

The Inhibitory Effect of S-777469, a Cannabinoid Type 2 Receptor Agonist, on Skin Inflammation in Mice

Takayo Haruna^a Masahiko Soga^a Yasuhide Morioka^a
Kinichi Imura^b Yoko Furue^e Mina Yamamoto^c Jun Hayakawa^b
Masashi Deguchi^a Akinori Arimura^d Kiyoshi Yasui^c

^aDrug Discovery and Disease Research Laboratory, ^bBiomarker R&D Department,
^cGlobal Innovation Office, ^dGlobal Project Management Department, SHIONOGI & Co., Ltd.,
and ^eShionogi TechnoAdvance Research Co., Ltd., Osaka, Japan

Keywords

S-777469 · Cannabinoid type 2 receptor ·
2-Arachidonoylglycerol · Inflammation · Dermatitis

Abstract

We investigated the effects of 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 (CB2) agonist, on 1-fluoro-2,4-dinitrobenzene (DNFB)-induced ear inflammation and mite antigen-induced dermatitis in mice. The oral administration of S-777469 significantly suppressed DNFB-induced ear swelling in a dose-dependent manner. In addition, S-777469 significantly alleviated mite antigen-induced atopic dermatitis-like skin lesions in NC/Nga mice. A histological analysis revealed that S-777469 significantly reduced the epidermal thickness and the number of mast cells infiltrating skin lesions. We demonstrated that S-777469 inhibited mite antigen-induced eosinophil accumulation in skin lesions and an endogenous CB2 ligand, 2-arachidonoylglycerol (2-AG)-induced eosinophil migration in vitro. Moreover, we confirmed that 2-AG levels significantly increased in skin

lesions of mite antigen-induced dermatitis model. Together, these results suggest that S-777469 inhibits skin inflammation in mice by blocking the activities of 2-AG.

© 2017 S. Karger AG, Basel

Introduction

Cannabinoids such as Δ^9 -tetrahydrocannabinol, the primary constituent of *Cannabis sativa*, and their natural and synthetic analogs have a wide range of pharmacological effects on the central nervous system (CNS) and immune system [1–3]. There are 2 primary types of cannabinoid receptors, cannabinoid type 1 (CB1) and cannabinoid type 2 (CB2), both of which couple with G-proteins. CB1 is found in abundance in the CNS, and evidence suggests that CB1 is involved in the regulation of higher brain functions such as memory, motion, and pain sensation [4]. CB2 is primarily found in peripheral organs and cells associated with the immune system, including spleen, tonsils, and thymus, and in B lymphocytes, natural killer cells, and macrophages/monocytes [5]. This suggests that CB2 plays a key role in the regula-

tion of inflammation. Indeed, several selective CB2 agonists with anti-inflammatory effects have been reported [6–10].

The principal endogenous CB2 ligand, 2-arachidonoylglycerol (2-AG), acts as an agonist [11–13]. Previous studies have reported that 2-AG induces the migration of several types of leukocytes, including human eosinophilic leukemia EoL-1 cells, macrophage-like HL-60 cells, human peripheral blood monocytes, mouse splenocytes, and microglia, via a CB2-dependent mechanism [14–17]. In addition, 2-AG and CB2 have been implicated in the inflammation in *in vivo* mouse models of hapten-induced contact dermatitis [18, 19], 12-O-tetradecanoylphorbol 13-acetate-induced acute inflammation [20], and carrageenan-evoked peritonitis [21].

Recently, we identified S-777469 (1-[[6-Ethyl-1-[4-fluorobenzyl]-5-methyl-2-oxo-1, 2-dihydropyridine-3-carbonyl]amino]-cyclohexanecarboxylic acid) to be a novel, selective CB2 agonist [22]. In a previous report, we demonstrated that S-777469 selectively binds and activates CB2, but not CB1, and that it exerted an anti-pruritic effect on mice without inducing CNS-associated side effects [23]. In this study, we investigated whether S-777469 has an anti-inflammatory activity. We examined the effect of S-777469 in mouse models of hapten-induced ear inflammation and mite antigen-induced atopic dermatitis. In addition, we investigated the effect of S-777469 on 2-AG-induced EoL-1 cell migration. Our results suggest that S-777469 inhibits inflammation via the inhibition of 2-AG-induced inflammatory cell migration.

Materials and Methods

Mice

Female Balb/c and NC/Nga mice were purchased from Charles River Japan (Yokohama, Japan). The mice were housed in an environment with controlled temperature (20–26°C), humidity (30–70%), and lighting. The lights were on from 08:00 to 20:00 for the Balb/c mice and from 06:00 to 18:00 for the NC/Nga mice. 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 was synthesized in our laboratories. The following drugs and reagents were also used in this study: 1-fluoro-2,4-dinitrobenzene (DNFB; Nacalai Tesque, Kyoto, Japan), prednisolone, acetone (Sigma-Aldrich, St. Louis, MO, USA), olive oil, methylcellulose (MC), sodium *n*-butyrate (Wako Pure Chemicals, Osaka, Japan), hydrophilic petrolatum (Maruishi, Osaka, Japan), and 2-AG (Cayman Chemical, Ann Arbor, MI, USA).

The optimal dosages or concentrations of S-777469 and prednisolone in each experiment were determined in preliminary experiments. The dose of prednisolone which exerted the highest effect without apparent side effects such as weight loss was determined.

DNFB-Induced Ear Model of Inflammation

Before first sensitization, the abdominal hair of Balb/c mice was shaved using an electric clipper after the mice were anesthetized with ether. The mice were sensitized on days 0 and 1 by painting the shaven abdomen with 30 μ L of 0.5% DNFB dissolved in acetone/olive oil (4:1). On days 6 and 8, mice were challenged by applying 10 μ L of 0.3% DNFB to both surfaces of right and left ears. The negative control mice were treated with an acetone/olive oil (4:1) mixture, and S-777469 and prednisolone suspended in 0.5% MC were orally administered once daily from days 6 to 9. On the challenge days (days 6 and 8), either S-777469 or the negative control 0.5% MC were administered within 30 min after the DNFB challenge. Ear swelling was used as an index of ear inflammation and was measured using a thickness gauge (SM-112; Teclock, Nagano, Japan). Ear thickness was measured daily from days 6 to 10. On the challenge days (days 6 and 8), ear swelling was measured before the DNFB challenge. The mean value of the absolute thickness of both ears was calculated.

Mite Antigen-Induced Model of Dermatitis

Mite antigen-induced dermatitis was established using a previously described protocol [24, 25]. On the day of first mite-antigen induction of dermatitis (day 0), mice were anesthetized with ether, and the hair on the dorsal-rostral skin of the NC/Nga mice was clipped using an electric clipper. Residual hair was depilated using a hair removal cream (Kracie, Tokyo, Japan). The antigen challenge consisted of a topical application of 100 mg of *Dermatophagoides farinae* body (Dfb) ointment to the dorsal shaved skin and the surface of both ears. On days 3, 7, 10, 14, and 17, hair that had regrown was removed using an electric clipper and an electric shaver. The skin barrier was disrupted by applying 150 μ L of 4% sodium dodecyl sulfate to the shaved dorsal skin and the surface of both ears 3 h before Dfb ointment was applied.

The following 4 parameters were assessed to score the degree of dermatitis: (1) erythema/hemorrhage; (2) scarring/dryness; (3) edema; and (4) excoriation/erosion. Each parameter was scored as either 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The dermatitis score was calculated as the sum of individual scores.

The effect of S-777469 on chronic dermatitis was evaluated in mice with a dermatitis score greater than 3 on day 14, and a score of 5–10 on day 21. S-777469 was administered once orally on day 21, and twice daily from days 22 to 33. In the prednisolone-treated group, prednisolone was orally administered once daily from days 21 to 33. Mice were sacrificed on day 34, and histopathological changes in the skin lesions were subsequently evaluated.

To investigate the effect of S-777469 on eosinophil infiltration into the dermis, the mice were re-challenged with Dfb ointment on day 21. S-777469 was administered immediately before, and 16 h after the application of the Dfb ointment. Mice in the control group were sacrificed on day 21 (the day of the last antigen challenge) while mice in the treated group were sacrificed on day 22 (24 h after the last antigen challenge).

Histopathological Analysis

Portions of the dorsal skin were fixed with 10% neutral formalin, embedded in paraffin, and sectioned with a thickness of 4 μm . The sections were stained with hematoxylin-eosin (H&E), toluidine blue (TB; pH 4.1), or direct fast scarlet (DFS).

The thickness of the epidermis was measured using a computer digitizing system consisting of a light microscope with camera (DXM1200; Nikon, Tokyo, Japan) and software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA). The computer program was configured to determine the epidermal thickness of the H&E-stained sections mounted on the slides. The slides were evaluated on a microscope with a 20 $\times$ objective lens. Fields of view (336.9 $\times$ 252.5 μm) lacking hair follicles were randomly selected, and 5 fields of view for each sample were evaluated.

Mast cells stained with TB and eosinophils stained with DFS in a 1 mm width from the epidermis to the panniculus adiposus were counted using a squared eyepiece micrometer under a microscope at a magnification of 400 $\times$.

Inhibition of 2-AG-Induced EoL-1 and HL-60 Cell

Chemotaxis by S-777469

EoL-1 and HL-60 cell chemotaxis was assayed using a Chemotaxicell (5 μm pore size; Kurabou, Osaka, Japan) in 24-well culture plates (Corning, Corning, NY, USA). EoL-1 cells differentiate into eosinophils after 5 days in culture with 0.5 mmol/L sodium n-butyrate, while HL-60 cells differentiate into monocytes after 6 days in culture with 100 nmol/L 1,25-(OH)₂ vitamin D₃ in a 37°C in a humidified atmosphere of 5% CO₂ [26, 27]. Differentiated EoL-1 or HL-60 cells were washed 3 times with pre-warmed CT-buffer (RPMI 1640 medium supplemented with 0.5% fetal bovine serum and 10 mmol/L HEPES 7.55). EoL-1 cells (4 $\times$ 10⁵ cells) and HL-60 cells (2.5 $\times$ 10⁶ cells) were suspended in 200 μL of CT-buffer and transferred to the Chemotaxicell (the upper well). 2-AG and/or S-777469, or DMSO in 600 μL of CT-buffer was added to each well of the culture plates in the lower well. After 4 h incubation at 37°C in a humidified atmosphere of 5% CO₂, cells that had migrated from the upper well to the lower well were counted using a CDA-500 particle counter (Sysmex, Hyogo, Japan).

Extraction of 2-AG

We obtained 8 mm punch biopsies from the dorsal skin of the mice. These biopsy samples were weighed, dissected, and immediately frozen in liquid nitrogen. Skin samples were homogenized in pre-chilled saline (9 times the volume [μL] of the skin sample) in a TissueLyser (25 Hz, 5 min). A solution of acetonitrile/methanol (1:1; 200 μL) was added to 50 μL of the homogenate, and the mixture was vortexed for 5 min. The supernatants were centrifuged (25°C, 12,000 rpm, 5 min), collected, and analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

LC-MS/MS Analysis of 2-AG Levels

The LC-MS/MS analysis was conducted using a Prominence system (Shimadzu Corporation, Kyoto, Japan) and an API 5000 triple quadrupole mass spectrometer (MDS-Sciex, Toronto, Canada). A 10 μL sample was injected into a Cadenza CD-C18 analytical column (3.0 $\times$ 50 mm, 3 μm ; Imtakt, Kyoto, Japan) at 60°C and eluted using a linear gradient with a mobile phase of acetonitrile/water/formic acid (500:500:1). The total run time was 14 min, and the flow rate was 0.5 mL/min.

To evaluate 2-AG, the mass spectrometer was operated in positive ion, electrospray ionization mode, and 2-AG-d₈ was used as an internal standard. Multiple reaction monitoring (MRM) for each analyte was conducted with the selected [M+H]⁺ ion, and transitions were monitored using m/z 379.2 > 287.0 for 2-AG and m/z 387.2 > 294.2 for 2-AG-d₈. Mass spectrometer parameters were optimized for MRM transitions, as indicated by the adjustment of the declustering potential to 91 V and collision energy to 19 eV.

Statistical Analysis

Data are expressed as mean $\pm$ SE or mean $\pm$ SD. Statistically significant differences were assessed using Welch's *t* test or Dunnett's test, with the exception of dermatitis scores. These were statistically analyzed using pair-wise comparisons based on repeated ANOVA measurements and a mixed-effects model. *p* values <0.05 were considered statistically significant.

Results

Effect of S-777469 on DNFB-Induced Ear Swelling

The shaved abdominal skin of Balb/c mice was sensitized with DNFB on days 0 and 1, and the mice were subsequently challenged on both surfaces of their right and left ears with DNFB on days 6 and 8. After the first DNFB challenge, ear thickness markedly increased in the vehicle-treated group compared with the negative control mice, and this effect persisted until day 10 (Fig. 1). To determine the effect of S-777469 on DNFB-induced ear swelling, S-777469 (1, 3, or 10 mg/kg) was orally administered once daily from days 6 to 9. The effect of S-777469 was compared with prednisolone (1 mg/kg). S-777469 (3 and 10 mg/kg) significantly inhibited ear swelling from days 7 to 10. Prednisolone also suppressed ear swelling, and the effect was significant from days 8 to 10 (Fig. 1). However, the efficacy of S-777469 (10 mg/kg) was superior to prednisolone (1 mg/kg).

Effect of S-777469 on Mite Antigen-Induced Dermatitis

Repeated application of Dfb ointment following barrier disruption induced atopic dermatitis-like chronic skin lesions in NC/Nga mice. Therefore, we evaluated the efficacy of S-777469 using this mouse model. In the vehicle-treated group, skin inflammation persisted for 13 days even though the mice received no additional applications of the Dfb ointment. S-777469 (10 and 30 mg/kg twice daily) reduced dermatitis scores in a dose-dependent manner, and the 30 mg/kg dose provided statistically significant improvements compared with the vehicle. Prednisolone (5 mg/kg once daily) also reduced dermatitis significantly (Fig. 2). H&E staining of the skin

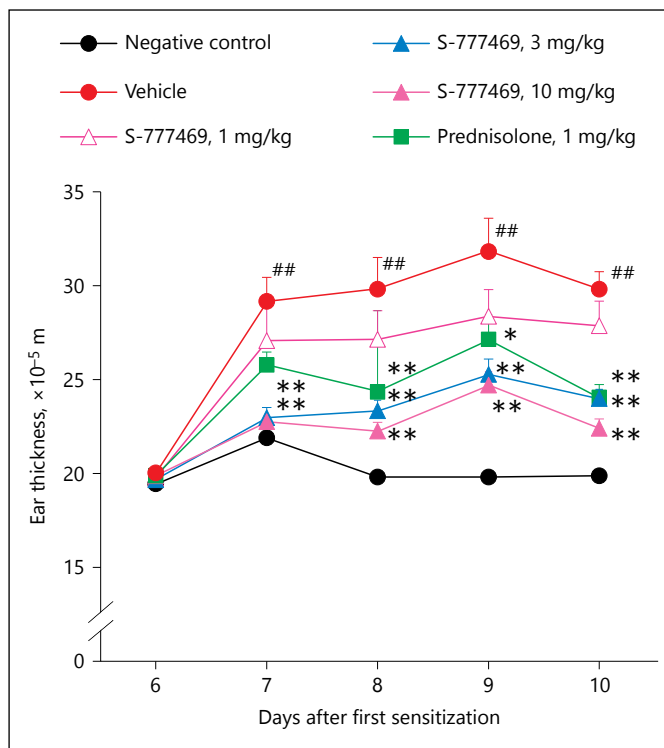


Fig. 1. The effect of S-777469 on DNFB-induced ear swelling. Mice were sensitized on days 0 and 1 by painting 30 μ L of 0.5% DNFB onto the shaved abdomen. On days 6 and 8, the mice were challenged by applying 10 μ L of 0.3% DNFB onto both surfaces of the right and left ears. The negative control mice were treated with acetone/olive oil (4:1). S-777469, prednisolone, and the vehicle (0.5% MC) were orally administered once daily from days 6 to 9 ($n = 7$). Data are presented as mean $\pm$ SE. $^{##} p < 0.01$ vs. negative control (Welch's t test), $^* p < 0.05$, $^{**} p < 0.01$ vs. vehicle (Dunnett's test).

lesions demonstrated that acanthosis (epidermal thickening) and inflammatory cell infiltration into the dermis occurred in the vehicle-treated group (Fig. 3). S-777469 significantly inhibited epidermal thickening in a dose-dependent manner compared with the vehicle control. S-777469 at doses of 10 and 30 mg/kg reduced the epidermal thickness by 34 and 52%, respectively, on day 34 (13 days after the first administration). In contrast, prednisolone did not inhibit epidermal thickening compared with the vehicle control (Fig. 3a, c). Moreover, S-777469 suppressed inflammatory cell infiltration in H&E-stained sections and significantly reduced the number of mast cells in TB-stained sections compared with the vehicle-treated group. S-777469 at doses of 10 and 30 mg/kg reduced the mast cell number by 25 and 40%, respectively, on day 34 (13 days after the first ad-

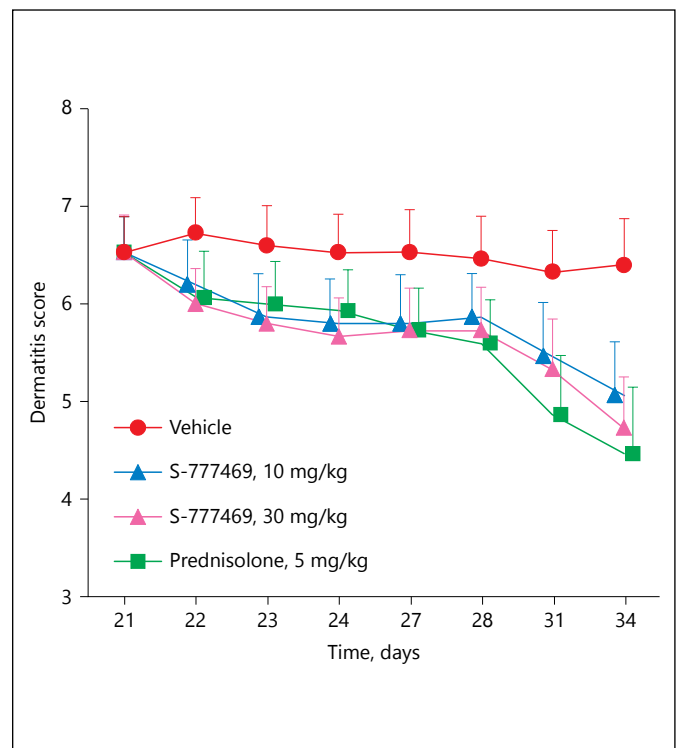


Fig. 2. The effect of S-777469 on mite antigen-induced dermatitis in NC/Nga mice. S-777469 and vehicle (0.5% MC) were administered twice daily, and prednisolone was administered once daily. The dermatitis score was evaluated on days 22, 23, 24, 27, 28, 31, and 34 ($n = 15$). Each value represents mean $\pm$ SE. The statistical analyses were conducted using pair-wise comparisons with the vehicle-treated group based on repeated ANOVA with a mixed-effects model. Multiple comparisons were conducted using Bonferroni's procedure. p values vs. the vehicle were as follows: S-777469 20 mg/kg/day, $p = 0.0523$; S-777469 60 mg/kg/day, $p = 0.0021$; prednisolone 5 mg/kg/day, $p < 0.0001$.

ministration). Similarly, the number of mast cells in the prednisolone-treated group significantly decreased by 24% (Fig. 3b, d).

Effect of S-777469 on Eosinophil Infiltration in the Mite Antigen-Induced Dermatitis Model

We further investigated the effect of S-777469 on eosinophil infiltration in mite antigen-induced dermatitis. In this model, eosinophils were transiently recruited into inflamed skin 24 h after the mite antigen challenge. Therefore, it was evaluated 24 h after the application of the Dfb ointment on day 21. S-777469 (1 and 10 mg/kg) was orally administered immediately before, and 16 h after the antigen challenge on day 21. In the vehicle-treated group, eosinophils infiltrated the re-challenged skin, and the number of eosinophils in the control and vehicle-treated

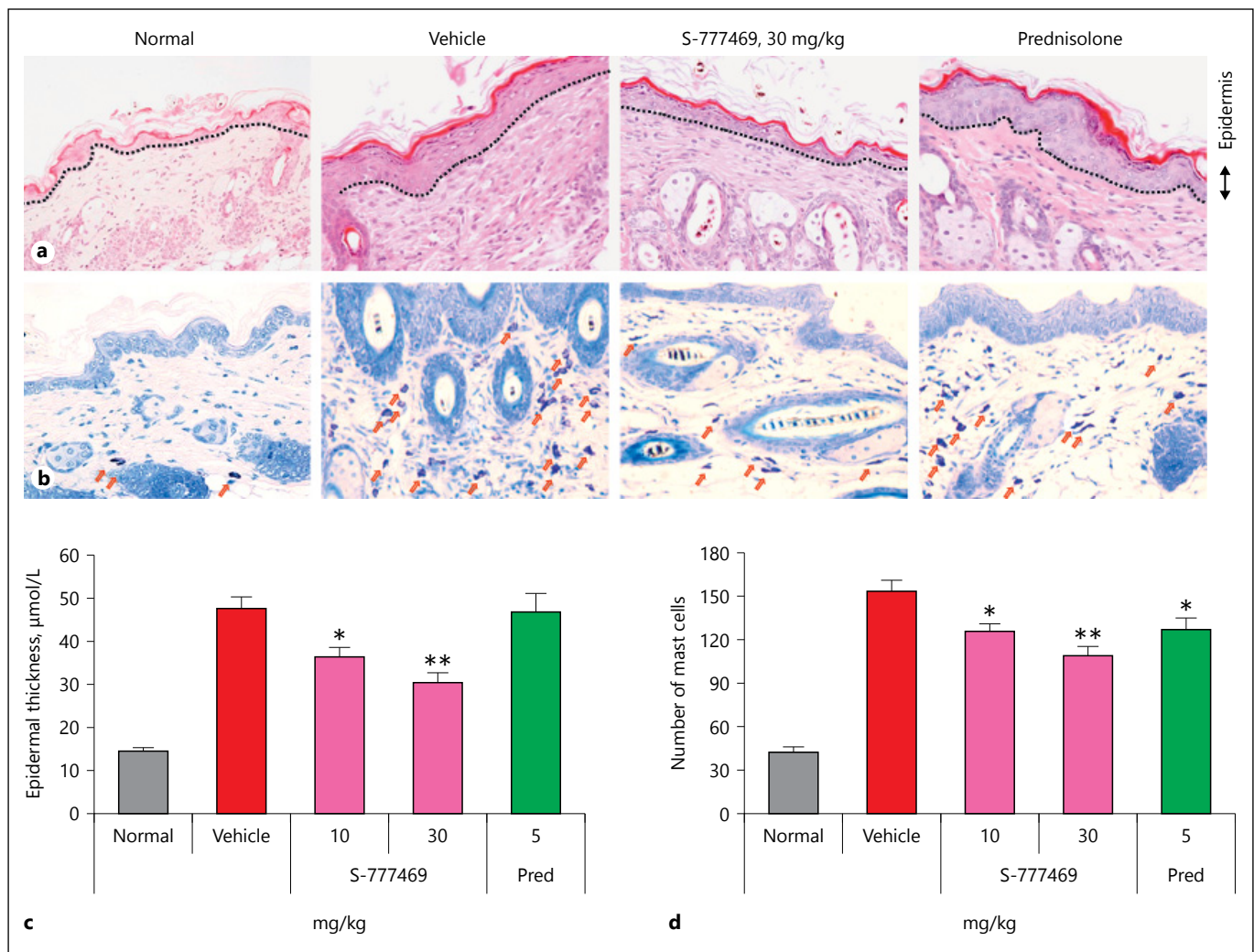


Fig. 3. The effects of S-777469 on epidermal thickness and mast cell number in the dorsal skin of the mite antigen-induced dermatitis model. S-777469 and vehicle (0.5% MC) were administered twice daily, and prednisolone was administered once daily. On day 13, histological changes in the dorsal skin samples stained with H&E (**a**) and TB (**b**), epidermal thickness (**a**, dotted line), and the

number of mast cells (**b**, arrows) were evaluated (**c**, **d**). Mast cells in a 1 mm section from the epidermis to the panniculus adiposus were counted. Original magnification: 200× (**a**) and 400× (**b**). Each value represents mean ± SE of 10 mice in the normal group and 15 mice in other groups. * $p < 0.05$, ** $p < 0.01$ vs. vehicle (Dunnett's test).

groups were 31.5 ± 7.2 and 57.7 ± 7.5 cells/mm, respectively. Eosinophil infiltration was inhibited by 29 and 88% with S-777469 at doses of 1 and 10 mg/kg, respectively, and the effect of the 10 mg/kg dose was statistically significant (Fig. 4).

2-AG Levels in Mite Antigen-Induced Dermatitis

To determine if the endogenous CB2 ligand 2-AG is involved in chronic inflammation, we evaluated 2-AG levels in mite antigen-induced dermatitis skin lesions on day 21. As shown in Figure 5, LC-MS/MS analysis re-

vealed that 2-AG levels significantly increased in the skin lesion samples from the mite antigen-induced dermatitis model (18,924 pg) compared with skin samples from normal mice (7,440 pg).

Effect of S-777469 on 2-AG-Induced Migration of EoL-1 and HL-60 Cells

As it has previously been reported that 2-AG induces the migration of EoL-1 human eosinophilic leukemia cells via the CB2 receptor [14], we investigated whether S-777469 can inhibit 2-AG-induced EoL-1 cell chemo-

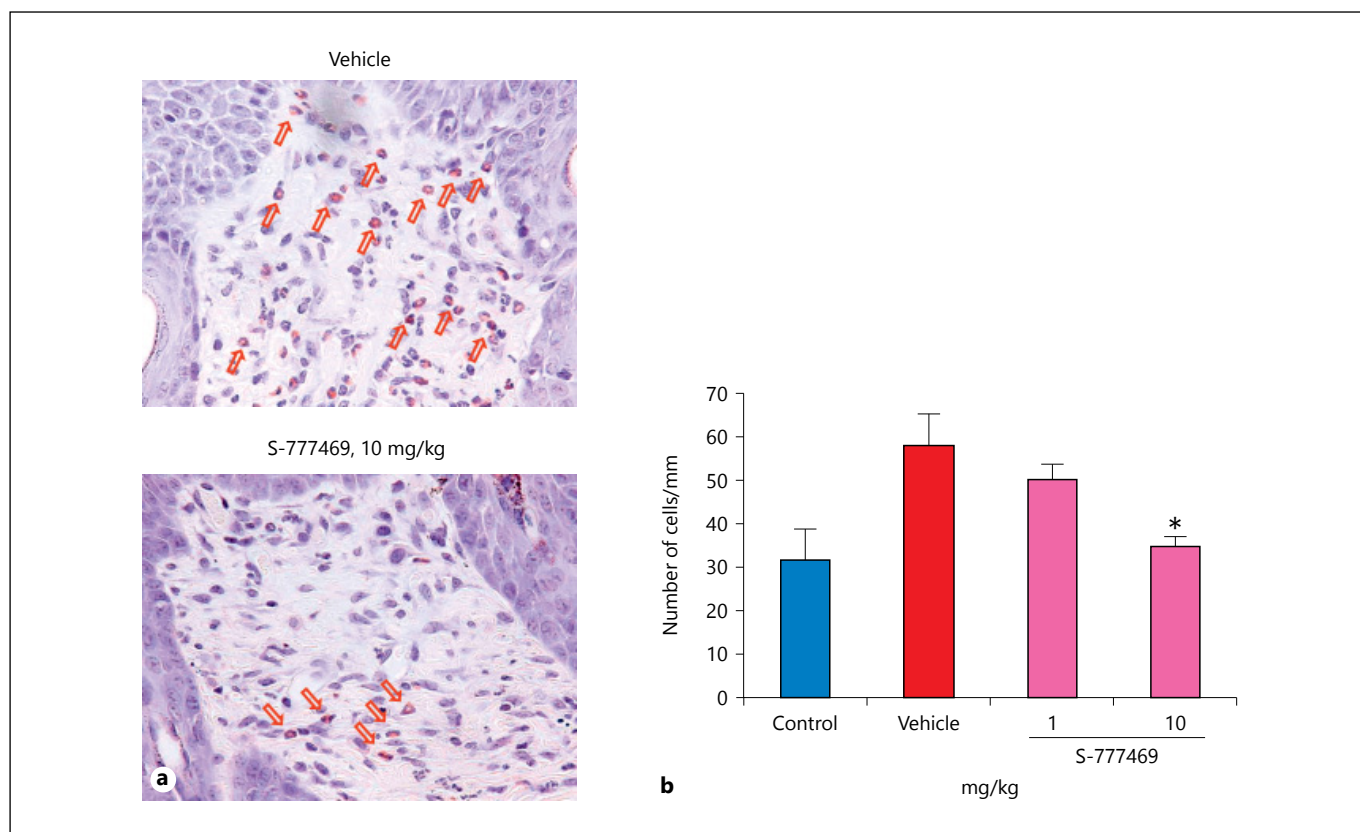
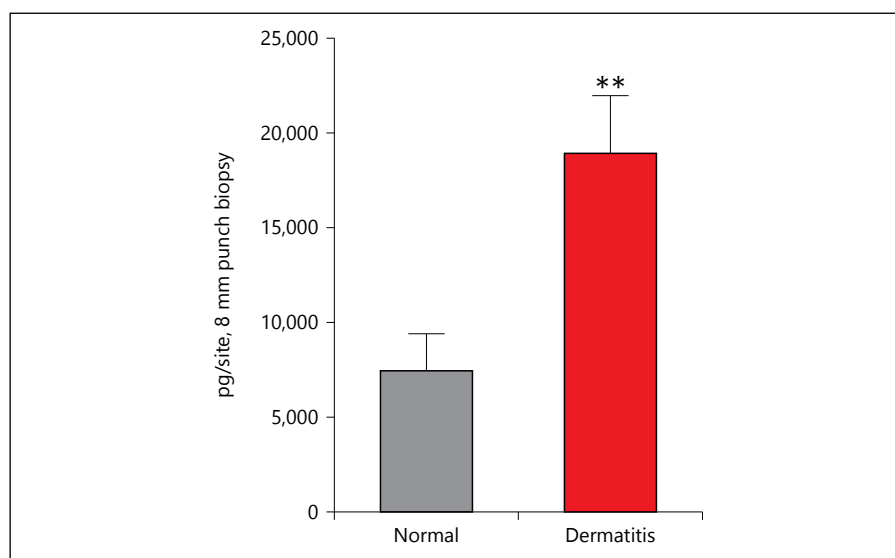


Fig. 4. The effect of S-777469 on eosinophil infiltration in the mite antigen-induced dermatitis model. S-777469 and vehicle (0.5% MC) were administered twice – immediately before and 16 h after the last antigen challenge on day 21. Histological changes in dorsal skin samples stained with DFS (**a**) and the number of eosinophils (**b**) were evaluated 24 h after the last antigen challenge. In the

control group, eosinophils were counted prior to the last antigen challenge. The eosinophils in a 1 mm section from the epidermis to the panniculus adiposus were counted. Original magnification: 400×. Each value represents the mean ± SE. * $p < 0.05$ vs. vehicle (Dunnett's test).

Fig. 5. 2-AG in the skin of the mite antigen-induced dermatitis model. 2-AG levels in the skin of normal mice and the skin lesions of mice with dermatitis on day 21 were analyzed using LC-MS/MS analysis ($n = 10$ and $n = 16$). Each value represents mean ± SD. ** $p < 0.01$ vs. normal (Welch's t test).



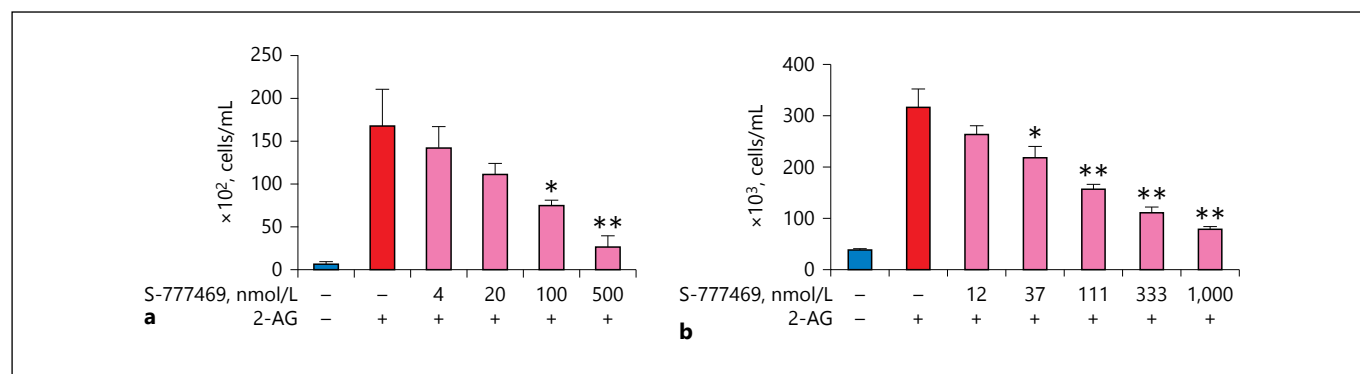


Fig. 6. The effect of S-777469 on 2-AG-induced EoL-1 and HL-60 cell migration. EoL-1 cells (4×10^5 cells) and HL-60 cells (2.5×10^6 cells) suspended in CT-buffer and 2-AG (0.5 $\mu\text{mol/L}$) were added to the lower and upper wells, respectively. EoL-1 cells or HL-60

cells were treated with S-777469 or DMSO. Cells that migrated from the upper well to the lower well were counted. Each value represents mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ vs. 2-AG-treated control (Dunnett's test).

taxis. 2-AG (0.5 $\mu\text{mol/L}$) strongly induced EoL-1 cell migration (Fig. 6a), while S-777469 suppressed this effect in a concentration-dependent manner. The inhibition rates observed with S-777469 at doses of 4, 20, 100, and 500 nmol/L were 16, 35, 58, and 88%, respectively (Fig. 6a). We conducted a similar cell migration study using monocytic HL-60 cells and showed that S-777469 suppressed HL-60 cell migration in a concentration-dependent manner. Inhibition rates observed with S-777469 at doses of 12, 37, 111, 333, and 1,000 nmol/L were 19, 35, 57, 74, and 85%, respectively (Fig. 6b). S-777469 (1 $\mu\text{mol/L}$), without 2-AG, hardly induced the migration of EoL-1 and HL-60 cells (data not shown).

Discussion

In this study, we investigated the anti-inflammatory effects of the selective CB2 agonist, S-777469. The oral administration of S-777469 significantly suppressed DNFB-induced ear inflammation in mice. In addition, S-777469 alleviated histological changes, including acanthosis and inflammatory cell infiltration, associated with the mite antigen-induced atopic dermatitis-like skin lesions. In addition, we confirmed that 2-AG levels increased in atopic dermatitis skin lesions. An in vitro cell migration assay demonstrated that S-777469 inhibited 2-AG-induced chemotaxis in eosinophilic (EoL-1) and monocytic (HL-60) cell lines. These results suggest that S-777469 exerts anti-inflammatory effects, potentially by suppressing 2-AG-induced inflammatory cell migration.

Cannabinoids exert immunosuppressive effects by inducing immune cell apoptosis, inhibiting cell proliferation,

suppressing cytokine production (tumor necrosis factor- α , granulocyte-macrophage colony-stimulating factor, and interferon- γ), and inducing regulatory T-cells [28]. These effects are thought to be mediated by CB2. Previous reports have demonstrated that the activation of CB2 has an anti-inflammatory effect in animal models of inflammation, including arachidonic acid-induced ear inflammation [8], carrageenan-induced paw edema [7], and experimental autoimmune encephalomyelitis [29]. These findings suggest that CB2 plays a key role in immunomodulation.

Previously, we have shown that S-777469, a CB2 selective agonist, exerts antipruritic effects without inducing CB1-associated CNS side effects [23]. Therefore, in this study, we used 2 distinct mouse models of skin inflammation to determine if S-777469 has anti-inflammatory activity. First, we examined the effect of S-777469 on conventional skin inflammation induced by a hapten, DNFB. An anti-inflammatory corticosteroid, prednisolone, significantly suppressed DNFB-induced increase in ear thickness. Orally administered S-777469 exerted a similar inhibitory effect in a dose-dependent manner. Next, we examined the effects of S-777469 on mite antigen-induced chronic dermatitis in NC/Nga mice [24]. The clinical, histological, and immunological features in this model are similar to those observed in patients with atopic dermatitis: S-777469 significantly suppressed dermatitis for 13 days. This reduction in dermatitis scores mediated by S-777469 was accompanied by improvements in various histological changes, such as epidermal thickening and inflammatory cell infiltration. To elucidate the mechanisms underlying the anti-inflammatory effects of S-777469, we evaluated the role of endogenous cannabinoids in mite antigen-induced skin inflammation.

Anandamide and 2-AG are the 2 primary endogenous cannabinoid receptor ligands. Sugiura et al. [30] proposed that 2-AG, but not anandamide, is a genuine endogenous ligand of the cannabinoid receptors. The former induces the migration of eosinophilic leukemia cells and macrophages via CB2 [14], and its levels markedly increased in inflamed mouse ears. Previous studies have shown that the topical application of 2-AG induces ear swelling in mice, and that this effect is suppressed by SR144528, a CB2 antagonist, and in CB2 knockout mice [18–20]. These studies suggest that 2-AG plays an important role in the inflammation response via CB2. The importance of CB2-mediated 2-AG response in inducing skin inflammation was also demonstrated using a CB2 selective agonist, HU308 and a non-selective CB agonist, Win55212-2 by Oka et al. [31]. Topical treatment of HU308 and Win55212-2 significantly inhibited 2-AG- and TPA-induced ear swelling. In addition, the migration of HL-60 cells elicited by 2-AG in vitro was also suppressed by the treatment of Win55212-2. On the contrary, HU308 and Win55212-2 did not cause ear swelling in mice. These results are very complicated, because CB2 agonists behave like CB2 antagonists. To demonstrate the reason why these CB2 agonists could inhibit CB2-mediated 2-AG responses, Oka et al. [31] examined the effect of Win55212-2 on 2-AG-induced calcium influx in HL-60 cells in vitro. The addition of 2-AG provoked a rapid increase in calcium influx in HL-60 cells. After the pretreatment of 2-AG or Win55212-2, these agonists suppressed 2-AG-induced calcium influx and desensitized the CB2 in HL-60 cells. Although rapid recovery from anergic state was observed in 2-AG-pretreated cells, continuous desensitization occurred in Win55212-2-pretreated cells. Based on these findings, Oka et al. [31] concluded that the sustained desensitization observed in Win55212-2-pretreated cells inhibited continuous stimulation of CB2 by 2-AG which is thought to be necessary for cellular responses such as cell migration and may contribute to the blocking effect of Win55212-2 against CB2-mediated 2-AG responses in vitro and in vivo. The data suggest that certain types of CB2 agonists which have a profile like Win55212-2 could exhibit anti-inflammatory effect in 2-AG-related and CB2-mediated inflammatory responses.

To confirm the involvement of 2-AG in mite antigen-induced dermatitis in mice, we measured 2-AG levels in skin lesions, and observed a significant increase in the levels compared with normal mice. The amount of 2-AG in DNFB model have not been evaluated. Some reports have shown that 2-AG increased in the DNFB-treated ears [32, 33]. We need to confirm this in our DNFB model. Next, we investi-

gated if S-777469 inhibits the effects of 2-AG. Results showed that S-777469 suppressed 2-AG-induced eosinophilic EoL-1 cell migration in vitro and inhibited the migration of eosinophils into the dermis 24 h after antigen administration in a mouse model of atopic dermatitis in vivo. In addition, we demonstrated that 2-AG-induced monocytic HL-60 cell migration was suppressed by S-777469 in vitro. Moreover, in our preliminary experiment, the pretreatment of S-777469 inhibited 2-AG-induced calcium influx in HL-60 cells for long duration the same as Win55212-2 (data not shown). These data suggest that the anti-inflammatory effects of S-777469 are mediated by the suppression of 2-AG-induced inflammatory cell migration through the same mechanism with Win55212-2.

We have previously demonstrated that S-777469 suppressed pruritogen-induced scratching behavior in rodents [23]. In addition, a single dose of S-777469 suppressed the nerve fiber sprouting into the epidermis 24 h after the administration of mite antigen in a mouse model of atopic dermatitis (data not shown). Moreover, we reported that cutting of toenails suppressed mite antigen-induced skin inflammation in NC/Nga mice [34]. Therefore, it is possible that the anti-inflammatory effects of S-777469 are partially mediated by the inhibition of pruritus. To clarify this point, further investigation, for example, whether S-777469 inhibits scratching behavior in NC/Nga atopic dermatitis model, is needed.

In conclusion, this demonstrated that S-777469, a selective CB2 agonist, suppressed DNFB- and mite antigen-induced skin inflammation in mice. The mechanism by which S-777469 exerts anti-inflammatory effects is associated with the inhibition of 2-AG functions, such as the induction of inflammatory cell migration.

Disclosure Statement

The authors have no conflict of interest to declare.

References

- 1 Pertwee RG: Cannabinoid receptor ligands: clinical and neuropharmacological considerations, relevant to future drug discovery and development. *Expert Opin Investig Drugs* 2000;9:1553–1571.
- 2 Piomelli D, Giuffrida A, Calignano A, Rodriguez de Fonseca F: The endocannabinoid system as a target for therapeutic drugs. *Trends Pharmacol Sci* 2000;21:218–224.
- 3 Porter AC, Felder CC: The endocannabinoid nervous system: unique opportunities for therapeutic intervention. *Pharmacol Ther* 2001;90:45–60.

- 4 Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG: International union of pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* 2002;54:161–202.
- 5 Galiegue S, Mary S, Marchand J, Dussosoy D, Carrière D, Carayon P, Bouaboula M, Shire D, Le Fur G, Casellas P: Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* 1995;232:54–61.
- 6 Clayton N, Marshall FH, Bountra C, O'Shaughnessy CT: CB1 and CB2 cannabinoid receptors are implicated in inflammatory pain. *Pain* 2002;96:253–260.
- 7 Quartilho A, Mata HP, Ibrahim MM, Vnderah TW, Porreca F, Makriyannis A, Malan TP Jr: Inhibition of inflammatory hyperalgesia by activation of peripheral CB2 cannabinoid receptors. *Anesthesiology* 2003;99:955–960.
- 8 Hanus L, Breuer A, Tchilibon S, Shiloah S, Goldenberg D, Horowitz M, Pertwee RG, Ross RA, Mechoulam R, Friede E: HU-308: a specific agonist for CB(2), a peripheral cannabinoid receptor. *Proc Natl Acad Sci U S A* 1999;96:14228–14233.
- 9 Kinsey SG, Mahadevan A, Zhao B, Sun H, Naidu PS, Razdan RK, Selley DE, Imad Damaj M, Lichtman AH: The CB2 cannabinoid receptor-selective agonist O-3223 reduces pain and inflammation without apparent cannabinoid behavioral effects. *Neuropharmacology* 2011;60:244–251.
- 10 Wang ZY, Wang P, Bjorling DE: Treatment with a cannabinoid receptor 2 agonist decreases severity of established cystitis. *J Urol* 2014;191:1153–1158.
- 11 Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K: 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun* 1995;215:89–97.
- 12 Sugiura T, Kondo S, Kishimoto S, Miyashita T, Nakane S, Kodaka T, Suhara Y, Takayama H, Waku K: Evidence that 2-arachidonoylglycerol but not N-palmitoylethanolamine or anandamide is the physiological ligand for the cannabinoid CB2 receptor. Comparison of the agonistic activities of various cannabinoid receptor ligands in HL-60 cells. *J Biol Chem* 2000;275:605–612.
- 13 Gonsiorek W, Lunn C, Fan X, Narula S, Lundell D, Hipkin RW: Endocannabinoid 2-arachidonyl glycerol is a full agonist through human type 2 cannabinoid receptor: antagonism by anandamide. *Mol Pharmacol* 2000;57:1045–1050.
- 14 Oka S, Ikeda S, Kishimoto S, Gokoh M, Yanagimoto S, Waku K, Sugiura T: 2-Arachidonoylglycerol, an endogenous cannabinoid receptor ligand, induces the migration of EoL-1 human eosinophilic leukemia cells and human peripheral blood eosinophils. *J Leukoc Biol* 2004;76:1002–1009.
- 15 Kishimoto S, Gokoh M, Oka S, Muramatsu M, Kajiwara T, Waku K, Sugiura T: 2-Arachidonoylglycerol induces the migration of HL-60 cells differentiated into macrophage-like cells and human peripheral blood monocytes through the cannabinoid CB2 receptor-dependent mechanism. *J Biol Chem* 2003;278:24469–24475.
- 16 Jordà MA, Verbakel SE, Valk PJ, Vankan-Berkhoudt YV, Maccarrone M, Finazzi-Agrò A, Löwenberg B, Delwel R: Hematopoietic cells expressing the peripheral cannabinoid receptor migrate in response to the endocannabinoid 2-arachidonoylglycerol. *Blood* 2002;99:2786–2793.
- 17 Walter L, Franklin A, Witting A, Wade C, Xie Y, Kunos G, Mackie K, Stella N: Nonpsychotropic cannabinoid receptors regulate microglial cell migration. *J Neurosci* 2003;23:1398–1405.
- 18 Mimura T, Oka S, Koshimoto H, Ueda Y, Watanabe Y, Sugiura T: Involvement of the endogenous cannabinoid 2 ligand 2-arachidonyl glycerol in allergic inflammation. *Int Arch Allergy Immunol* 2012;159:149–156.
- 19 Oka S, Wakui J, Ikeda S, Yanagimoto S, Kishimoto S, Gokoh M, Nasui M, Sugiura T: Involvement of the cannabinoid CB2 receptor and its endogenous ligand 2-arachidonoylglycerol in oxazolone-induced contact dermatitis in mice. *J Immunol* 2006;177:8796–8805.
- 20 Oka S, Yanagimoto S, Ikeda S, Gokoh M, Kishimoto S, Waku K, Ishima Y, Sugiura T: Evidence for the involvement of the cannabinoid CB2 receptor and its endogenous ligand 2-arachidonoylglycerol in 12-O-tetradecanoylphorbol-13-acetate-induced acute inflammation in mouse ear. *J Biol Chem* 2005;280:18488–18497.
- 21 Sugiura T, Oka S, Gokoh M, Kishimoto S, Waku K: New perspectives in the studies on endocannabinoid and cannabis: 2-arachidonoylglycerol as a possible novel mediator of inflammation. *J Pharmacol Sci* 2004;96:367–375.
- 22 Odan M, Ishizuka N, Hiramatsu Y, Inagaki M, Hashizume H, Fujii Y, Mitsumori S, Morioka Y, Soga M, Deguchi M, Yasui K, Arimura A: Discovery of S-777469: an orally available CB2 agonist as an antipruritic agent. *Bioorg Med Chem Lett* 2012;22:2803–2806.
- 23 Haruna T, Soga M, Morioka Y, Hikita I, Imura K, Furue Y, Yamamoto M, Imura C, Ikeda M, Yamauchi A, Deguchi M, Shichijo M, Arimura A, Yasui K: S-777469, a novel cannabinoid type 2 receptor agonist, suppresses itch-associated scratching behavior in rodents through inhibition of itch signal transmission. *Pharmacology* 2015;95:95–103.
- 24 Yamamoto M, Haruna T, Yasui K, Takahashi H, Iduhara M, Takaki S, Deguchi M, Arimura A: A novel atopic dermatitis model induced by topical application with dermatophagoides farinae extract in NC/Nga mice. *Allergol Int* 2007;56:139–148.
- 25 Yamamoto M, Haruna T, Imura K, Hikita I, Furue Y, Higashino K, Gahara Y, Deguchi M, Yasui K, Arimura A: Inhibitory effect of a potent and selective cytosolic phospholipase A2alpha inhibitor RSC-3388 on skin inflammation in mice. *Pharmacology* 2008;81:301–311.
- 26 Saito H, Hayakawa T, Mita H, Akiyama K, Shida T: Effect of butyric acid on induction of differentiation into eosinophil-like cells in human eosinophilic leukemia cells, EoL-1 cell line: possible role of granulocyte-macrophage colony-stimulating factor as an autocrine differentiating factor. *Int Arch Allergy Immunol* 1993;100:240–247.
- 27 Murao S, Gemmell MA, Callahan MF, Anderson NL, Huberman E: Control of macrophage cell differentiation in human promyelocytic HL-60 leukemia cells by 1,25-dihydroxyvitamin D3 and phorbol-12-myristate-13-acetate. *Cancer Res* 1983;43:4989–4996.
- 28 Nagarkatti P, Pandey R, Rieder SA, Hegde VL, Nagarkatti M: Cannabinoids as novel anti-inflammatory drugs. *Future Med Chem* 2009;1:1333–1349.
- 29 Arévalo-Martín A, Vela JM, Molina-Holgado E, Borrell J, Guaza C: Therapeutic action of cannabinoids in a murine model of multiple sclerosis. *J Neurosci* 2003;23:2511–2516.
- 30 Sugiura T, Kishimoto S, Oka S, Gokoh M: Biochemistry, pharmacology and physiology of 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand. *Prog Lipid Res* 2006;45:405–446.
- 31 Oka S, Wakui J, Gokoh M, Kishimoto S, Sugiura T: Suppression by WIN55212-2, a cannabinoid receptor agonist, of inflammatory reactions in mouse ear: Interference with the actions of an endogenous ligand, 2-arachidonoylglycerol. *Eur J Pharmacol* 2006;538:154–162.
- 32 Karsak M, Gaffal E, Date R, Wang-Eckhardt L, Rehnelt J, Petrosino S, Starowicz K, Steuder R, Schlicker E, Cravatt B, Mechoulam R, Buettner R, Werner S, Di Marzo V, Tüting T, Zimmer A: Attenuation of allergic contact dermatitis through the endocannabinoid system. *Science* 2007;316:1494–1497.
- 33 Mimura T, Oka S, Koshimoto H, Ueda Y, Watanabe Y, Sugiura T: Involvement of the endogenous cannabinoid 2 ligand 2-arachidonyl glycerol in allergic inflammation. *Int Arch Allergy Immunol* 2012;159:149–156.
- 34 Yamamoto M, Haruna T, Ueda C, Asano Y, Takahashi H, Iduhara M, Takaki S, Yasui K, Matsuo Y, Arimura A: Contribution of itch-associated scratch behavior to the development of skin lesions in dermatophagoides farinae-induced dermatitis model in NC/Nga mice. *Arch Dermatol Res* 2009;301:739–746.

Copyright of Pharmacology is the property of Karger AG and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.