



Immunopharmacology and Inflammation

Oral chymase inhibitor SUN13834 ameliorates skin inflammation as well as pruritus in mouse model for atopic dermatitis

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ABSTRACT

Chymase is a chymotrypsin-like serine protease exclusively stored in secretory granules of mast cells and has been thought to participate in allergic diseases. It has already been shown that chymase inhibitor SUN13834 improves dermatitis in NC/Nga mice that spontaneously develop dermatitis resembling atopic dermatitis. In the present study, effect of chymase inhibitor SUN13834 on itch, the major feature of atopic dermatitis, was examined using a mouse dermatitis model induced by repeated topical application of 2,4-dinitrofluorobenzene (DNFB). Oral administration of SUN13834 once a day for 5 weeks inhibited not only skin swelling but accumulation of inflammatory cells including mast cells and eosinophils in the skin of the mice. In addition, SUN13834 also decreased significantly at 10 and 50 mg/kg the amount of scratching behavior induced by the DNFB challenge. This result indicates for the first time that mast cell chymase may be involved in itch induction. In conclusion, SUN13834 is thought to be useful as therapeutic agent for atopic dermatitis.

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1. Introduction

Atopic dermatitis is a common chronic inflammatory skin disease associated with high levels of serum immunoglobulin E (IgE) (Rudikoff and Lebowitz, 1998). The most prominent feature for patients with atopic dermatitis is severe itching, because scratching behavior responding to itch further aggravates skin symptoms, which is generally termed as “itch-scratch cycle” (Mihara et al., 2004). Mast cells that express high-affinity receptor for IgE are significantly increased in the skin of atopic dermatitis compared with the normal skin (Badertscher et al., 2005; Mihm et al., 1976) and therefore thought to play a part in pathogenesis of atopic dermatitis.

Chymase is a chymotrypsin-like serine protease stored within the mast cell granules and secreted upon stimulation of the cells (Welle, 1997). Although the precise physiological role of chymase is not known, evidence suggests that this enzyme might play a crucial role in pathogenesis of atopic dermatitis, e.g. i) an intradermal injection of chymase to mouse skin elicits edema as well as inflammatory cell accumulation (Tomimori et al., 2002a,b), ii) there are several reports suggesting a correlation between a certain single nucleotide polymorphism (SNP) of chymase gene and incidence of atopic dermatitis (Mao et al., 1996; Tanaka et al., 1999; Weidinger et al., 2005). In addition, a chymase inhibitor SUN C8257 has improved

skin inflammation in three animal models for atopic dermatitis (Tomimori et al., 2002b,c; Watanabe et al., 2002). However, the effect of this compound was shown only when either administered intraperitoneally (Tomimori et al., 2002b,c) or given as drinking water (Watanabe et al., 2002), as it has low oral bioavailability and short half-life *in vivo*.

Recently, we have synthesized a novel chymase inhibitor with improved profile (Maruoka et al., 2007; Tanaka et al., 2007) and shown that oral administration of the representative compound SUN13350 (free form of SUN13834) improves skin inflammation in NC/Nga mice (Watanabe et al., 2007) that spontaneously develop dermatitis in conventional condition (Matsuda et al., 1997; Tsudzuki et al., 1997). In the present study, effect of SUN13834 on pruritus was examined using a mouse 5-week atopic dermatitis model induced by repeated painting of 2,4-dinitrofluorobenzene (DNFB). The results demonstrated that SUN13834 reduces scratching behavior in the model. This is the first paper which reports the data suggesting a possible involvement of chymase in itch response.

2. Materials and methods

2.1. Mice

C3H/HeN (8 weeks old, female) and BALB/c mice (6 weeks old, female) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and Charles River Japan, Inc. (Tokyo, Japan), respectively. All animal experiments were approved by the Ethics Committee for Animal

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Experiments of Biomedical Research Laboratories, Asubio Pharma Co., Ltd. and were performed in accordance with the Guideline for Animal Experiments of the laboratories.

2.2. Chymase inhibitors

SUN13834 (6-(5-chloro-2-methoxybenzyl)-4-alkylaminocarbonyl-1,4-diazepane-2,5-dione) was synthesized in Biomedical Research Laboratories of Asubio Pharma as described (Maruoka et al., 2007; Tanaka et al., 2007). The K_i values of SUN13834 for mouse chymase (mMCP-4) and human skin-derived chymase were 0.063 μ M and 0.16 μ M, respectively. The inhibition of chymase by SUN13834 is specific in that it has little effect on other enzymes such as tryptase, cyclooxygenase-1 (COX-1) and angiotensin converting enzyme (ACE). SUN13834 was suspended in 0.5% of hydroxy propyl cellulose (HPC) (Nippon Soda Co., Ltd., Tokyo, Japan) and orally administered once daily for 5 weeks. In the case of the day of DNFB application, SUN13834 was administered 1 h prior to the application.

2.3. Induction of dermatitis and pruritus by repeated painting with DNFB in mice

Allergic dermatitis was induced by repeated topical application of DNFB as described (Tomimori et al., 2002a). Briefly, 25 μ l of 0.15% DNFB in acetone/olive oil (3:1) was applied topically to outer and inner sides of right ears of C3H/HeN mice 6 times at seven-day intervals. Skin thickness of the ear was measured 0, 24, 48 and 72 h after each painting using a thickness dial gauge (Digimatic Indicator, Mitsutoyo Corp., Tokyo, Japan) and the increase in skin thickness was determined by subtracting skin thickness before the first painting from that of each point. The same volume of acetone/olive oil (3:1) was painted in control mice. Ear swelling was calculated as the values subtracted the increase in skin thickness painted acetone/olive oil only from that induced by DNFB-painting. Pruritus was induced by repeated painting with DNFB in a similar way, except that the hapten was applied to the shaved back of mice. That is, 20 μ l of 0.1% DNFB in acetone/olive oil (3:1) was applied to the back of BALB/c mice 6 times at seven-day intervals. The same volume of acetone/olive oil (3:1) was painted in control mice.

2.4. Evaluation of pruritus

Pruritus was evaluated by counting automatically scratching behavior using MicroAct (Neuroscience, Inc., Tokyo Japan) as reported previously (Inagaki et al., 2006). A magnet (1 mm in diameter, 3 mm long, Teflon-coated) was subcutaneously inserted into each hind paw of mouse, and movement of the hind limb with magnets was measured in an observation chamber (11 cm in diameter, 18 cm high), which was surrounded by a round coil. The electric current induced by the movement of magnets attached to the paws was recorded, and characteristic waves corresponding to scratching behaviors were detected using a computer. The number of scratching behavior was measured for 1 h immediately (at 0–1 h) and at 24–25 h after each painting with DNFB.

2.5. Histological analysis

Ear samples were taken at the end of the experiment (72 h after 6th application of DNFB) and fixed in 10% (v/v) buffered formalin, embedded in paraffin, sectioned at 5 μ m, and then stained with toluidine blue and Fast Green FCF (Fluka, Sigma-Aldrich Japan) for counting mast cells and eosinophils, respectively. The numbers of mast cells and eosinophils were determined by counting the stained cells in ten high-power fields ($\times 400$ for count) for each section using an ocular grid. The histological analysis was also performed after the sections were stained with hematoxylin and eosin. For measurement

of the number of neutrophils, T lymphocytes and macrophages, immunohistochemical study was performed using specific antibodies (anti-Gr-1 antibody for neutrophils, R&D Systems Inc., MN; anti-CD3 ϵ antibody for T lymphocytes, Santa Cruz Biotechnology Inc., CA, anti-F4/80 antibody for macrophages, Abcam K.K, Tokyo Japan). Visualization of the antibody bound to the cells was performed using Simple Stain Kit (Nichirei Co., Tokyo Japan) and DAB+ Liquid (DAKO Japan Inc., Tokyo Japan). The numbers of the cells were determined by counting the stained cells in ten high-power fields ($\times 400$ for count) for each section using an ocular grid.

2.6. Statistical analysis

The statistical analysis was performed with Aspin–Welch's *t*-test, Dunnett's multiple comparison test or Student's *t*-test using EXSAS Version 7.5.2 (Arm Systex Co., Ltd.) or JMP Version 6.0.3 (SAS Institute Inc., Cary, NC), respectively. The *P*-value less than 0.05 was considered significant.

3. Results

3.1. Effect of chymase inhibitor SUN13834 on skin swelling induced by repeated painting of DNFB

A topical application of DNFB to the skin of mice elicited transient skin reaction, and repeating the challenge weekly 6 times enhanced the response to DNFB (Fig. 1A). Calculating the area under the curve (AUC) of the skin data revealed that the skin swelling in the DNFB-treated mice was statistically significant compared with the control mice (Fig. 1B). Oral administration of chymase inhibitor SUN13834 reduced the DNFB-induced skin swelling in a dose-dependent manner, and statistical significance was detected at 2 mg/kg and higher (Fig. 1B). The effect of SUN13834 was evident judging from the microscopic observation of the skin sections, e.g. the thickness of the dermis was obviously increased by the DNFB application, and the increase was significantly inhibited by SUN13834 administration (Fig. 2). This result is in agreement with the previous study using the other chymase inhibitors in the same model (Tomimori et al., 2002a).

3.2. Effect of chymase inhibitor SUN13834 on inflammatory cell accumulation in DNFB-induced dermatitis model

Repeated DNFB painting has also been known to accumulate inflammatory cells in the inflamed dermis (Nagai et al., 1997). Fig. 3A shows that the DNFB treatment increased the number of mast cells in

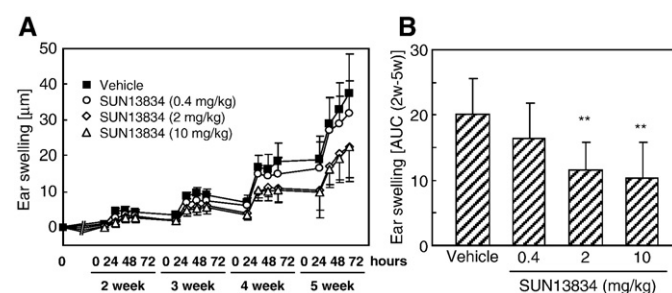


Fig. 1. Effect of SUN13834 on skin swelling in dermatitis model induced by repeated painting with DNFB in mice. Dermatitis was elicited by repeated painting of 0.15% DNFB (in acetone/olive oil) on outer and inner sides of mouse ear 6 times at seven-day intervals. The same volume of acetone/olive oil was painted in control mice. SUN13834 was orally administered daily for 5 weeks from the day of first DNFB application. Skin thickness of the ear was measured and ear swelling was calculated as described in Materials and methods. A: Time course of change in ear swelling, B: AUC data calculated with the data of A. Data are mean $\pm$ S.D. **, $P < 0.01$ as compared with vehicle group (Dunnett's test) ($n = 10$).

the skin of mouse, and oral administration of SUN13834 suppressed the increase in the number of the cells. Examination of eosinophil infiltration showed similar results (Fig. 3B). Statistical significance of the inhibition of cell infiltration by SUN13834 was shown at 2 and 10 mg/kg for both mast cells and eosinophils. Similarly, SUN13834 decreased the numbers of other inflammatory and immune cells including neutrophils (Fig. 3C), T lymphocytes (Fig. 3D) and macrophages (Fig. 3E). These results suggest that the inhibitory effect of SUN13834 on skin thickening might be associated with inflammatory cell accumulation at the affected sites.

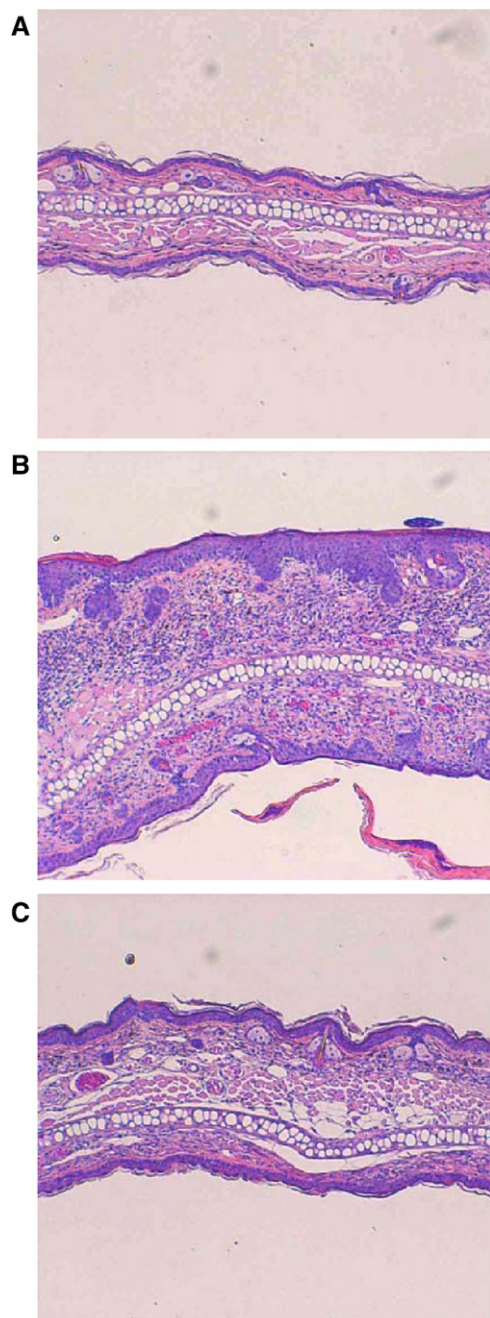


Fig. 2. Histological analysis of effect of SUN13834 in dermatitis model induced by repeated painting with DNFB in mice. Dermatitis was elicited by repeated painting of 0.15% DNFB (in acetone/olive oil) on outer and inner sides of mouse ear 6 times at seven-day intervals. The same volume of acetone/olive oil was painted in control mice. SUN13834 was orally administered daily for 5 weeks from the day of first DNFB application. The sections were prepared as described in Materials and methods, and stained with hematoxylin and eosin. A, Control; B, vehicle; C, SUN13834 (10 mg/kg). Original magnification, $\times 50$.

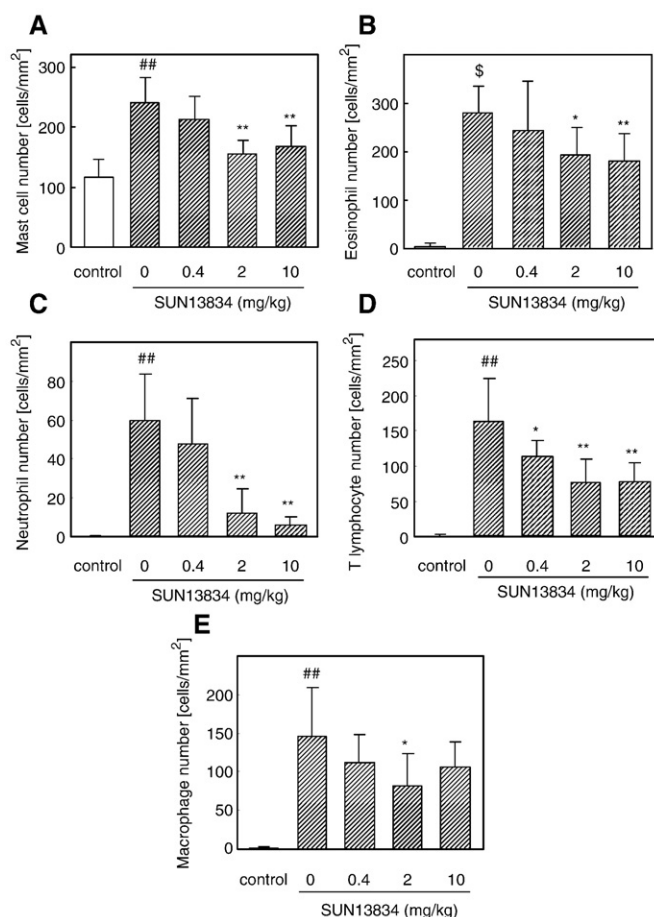


Fig. 3. Effect of SUN13834 on inflammatory cell accumulation in the skin in dermatitis model induced by repeated painting with DNFB in mice. Dermatitis was elicited by repeated painting of 0.15% DNFB (in acetone/olive oil) on outer and inner sides of mouse ear 6 times at seven-day intervals. The same volume of acetone/olive oil was painted in control mice. SUN13834 was orally administered daily for 5 weeks from the day of first DNFB application. The cell numbers in dermis were counted as described in Materials and methods. A, Mast cells; B, eosinophils; C, neutrophils; D, T lymphocytes; E, macrophages. Data are mean $\pm$ S.D. ^{##}, $P < 0.01$ as compared with the control group (Student's *t*-test); ^{\$}, $P < 0.01$ as compared with the control group (Aspin-Welch's *t*-test); ^{*}, $P < 0.05$, ^{**}, $P < 0.01$ as compared with vehicle group (Dunnett's test) ($n = 10$).

3.3. Scratching behavior induced by repeated painting of DNFB

As shown in Fig. 4, topical application of DNFB on the back skin of mouse elicited scratching behavior. For example, when the scratching behavior was counted for 1 h immediately after the DNFB painting, the number of scratching behavior in the DNFB-treated mice was significantly increased as compared with the control even at the first painting and increased further in a time-dependent manner for 3 weeks, reaching to plateau thereafter (Fig. 4A). The scratching number increased similarly when counted at 24 h after the DNFB challenge (Fig. 4B). However, the increase was gradual and the numbers of scratching behavior at 24 h were less than that at 0–1 h after the painting.

3.4. Effect of chymase inhibitor SUN13834 on scratching behavior in DNFB-induced dermatitis model – 1) time course analysis

Oral administration of SUN13834 inhibited the increase in the number of scratching behavior, and significant effect was detected at 3–5 weeks for 50 mg/kg and at 5 weeks for 2–10 mg/kg, when the scratching number was counted for 1 h immediately after the DNFB painting (Fig. 4A). Although the inhibitory effect of SUN13834 was also observed on the evaluation at 24 h after the painting, statistical

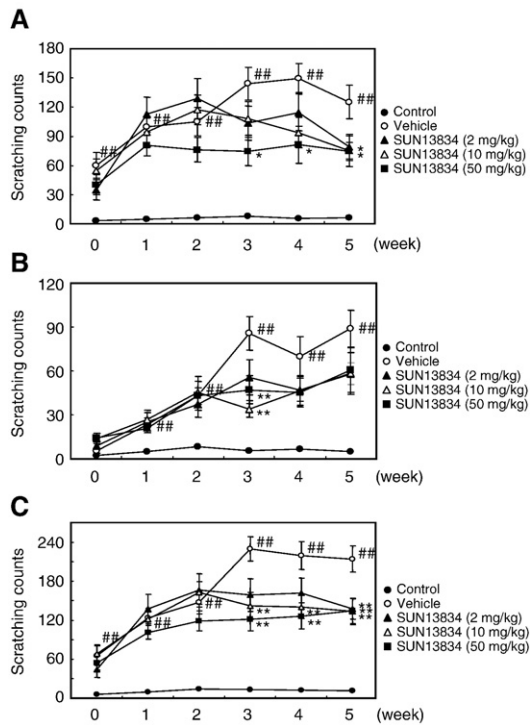


Fig. 4. Effect of SUN13834 on scratching behavior in dermatitis model induced by repeated painting with DNFB in mice (time course). Dermatitis was elicited by repeated painting of 0.1% DNFB (in acetone/olive oil) to the shaved back of mouse 6 times at seven-day intervals. The same volume of acetone/olive oil was painted in control mice. SUN13834 was orally administered daily for 5 weeks from the day of first DNFB application. The scratching behavior was evaluated as described in Materials and methods. Data are mean $\pm$ S.E.M. ($n=12$ except for the group of SUN13834 at 50 mg/kg, in which one mouse was excluded from the experiment because of the removal of magnet from the paw, resulting in $n=11$). A: Scratching behavior at 0–1 h after each DNFB challenge; B: scratching behavior at 24–25 h after each DNFB challenge; C: total count of scratching behavior for 2 h (0–1 h and 24–25 h after each challenge); ##, $P<0.01$ as compared with the control group (Student's *t*-test), *, $P<0.05$, **, $P<0.01$ as compared with vehicle group (Dunnett's test).

significance was detected at 10 and 50 mg/kg only at 3 weeks (Fig. 4B). When the data were analyzed by the sum of the scratching numbers at 0–1 h and 24–25 h, significant inhibition by SUN13834 was indicated at doses of 10 and 50 mg/kg at 3–5 weeks, whereas SUN13834 at 2 mg/kg showed a significant inhibition only at 5 weeks (Fig. 4C).

3.5. Effect of chymase inhibitor SUN13834 on scratching behavior in DNFB-induced dermatitis model – 2) analysis by AUC

When the effect of SUN13834 on itch was further analyzed by AUC of the skin data, it was shown that the inhibitory effect of SUN13834 on the scratching was statistically significant at 10 and 50 mg/kg immediately after the DNFB painting (Fig. 5A). On evaluation at 24 h, statistical significance in the inhibition was detected at 2 and 10 mg/kg (Fig. 5B). When the analysis was performed further by the total scratching numbers at 0–1 h and 24–25 h, the inhibitory effect of SUN13834 on the scratching was dose-dependent and statistically significant at 10 and 50 mg/kg (Fig. 5C). The treatment with SUN13834 at 2 mg/kg showed a tendency to inhibit the scratching, but statistical significance was not detected.

4. Discussion

The data in the present study showed that chymase inhibitor SUN13834 inhibits skin swelling as well as inflammatory cell accumulation in the skin in the mouse dermatitis model induced by multiple painting with DNFB (Figs. 1–3). These findings are consistent

with our previous data that other chymase inhibitors show similar effects in this model (Tomimori et al., 2002a), and that SUN13350 (free form of SUN13834) improves skin clinical score of NC/Nga mice that spontaneously develop dermatitis (Watanabe et al., 2007). The mechanisms underlying the effect of chymase inhibitor on cell accumulation is not completely known. However, chymase is known to be a chemoattractant for leukocytes (Tani et al., 2000; Tomimori et al., 2002a) and to stimulate the migration of the cells *in vitro*. In addition, it has been reported that chymase is involved in processing of stem cell factor (Longley et al., 1997; Tomimori et al., 2002b), a cytokine for mast cell proliferation, and it is thought therefore that inhibitory effect of SUN13834 on mast cell number in the skin might be due to the suppression of the processing.

The most interesting finding in this study is that chymase inhibitor reduces scratching behaviors in the DNFB-induced pruritus model. The mechanism of the DNFB-induced scratching behaviors is not clear at present. However, the scratching behaviors observed immediately and at 24 h after the challenge may be mediated by different mechanisms, because the effect of SUN13834 was dose-dependent at 2–50 mg/kg at 0–1 h after the challenge, whereas the effect was almost the same at these doses at 24–25 h (Fig. 5A and B). Supporting this idea is that the scratching behavior was evident at the first painting of DNFB at 0–1 h but not at 24–25 h (Fig. 4A and B). Such a trend in the time course was also reported by Inagaki et al. (2006) in a similar DNFB-induced itch model, while the procedure to induce itch is not entirely the same.

The mechanism in which chymase participates in scratching behavior is also uncertain, but it is important to note that an intradermal injection of rat mast cell chymase elicits itching in human skin (Hagermark et al., 1972), suggesting that chymase may be pruritogenic.

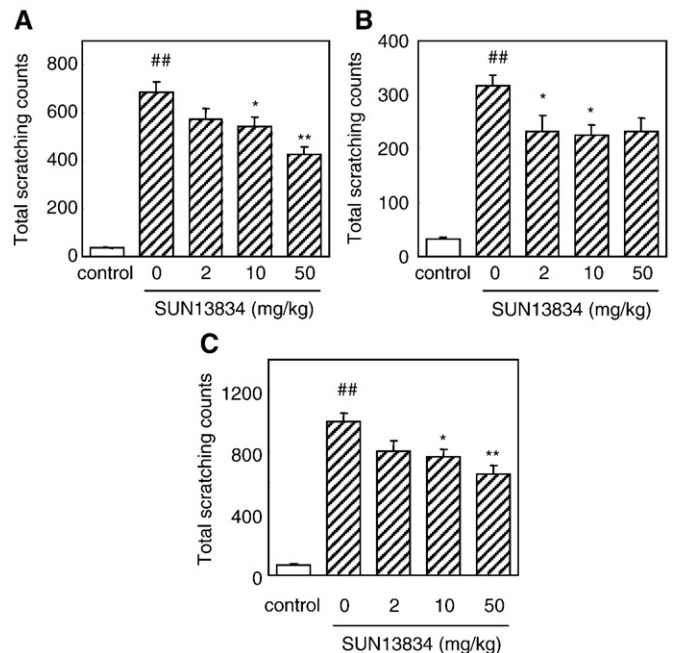


Fig. 5. Effect of SUN13834 on scratching behavior in dermatitis model induced by repeated painting with DNFB in mice (AUC). Dermatitis was elicited by repeated painting of 0.1% DNFB applied to the back of mouse 6 times at seven-day intervals. The same volume of acetone/olive oil was painted in control mice. SUN13834 was orally administered daily for 5 weeks from the day of first DNFB application. The scratching behavior was evaluated as described in Materials and methods, and the data are shown as AUC of the kinetics in Fig. 4. Data are mean $\pm$ S.E.M. ($n=12$ except for the group of SUN13834 at 50 mg/kg, in which one mouse was excluded from the experiment because of the removal of magnet from the paw, resulting in $n=11$). A: Scratching behavior at 0–1 h after each DNFB challenge; B: scratching behavior at 24–25 h after each DNFB challenge; C: total counts of scratching behavior for 2 h (0–1 h and 24–25 h after each challenge) ## $P<0.01$ as compared with the control group (Student's *t*-test), *, $P<0.05$, **, $P<0.01$ as compared with vehicle group (Dunnett's test).

A possible explanation for the action of chymase would be that chymase might induce itch via activation of some protease-activating receptor (PAR) on the cells such as neurons. This idea is based on the following facts; i) chymase stimulates inflammatory cells to migrate (Tomimori et al., 2002a; Watanabe et al., 2002) or produce a chemokine (Terakawa et al., 2006), suggesting direct action of chymase to the cells, ii) chymase activates ERK and p38 probably through G-protein-coupled receptor (Terakawa et al., 2005), iii) cathepsin G (an enzyme related to chymase) interacts with PAR-4 on platelets (Sambrano et al., 2000), iv) PAR-2 expression was markedly enhanced on primary afferent nerve fibers in the skin of the patients with atopic dermatitis and injection of PAR-2 agonists provokes itch (Steinhoff et al., 2003).

An alternative possible mechanism for involvement of chymase in itch could be indirect action. For example, endothelin-1 (ET-1) is known to cause itch in mice (Trentin et al., 2006), and ET-1 can be generated by chymase by converting extracellular big ET-1 (the inactive precursor of ET-1) into a 21-residue peptide named ET-1 (1–31), which is subsequently cleaved to yield ET-1 by neutral endopeptidase and ET-converting enzyme (Nakano et al., 1997). Since the ET-1-induced itch seems mediated through ET(A) receptors (Trentin et al., 2006), it is interesting to elucidate whether ET(A) receptor antagonists inhibit the chymase-induced itch.

The most interesting finding in recent studies concerning pruritus is the involvement of IL-31. That is, transgenic mice overexpressing IL-31 were shown to develop severe pruritus and skin lesions (Dillon et al., 2004), and IL-31 expression is known to be elevated in atopic dermatitis (Sonkoly et al., 2006) as well as its animal model (Takaoka et al., 2005). As far as we know, however, chymase is not considered to influence on the expression of IL-31, though it is possible that it inhibits the expression as a result of improving the dermatitis.

In the dermatitis model induced by repeated DNFB painting, tacrolimus significantly inhibited the scratching behavior, while dexamethasone failed to show the effect (Inagaki et al., 2006). In addition, dexamethasone inhibited the accumulation of inflammatory cells, but tacrolimus did not. These results suggest that inflammatory cell accumulation is not related to the itch induction in this model. Their data indicated that anti-itch effect of tacrolimus is mediated, at least in part, by inhibition of nerve fiber extension (Inagaki et al., 2006). This finding also suggests that the improvement of pruritus by SUN13834 is not due to the inhibition of cell accumulation.

The effect of SUN13834 was examined by administering it at the initiation of the experiment, and it is interesting and important to know whether this compound also has a therapeutic effect. Previously, we have shown that SUN13834 improves clinical skin score of NC/Nga mice when administered before disease develops (Watanabe et al., 2007). The compound was also effective in the model if given after the dermatitis was evident, but the effective dose was higher than that for the prophylactic effect (our unpublished data). Thus it is possible that SUN13834 could show therapeutic effect if it is administered at higher doses.

There are a number of pruritogenic stimuli, in which histamine is one of the most potent mediators (Behrendt and Ring, 1990). However, there is no clear evidence to support the effectiveness of non-sedative antihistamine agents in atopic dermatitis (Klein and Clark, 1999), showing that histamine is not the major trigger for pruritus at least in atopic dermatitis. Neither skin inflammation nor scratching behavior was susceptible to antihistamines in our DNFB-induced model used in the present study (data not shown). Thus, this model is thought to be useful for evaluating anti-itch agents for atopic dermatitis.

The inhibitory activity of SUN13834 is specific for chymase and this compound exhibits little inhibition in other enzymes (Maruoka et al., 2007). In addition, SUN13834 is stable relative to our prototype inhibitors (Fukami et al., 2000), and thus thought to be useful as oral agent. In fact, it inhibited skin inflammation and pruritus in our model as demonstrated in this paper. Taken together, SUN13834 is expected to be used as a novel therapeutic agent for atopic dermatitis.

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