

A Novel CC Chemokine Receptor 4 Antagonist RS-1269 Inhibits Ovalbumin-Induced Ear Swelling and Lipopolysaccharide-Induced Endotoxic Shock in Mice

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Abstract: There is growing evidence that chemokines recruit leukocytes in allergic, inflammatory and immune responses. CC chemokine receptor 4 (CCR4) is implicated as a preferential marker for T helper 2 cells, and the cells selectively respond to CC chemokine ligand 17 (CCL17) and CCL22. We searched for compounds having a profile as a CCR4 antagonist from an in-house library and have previously reported that 3-{2-[(2*R*)-2-phenyl-4-(4-pyridin-4-ylbenzyl)morpholin-2-yl]ethyl}quinazoline-2,4-(1*H*,3*H*)-dione (named RS-1154) was capable of significantly inhibiting the binding of [¹²⁵I]CCL17 to human CCR4-expressing CHO cells. From further synthesis of its derivatives, we newly focused on 3-(isobutylamino)-*N*-{2-[(2*R*)-2-phenyl-4-(4-pyridin-4-ylbenzyl)morpholin-2-yl]ethyl}benzamide (RS-1269), which showed potency comparable to RS-1154 in inhibiting CCL17-induced migration of DO11.10 mice-derived T helper 2 cells with an IC₅₀ value of 5.5 nM *in vitro*. We then investigated the pharmacological effects of RS-1269 on ovalbumin-induced ear swelling and lipopolysaccharide-induced endotoxic shock in mice. The ear thickness was significantly decreased by oral administration of RS-1269 at the dose of 30 mg/kg. Treatment with lipopolysaccharide significantly increased the serum level of tumour necrosis factor- α . Compared with an anti-CCL17 antibody, RS-1269 significantly inhibited the production at the dose of 100 mg/kg. These results raise the possibility that RS-1269 or one of its derivatives has potential to serve as a prototype compound to develop therapeutic agents for atopic dermatitis and inflammatory diseases.

Chemokines and their receptors mainly regulate the trafficking of leukocytes by making and recognizing a chemoattractant gradient [1,2]. Moreover, this system is also involved in cellular activation, differentiation of cells and inflammatory mediator release. Disruption of this elaborate balance triggers events leading to the development of many allergic diseases, such as atopic dermatitis, asthma, allergic rhinitis, etc. Therefore, chemical compounds or antibodies targeting the receptors and ligands have been intensively investigated to develop medicines against the above diseases [3,4]. Among chemokines, CC chemokine ligand 17 (CCL17/thymus and activation-regulated chemokine: TARC) and CCL22 (macrophage-derived chemokine: MDC) are well-known ligands which selectively bind to CC chemokine receptor 4 (CCR4). Because CCR4 is preferentially expressed on T helper 2 (Th2) cells that produce interleukin (IL)-4, IL-5 and IL-10 [5,6], the ligands and CCR4 are involved in Th2-predominant allergic inflammation [7]. Therefore, considerable effort has been made to develop CCR4 antagonists [2], which are expected to inhibit the pathology of allergic diseases by

inhibiting CCR4-dependent cellular migration to inflammatory sites.

Atopic dermatitis is a chronically relapsing inflammatory skin disease characterised by pruritic and eczematous lesions. An expansion of the population of Th2 cells and a decrease of Th1 cells is involved, at least in the initial stages [8]. However, Th1 cytokines play an important role in the chronic inflammation followed by the infiltration of eosinophils and macrophages [9]. Clinical data demonstrate that CCL17, CCL22 and CCR4 are highly connected to the pathogenesis of atopic dermatitis. CCL17 [10] and CCL22 [11] are mainly released from keratinocytes and dendritic cells, respectively, in atopic dermatitis patients, and the serum levels of the ligands are correlated to the disease severity [12]. The increase of the ligands attracts CCR4-expressing CD4⁺ cells [13,14], skin-homing cutaneous lymphocyte-associated antigen (CLA⁺) lymphocytes [15,16] and keratinocytes [17] to the lesions of atopic dermatitis, and also results in exacerbated inflammation with intense pruritus. The involvement of CCL17 in the pathogenesis of atopic dermatitis has also been experimentally confirmed by generating CCL17-transgenic mice [18].

We previously screened an in-house library to find compounds which have a profile of inhibiting binding of [¹²⁵I] CCL17 to human CCR4-expressing CHO cells [19]. From

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the screening hits, we reported that 3-{2-[(2*R*)-2-phenyl-4-(4-pyridin-4-ylbenzyl)morpholin-2-yl]ethyl}quinazoline-2,4 (1*H*,3*H*)-dione (named RS-1154, fig. 1A) is capable of significantly inhibiting CCL17-dependent chemotaxis of DO11.10 mice-derived T helper 2 cells. Furthermore, RS-1154 suppresses ovalbumin-induced ear swelling in mice, which is convenient to evaluate atopic dermatitis-like allergic responses by measuring the thickness of the ears [20,21]. In

this study, we report the newly optimised compound 3-(isobutyrylamino)-*N*-{2-[(2*R*)-2-phenyl-4-(4-pyridin-4-ylbenzyl)morpholin-2-yl]ethyl}benzamide (RS-1269, fig. 1A).

Materials and Methods

Animals. This research was carried out according to the Guidelines for the Care and Use of Laboratory Animals of Daiichi Sankyo Co., Ltd. Five- or six-week-old female BALB/c mice and DO11.10 mice were obtained from Charles River Japan, Inc. (Yokohama, Japan) and CLEA Japan, Inc. (Tokyo, Japan), respectively, and housed under specific pathogen-free conditions. The mice were acclimatised for at least 7 days in a breeding room before the start of the experiments. In the room, the environmental conditions were maintained in a 12-hr light:dark cycle at a room temperature of 21–23°C. The mice were fed an FR-2 solid diet (Funabashi Farm Co., Funabashi, Japan) and allowed access to tap water *ad libitum*.

Reagents. RS-1269 (purity >95% by HPLC analysis) was synthesized in our laboratory and diluted in a solution consisting of 70% dimethyl sulfoxide and 30% methanol at a concentration of 5 mM in *in vitro* experiments. In the *in vivo* experiments, RS-1269 was suspended in saline containing 0.5% methyl cellulose. Mouse IL-4, anti-mouse IL-12 antibody, mouse CCL17, anti-mouse CCL17 antibody (rat IgG2A, clone 110946), anti-mouse CCL22 antibody (rat IgG2A, clone 158113), isotype control (rat IgG2A, clone 54447) and a tumour necrosis factor- α (TNF- α) ELISA kit were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Ovalbumin and lipopolysaccharide (LPS) was purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

Binding assay of CCR4-expressing CHO cells. The binding assay was performed as described previously [19]. Briefly, CHO cells constitutively expressing the chimeric Gq α subunit protein were employed. The cells were transfected with a pCneo vector containing human CCR4 cDNA and selected in Ham's F12 medium with hygromycin B. After selection, CHO cells stably expressing CCR4 were plated at a density of 40,000 cells per well on 96-well plates and cultured in a humidified atmosphere (5% CO₂–95% room air) at 37°C. One day after plating, the medium was changed to binding buffer (final concentrations: 0.15 nM [¹²⁵I]CCL17, various concentrations of reagents, 20 mM HEPES [pH 7.6], 5 mM MgCl₂, 0.1% NaN₃ and 0.5% bovine serum albumin) and then the culture was incubated at room temperature for 90 min. The reaction was terminated and the radioactivity counts in the homogenates were measured using a γ counter.

Chemotaxis assay of Th2 cells. The chemotaxis assay of Th2 cells was conducted according to the methods previously described [19]. Briefly, CD4⁺ T cells were isolated from single-cell suspensions of DO11.10 spleen cells. The cells were plated with 2.5 μ g/ml anti-CD3 antibody, 1 μ g/ml anti-CD28 antibody, 50 ng/ml (3.6 nM) IL-4 and 0.5 μ g/ml anti-IL-12 antibody in RPMI 1640 medium, and cultured in a humidified atmosphere (5% CO₂–95% room air) at 37°C for 7 days. The differentiated Th2 cells were loaded into the upper chamber and CCL17 was diluted at the concentration of 300 ng/ml in the lower chamber. Ninety min., after incubation at 37°C, the migrated cells in the lower chamber were collected and counted.

Ovalbumin-induced ear swelling assay. Mice were intraperitoneally sensitised with 500 μ l immunizing solution on days 0 and 14 to generate ovalbumin-induced ear swelling. The immunizing solution consisted of 10 μ g of ovalbumin and 4 mg of alum in saline. After being housed for another 7 days, the right ears of the sensitised mice were subcutaneously challenged with 10 μ l ovalbumin solution on day 21, and the left ears were left untreated. The solution contained 1 μ g of ovalbumin in 10 μ l of saline. To investigate the effect of reagents, RS-1269 was orally administered at the dose of 30 mg/kg a total of

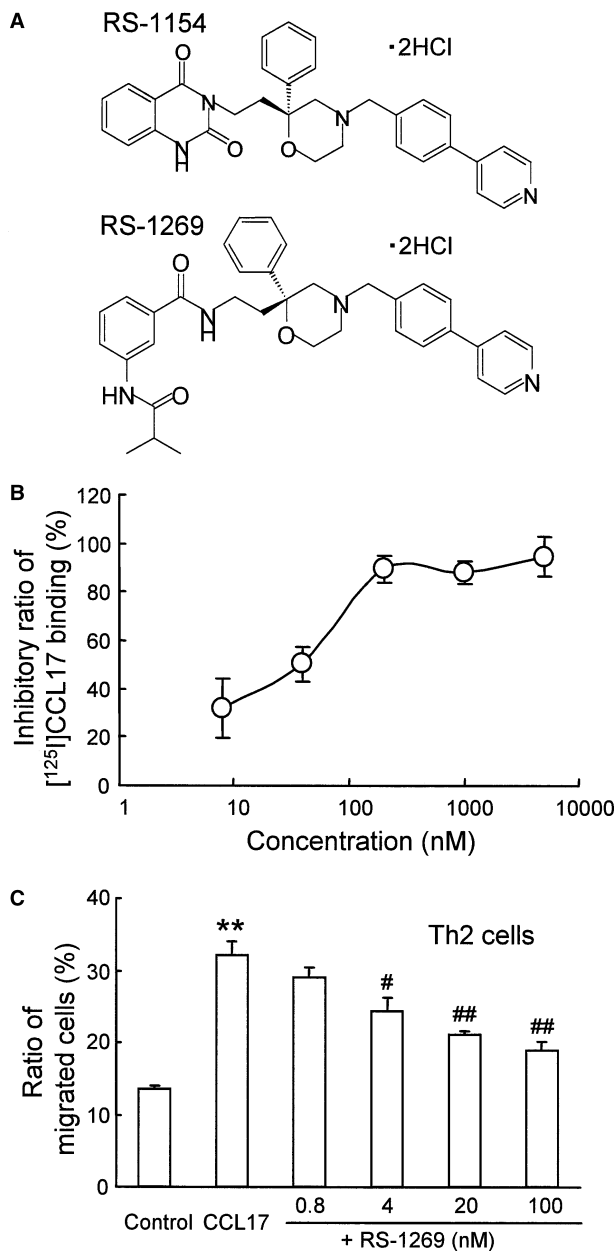


Fig. 1. The chemical structure of RS-1269 and its *in vitro* potency. (A) RS-1269: C₃₅H₃₈N₄O₃ · 2HCl, molecular weight 635.62. (B) Inhibitory effect of RS-1269 on the binding of [¹²⁵I]CCL17. The IC₅₀ value was 27.0 nM, n = 4. (C) Inhibitory effect of RS-1269 on CCL17-induced chemotaxis of Th2 cells. The IC₅₀ value was 5.5 nM. ***p* < 0.01 versus control, #*p* < 0.05, ##*p* < 0.01 versus CCL17, n = 3.

two times, once each on days 14 and 21, 30 min. before immunization and challenge, respectively. The right ear thickness was measured using a dial thickness gauge in millimetre to two decimal places.

LPS-induced TNF- α assay. REC-1269 was orally administered 30 min. before the intraperitoneal administration of 400 μ g/animal LPS. At the same time, anti-CCL17 antibody or anti-CCL22 antibody in saline was intravenously administered at the dose of 20 μ g per animal. Ninety min., after the LPS administration, serum was obtained. The concentration of TNF- α in the serum was measured according to the manufacturer's instructions.

Statistical analysis. All data in this study are expressed as means $\pm$ S.E.M. Significant differences of data were calculated by Tukey's test after an ANOVA. Probability values of $p < 0.05$ were considered to represent significant differences. The IC₅₀ values were calculated using the program Prism (GraphPad Software, Inc., San Diego, CA, USA).

Results

Inhibition of [¹²⁵I]CCL17 binding to human CCR4.

In our previous report, RS-1154 showed competitive displacement activity during the binding of [¹²⁵I]CCL17 with an IC₅₀ value of 27.7 nM [19]. RS-1269 also had strong affinity to displace the binding and the IC₅₀ value was determined to be 27.0 nM in CCR4-expressing CHO cells (fig. 1B). To investigate the selectivity for CCR4, we also performed competitive displacement of RS-1269 in the binding of [¹²⁵I]CCL3 (macrophage inflammatory protein-1 α : MIP-1 α) to CCR1 and [¹²⁵I]CCL2 (monocyte chemoattractant protein-1: MCP-1) to CCR2b. However, RS-1269 did not displace the binding of these ligands even in the range from 10 nM to 40 μ M.

Inhibition of CCL17-induced migration of Th2 cells.

To check the pharmacological properties of RS-1269 at a cellular level, a chemotaxis assay was conducted to investigate the functional inhibitory effect of RS-1269 by using DO11.10 spleen cell-derived Th2 cells. The ratio of migrated Th2 cells significantly increased by the presence of 300 ng/ml (40 nM) CCL17, implying that the Th2 cells functionally express CCR4 (fig. 1C). The effect of RS-1269 was tested in the range of 0.8–100 nM and RS-1269 dose-dependently inhibited CCL17-induced migration of Th2 cells with an IC₅₀ value of 5.5 nM. In addition, we also checked the selectivity by using DO11.10 spleen cell-derived Th1, but RS-1269 did not inhibit 300 ng/ml (38 nM) CCL3 and 300 ng/ml (34 nM) CXCL10-induced chemotaxis even at the concentration of 10 μ M (data not shown).

Inhibition of ovalbumin-induced ear swelling.

In the ear swelling assay, we already demonstrated that subcutaneous challenge with ovalbumin to ovalbumin-sensitized mice induced bi-phasic reactions and that the swelling was inhibited in both phases by an anti-CCL22 antibody or an anti-CCL17 antibody [19]. The data indicate that the swelling is, at least in part, CCR4-dependent inflammation. RS-1269 was orally administered at the dose of 30 mg/kg

and the ear thickness at 3 hr after the challenge in the RS-1269-administered group was significantly lower than that in the saline-administered group by 43.7% (fig. 2A,B). In addition, 20 μ g/animal an anti-CCL17 antibody, 20 μ g/animal an anti-CCL22 antibody and 30 mg/kg RS-1154 inhibited the ear swelling at 3 hr after the challenge by 53.2%, 25.8% and 29.4%, respectively [19].

Inhibition of LPS-induced TNF- α production.

It is reported that the serum level of TNF- α is reduced in CCR4-deficient mice compared with wild-type mice after treatment with LPS [22]. Therefore, we examined the effect of RS-1269 administration on LPS-induced TNF- α production. Treatment with LPS significantly increased the serum level of TNF- α , which was over 2000 pg/ml (118 pM) (fig. 3A). Administration of an anti-CCL17 antibody slightly decreased this increase, although the effects were not significant. Compared with the antibodies, RS-1269 significantly inhibited the LPS-induced TNF- α production at the dose of 100 mg/kg (fig. 3B).

Discussion

In this study, we have demonstrated that RS-1269, a newly synthesized CCR4 antagonist, inhibits binding of [¹²⁵I]CCL17 on human CCR4-expressing CHO cells and

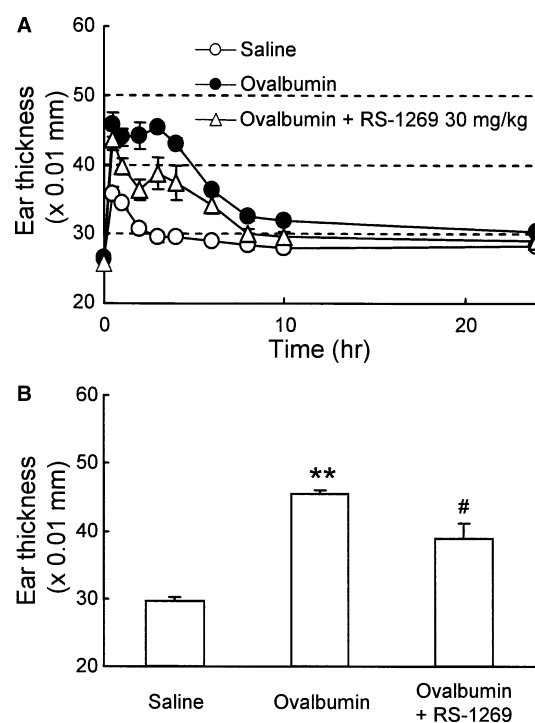


Fig. 2. Inhibitory effect of RS-1269 on ovalbumin-induced ear swelling. (A) Time course of ear thickness after ovalbumin injection. Saline-challenged group (open circles), ovalbumin-challenged group (closed circles) and 30 mg/kg RS-1269-administered ovalbumin-challenged group (open triangles). (B) Ear thickness 3 hr after ovalbumin injection. ** $p < 0.01$ versus saline, # $p < 0.05$ versus ovalbumin, $n = 5$ –16 from three independent experiments.

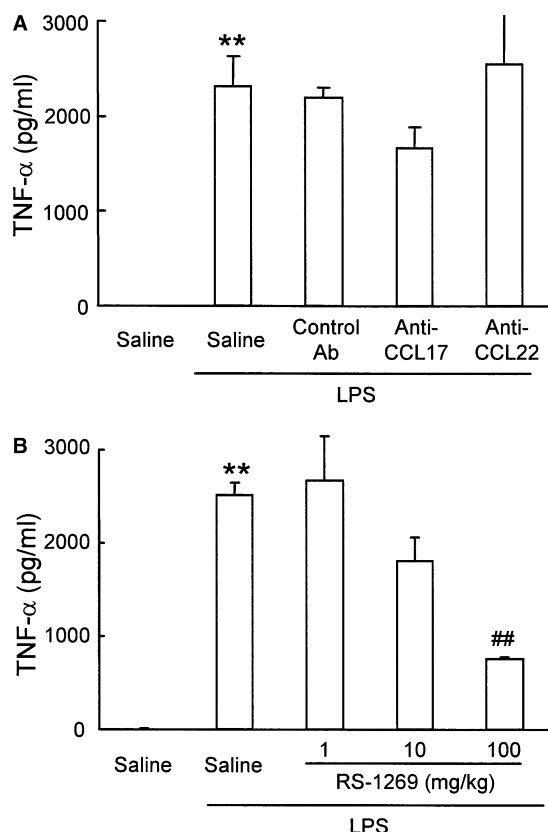


Fig. 3. Effect of antibodies and RS-1269 on LPS-induced TNF- α production. (A) Effect of anti-CCL17 and anti-CCL22 antibodies on LPS-induced TNF- α production. Each antibody was intravenously administered at the dose of 20 μ g per animal. (B) Inhibitory effect of RS-1269 on LPS-induced TNF- α production. ** p < 0.01 versus saline, ## p < 0.01 versus LPS, n = 4–5.

suppresses chemotaxis of Th2 cells. Orally administered RS-1269 ameliorates ovalbumin-induced ear swelling in mice and the potency of RS-1269 to inhibit ear swelling is superior to that of previously reported RS-1154. Taken together, these results imply that RS-1269 is a lead compound for clinical candidates for allergic diseases such as atopic dermatitis. Moreover, RS-1269 also inhibited LPS-induced TNF- α production, suggesting that excessive amplification of innate immunity such as sepsis is alleviated by CCR4 antagonists.

A challenge with ovalbumin to ovalbumin-sensitized mice induces ear swelling with a typical bi-phasic reaction [23,24]. In the early-phase reaction, the swelling peaks 1 hr after an allergen challenge and the response is triggered by allergen-specific IgE cross-linking followed by chemical mediators release from mast cells and basophils. The late phase reaction is observed approximately 6 hr after the allergen challenge and the infiltration of inflammatory cells such as T cells, eosinophils and monocytes is involved in the allergic responses. Although the detailed mechanisms are not clear yet and the ear swelling lasts for several hours, contrary to the chronic inflammation in atopic dermatitis, the late phase reaction would reflect one of the properties of atopic dermatitis. Oral administration of RS-1269 significantly inhibited

the late phase reaction of ear swelling compared with the early-phase. This result also indicates that CCL17- and CCL22-dependent recruitment of CCR4⁺ Th2 cells into ovalbumin-challenged ears is more important in the late phase. As mentioned in section 'Results', we have already demonstrated that intravenous administration of anti-CCL17 and anti-CCL22 antibodies ameliorated ear swelling in the late phase [19]. Thus, an ovalbumin-induced ear swelling assay would be an easy way to evaluate the potency of CCR4 antagonists *in vivo*.

The chemokine system is orchestrally regulated by approximately 50 chemokine ligands and 20 chemokine receptors [7]. CCR4 is mainly considered responsible for the excessive recruitment of Th2 cells, but this notion has become more complicated by the conflicting results. For example, neither lymphocyte accumulation to the airway nor airway hyper-responsiveness was alleviated in ovalbumin-challenged CCR4-deficient mice [22] although the hyper-responsiveness was attenuated when using *Aspergillus* antigens instead of ovalbumin [25]. One reason is that CCR4 plays a different role in different cell types and the outcome is dependent on the balance of all the CCR4-expressing cells. Indeed, CD4⁺ CD25⁺ regulatory T cells were shown to strongly express CCR4 [26,27], which functions to attenuate activated T cells at inflammatory sites. Natural killer cells [28], macrophages [29] and dendritic cells [30] also express CCR4. Further studies will be required to elucidate the complexity of the CCR4-dependent allergic responses and whether the other chemokine systems are also concerned in the migration of Th2 cells.

Lipopolysaccharide, as an LPS-CD14 complex, induces host cellular activation via Toll-like receptor 4 [31]. Most Toll-like receptor-dependent responses are dependent on NF- κ B, and translocation of NF- κ B to the nucleus triggers the transcription of inflammatory cytokines and chemokines. CCR4-deficient mice were shown to be resistant to mortality after administration of LPS, which accompanies reduced pro-inflammatory cytokines and peritoneal macrophage recruitment [22]. The precise mechanisms are still unknown; however, the down-regulation of NF- κ B signalling in CCR4-deficient macrophages seems involved in the resistance [32]. In accordance with the above reports, RS-1269 also inhibited LPS-induced TNF- α in mice. In the context of CCR4-dependent chemotaxis, the contribution of CCR4 to acquired immunity has been the main focus of investigation, but one must also consider the role of CCR4 in innate immunity. In particular, the influence of CCR4 on the Toll-like receptor signalling is worthy of further exploration.

In summary, we have obtained the newly synthesized compound RS-1269, which inhibits the CCL17-induced chemotaxis of Th2 cells *in vitro*, and also has a pharmacological profile that includes amelioration of ovalbumin-induced ear swelling *in vivo*. The potency *in vivo* was superior to that of our previously reported compound, RS-1154. The improvement of RS-1269 would be at least due to pharmacokinetic profiles because free-form plasma concentration 4 hr after single oral administration of 100 mg/kg RS-1269 and 100 mg/kg RS-1154 is 35.0 and 5.4 nM, respectively (our

unpublished data). Considering therapeutic concentrations, the free-form plasma concentration should be over an IC_{50} value of 5.5 nM in a migration assay (fig. 1C). Recently, a new pyrimidine derivative, K327, was reported to suppress ovalbumin-induced lung allergic inflammation in mice [33]. The chemical structure of K327 is much different from RS-1269; however, the information might be useful to optimise RS-1269, and investigate further structure–activity relationships and the potency of RS-1269 against lung inflammation. The data reported here will help pave the way to develop successful morpholine structure-containing CCR4 antagonists.

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