more than half of the whole number of DRG neurons was immunohistochemically CB_2 positive.

Effects of S-777469 on Nerve Activity

To clarify the mechanism underlying its antipruritic effects, we examined the effects of S-777469 on nerve activity related to the itch-associated response. Intradermal injection of histamine induced an increase in nerve firing, and S-777469 (10 mg/kg) significantly inhibited the cutaneous nerve firing evoked by peripherally administered histamine (fig. 5).

Discussion

In the present study, we investigated the antipruritic effects of S-777469, a CB_2-selective agonist, on scratching behavior induced by several pruritogens in rodents, in comparison to the H_1-antihistmine fexofenadine. S-777469, administered orally, significantly suppressed compound 48/80-, histamine-, and substance P-induced as well as serotonin-induced scratching behavior in mice and rats, respectively. By contrast, fexofenadine clearly inhibited histamine-induced scratching behavior but had no effect on scratching behavior induced by substance P and serotonin. The inhibitory effects of S-777469 on itch-associated scratching behavior were antagonized by treatment with SR144528, a CB_2-selective antagonist, and S-777469 inhibited histamine-induced nerve firing, indicating that the antipruritic effects of S-777469 are mediated by CB_2 via the suppression of itch signal transmission.

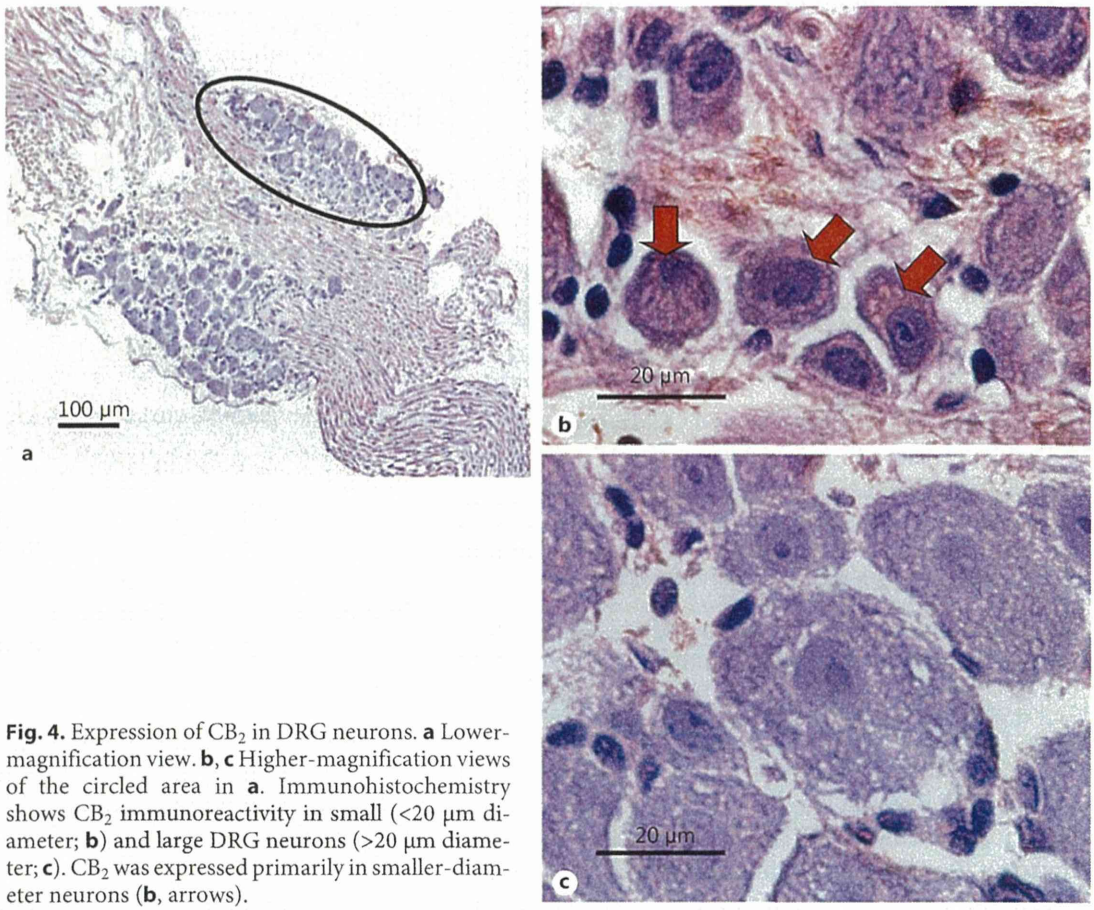


Fig. 4. Expression of CB_2 in DRG neurons. **a** Lower-magnification view. **b**, **c** Higher-magnification views of the circled area in **a**. Immunohistochemistry shows CB_2 immunoreactivity in small (<20 μm diameter; **b**) and large DRG neurons (>20 μm diameter; **c**). CB_2 was expressed primarily in smaller-diameter neurons (**b**, arrows).

Chronic itch is a troublesome symptom observed in several disorders including atopic dermatitis, and no efficacious drugs have thus far been developed that relieve this symptom. The pathogenesis of itch is very complicated, and several molecules that act on itch-relevant neural transmission are thought to be involved [21–23]. Cannabinoids are candidate molecules for the mediation of itch transmission, as shown in a previous report demonstrating that the topical application of CB agonists exert antipruritic effects on histamine-induced itch as well as on atopic eczema and uremic pruritus in humans [17]. Among CB, CB_1 is mainly found in the CNS, whereas CB_2 is predominantly expressed in the periphery, such as in sensory nerves and immune cells [6–10]. These findings prompted us to explore CB_2-selective agonists as antipruritic drugs, without side effects on the CNS mediated by CB_1, and we discovered S-777469. In that previous study [19], we examined the in vitro activity of S-777469 for CB_1 and CB_2 and demonstrated that S-777469 is a CB_2-selective agonist with no side effects on the CNS. In addition, the effects of S-777469 on various other receptors or enzymes were evaluated. A total of 38 types of receptors, including opiates, chemical mediators, neurotransmitters, chemokines, and 11 enzymes related to inflammation or the metabolism of neurotransmitters, were investigated. S-777469 (10 μmol/l) did not markedly inhibit ligand binding to any of these receptors and any enzyme activities (data not shown). These results further confirmed the CB_2 selectivity of S-777469.

Using a pruritogen-induced scratching behavior model in rodents as an itch-related response [24], we evaluated the antipruritic effects of S-777469. First of all, we investigated the contribution of CB_2 to the effects of S-777469 on compound 48/80-induced scratching behavior in mice. S-777469 significantly inhibited the response, and this effect is suggested to be mediated by CB_2, based on the result that SR144528 reversed this effect. Next, we characterized the antipruritic effects of S-777469

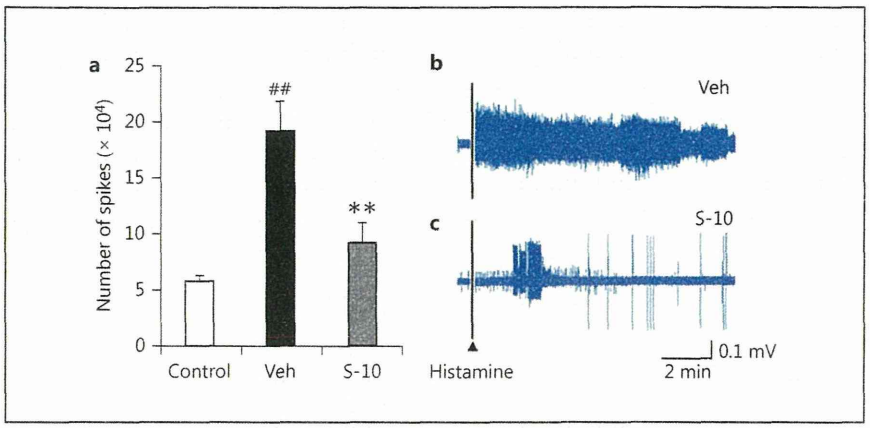


Fig. 5. Effects of S-777469 on histamine-induced nerve firing in mice. Cutaneous nerve activity was recorded for 30 min after the intradermal injection of histamine. The total number of spikes was counted to determine the nerve activity (**a**), and the typical data on nerve firing in the vehicle-treated group (**b**) and the S-777469-treated group (**c**) are shown. Histamine was injected at a dose of 12.5 μmol/site, as indicated by the arrowhead. n = 13–15. All data represent means ± SE. Control: negative control. Veh: vehicle. S-10: S-777469 at 10 mg/kg. ## $p < 0.01$ vs. control (Welch's t test); ** $p < 0.01$ vs. Veh (Welch's t test).

by comparing them with those of the H_1-antihistamine fexofenadine, using several other pruritogen-induced responses. Fexofenadine clearly suppressed histamine-induced scratching behavior in mice but did not affect substance P- and serotonin-induced responses in mice and rats, respectively. The results of our current study regarding the effects of fexofenadine are pharmacologically reasonable in that a selective antagonist attenuated the corresponding agonist-induced responses. On the other hand, S-777469 significantly inhibited histamine-, substance P-, and serotonin-induced scratching behavior in rodents. However, S-777469 had no effects on pain-related behaviors using the formalin test in mice and mechanical stimuli (pinching the tail with tweezers) in rats (data not shown). S-777469 did not show antagonistic activities for histamine, substance P, or serotonin to receptors and had no effect on general activity and behavior at doses of up to 2,000 mg/kg in rats (data not shown), whereas cannabinoids may be involved in itch transmission [21, 22]. Therefore, to clarify the mechanism of action of the antipruritic effect of S-777469, we next examined the contribution of CB_2 to itch transmission.

CB_2 is expressed on DRG neurons in humans and rodents [25, 26] as well as in skin sensory neurons in humans [27]. CB_2 agonists inhibit capsaicin-induced increases in intracellular calcium in DRG neurons [26] and inhibit C fiber activity enhancements in response to electrical stimulation [28]. In an in vivo study, the cutaneous nerve firing evoked by peripherally given pruritogens at least partly included itch-associated responses [29]. Therefore, we conducted experiments confirming the expression of CB_2 on DRG neurons and the effects of S-777469 on nerve activity in mice. Immunohistochemical examination revealed that CB_2 was expressed on mouse DRG neurons, particularly on a subset of small-sized DRG. We have not investigated whether CB_2 is co-expressed with receptors of histamine (H_1, H_4), serotonin ($5\text{-}HT_2$), substance P (NK1), or leukotriene B_4 (it was suggested that leukotriene B_4 is involved in substance P-induced itch [30]). Since previous reports have demonstrated that each receptor was expressed in DRG neurons [31–34], additional research on the coexpression of CB_2 with other receptors is needed. Additionally, S-777469 suppressed histamine-induced nerve firing in mice. In a preliminary experiment, S-777469 (10 mg/kg) also significantly inhibited serotonin-induced nerve firing in mice (data not shown). These results suggest that S-777469 inhibits scratching induced by multiple pruritogens through the suppression of the activity of primary afferent fibers. Moreover, CB_2 agonists (AM-1241 and L-768242) dose-dependently inhibited the capsaicin-induced, calcitonin gene-related peptide release from rat spinal cord slices [35]. That report indicates that S-777469 may control itch through direct neuronal actions on neurotransmitter release. Further studies are needed to clarify the inhibitory mechanism of itch-related but not pain-related responses by S-777469.

For the treatment of chronic itch, medications that can be given chronically are required. Therefore, we next investigated whether the antipruritic effect of S-777469 persisted after repeated administration. S-777469 administered repeatedly inhibited the compound 48/80-induced scratching behavior as potently as when it was given the first time, and it had no effect on general activity. In addition, in a 1-month repeated-oral-dose toxicity study in rats (200, 600, and 2,000 mg/kg/day), S-777469 also had no effect on general activity (data not shown). Previous reports have demonstrated that CB_2 is internalized after