RS-1748, a Novel CC Chemokine Receptor 4 Antagonist, Inhibits Ovalbumin-Induced Airway Inflammation in Guinea Pigs

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CC chemokine receptor 4 (CCR4) is generally recognized as a preferential marker for T helper 2 cells, and we have previously reported morpholine-derivative CCR4 antagonists, RS-1154 and RS-1269. Here, we investigate the pharmacological profiles of a novel pyrimidine-derivative CCR4 antagonist, 2-[4-[2-(diethylamino)ethoxy]phenyl]-N-(2,4-difluorobenzyl)-5-fluoropyrimidin-4-amine (RS-1748), which showed potency to inhibit the bindings of [125I]CCL17 and [35S]GTPγS to human CCR4-expressing Chinese hamster ovary (CHO) cells with IC50 values of 59.9 nM and 18.4 nM, respectively. Furthermore, RS-1748 inhibited ovalbumin-induced airway inflammation in guinea pigs at a dose of 10 mg/kg. These results indicate that RS-1748 would be a promising lead compound for developing a therapeutic agent against asthma.

Key words CC chemokine receptor 4; RS-1748; airway inflammation

MATERIALS AND METHODS

Materials Five-weeks old, male Hartley guinea pigs were purchased from Japan SLC, Inc. (Shizuoka), and all experiments were carried out according to the Guidelines for the Care and Use of Laboratory Animals of Daiichi Sankyo Co., Ltd. RS-1748 was synthesized in our laboratory. In the in vitro experiments, RS-1748 was diluted in a solution consisting of 70% dimethyl sulfoxide and 30% methanol at a concentration of 5 mM. In the in vivo experiments, RS-1748 was suspended in 0.5% methyl cellulose #400 just before use. Human CCR4-expressing Chinese hamster ovary (CHO) cells were generated as previously reported.7,8) Dihydrofolate reductase-deficient CHO cells were transfected with the plasmid pcDNA3.1(−) carrying guinea pig CCR4 cDNA, the plasmid carrying chimeric Gqi5α and the plasmid pSV2-dhfr. Stably CCR4-transfected CHO cells were employed for the following experiments.

Calcium Mobilization Assay and Binding Assay For calcium mobilization assay, human CCR4-expressing CHO cells were plated in Ham’s F12 medium containing 10% fetal bovine serum at a density of 3.5×103/well and guinea pig CCR4-expressing CHO cells were plated in modified Eagle’s medium containing 10% fetal bovine serum at a density of 2.5×105/wells, and cultured in a humidified atmosphere at 37°C for 2 d in 384-well plates. Cells were washed with washing buffer (25 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) [pH 7.4] and 2.5 mM probenecid in HBSS) and loaded with loading buffer (4 μM Fluo3-AM and 0.04% pluronic acid in washing buffer) for 60 min. After washing, washing buffer with or without RS-1748 was added and the cells were cultured for another

Fig. 1. The Chemical Structure of RS-1748

C23H25F3N4O·2HCl, molecular weight 503.39.

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20 min. Without washing, human CCL22 in washing buffer containing 0.1% albumin was applied. The final concentration of CCL22 was 50 nm for human CCR4-expressing CHO cells and 100 nm for guinea pig CCR4-expressing CHO cells. Changes in the fluorescence were measured by a fluorometric imaging plate reader (FLIPR, Molecular Devices Corp.) at excitation wavelength of 488 nm.9) The binding assay of human CCR4-expressing CHO cells was performed as described previously.7,8) For [35S]GTPγS binding assay, the membrane fraction of human CCR4-expressing CHO cells was prepared. Aliquots of the diluted membranes equivalent to 20 μg/well protein were incubated at room temperature for 60 min in 100 μl of assay buffer (50 mM HEPES [pH 7.5], 100 mM NaCl, 5 mM MgCl2, 5 mM human CCL22, 5 mM GDP, 3.5 mM [35S]GTPγS, 0.1% albumin, 0.05 mg/ml saponins and various concentrations of RS-1748) in 96-well plates. The radioactivity was counted by a liquid scintillation analyzer.

Airway Inflammation Assay Ovalbumin-induced airway inflammation was assayed as previously reported10) with some modifications. All guinea pigs were systemically sensitized with immunizing solution on Days 0 and 7. A challenge procedure consists of reagent oral administration (vehicle or RS-1748, 10 mg/2 ml/kg, 0 min), salbutamol inhalation (10 μg/ml, 30—35 min) and ovalbumin inhalation (10 mg/ml, 35—42 min). In some groups, saline inhalation was conducted instead of ovalbumin. This challenge was performed on Days 16, 23 and 30, and bronchoalveolar lavage fluid was collected on Day 31.

Statistical Analysis All data in this study are expressed as means±S.E.M. Significant differences of data were calculated by a Tukey’s test after an analysis of variance. The IC50 values were calculated using the program Prism (GraphPad Software, Inc., San Diego, CA, U.S.A.).

RESULTS

The application of CCL22 induced significant mobilization of intracellular Ca2+ concentration ([Ca2+]i) (Fig. 2A). The fluorescence change peaked at 15 s after the application of CCL22 and the fluorescence gradually decreased. In human CCR4-expressing CHO cells treated with RS-1748 for 20 min before the application of CCL22, the fluorescence change was smaller than that of non-RS-1748-treated cells. The effects of RS-1748 were investigated in the range of 0.256—20000 nm. RS-1748 dose-dependently inhibited the inhibitory effects of RS-1748 were investigated in the range of 0.256—20000 nm. RS-1748 dose-dependently inhibited the binding of [125I]CCL17. We next investigated the in vivo and in vitro effects of RS-1748 on CCL22-induced [Ca2+]i mobilization of human CCR4-expressing CHO cells. Cells were treated with RS-1748, 20 min before the application of CCL22. The arrowhead indicates the timing of application of CCL22. (B) Inhibitory effects of RS-1748 on CCL22-induced [Ca2+]i mobilization of human CCR4-expressing CHO cells. (C) Inhibitory effects of RS-1748 on CCL22-induced [Ca2+]i mobilization of guinea pig CCR4-expressing CHO cells. n=4.

DISCUSSION

In this study, we succeeded in obtaining a newly synthesized orally active compound, RS-1748, which has the potential to inhibit [Ca2+]i, mobilization of CCR4-expressing CHO cells, the binding of [125I] CCL17 to CCR4-expressing CHO cells and the binding of [35S]GTPγS to CCR4-expressing CHO cells in vitro, and to decrease ovalbumin-induced airway inflammation in vivo. The above profile of RS-1748 is similar to that of previously reported morpholine derivatives, RS-1154 and RS-1269, although the chemical structures of the two compounds are quite different from each other. The loss of species cross-reactivity should be overcome in developing chemokine antagonists; however, the antagonism of RS-1748 was confirmed in guinea pig CCR4 as human CCR4. This point would give RS-1748 an advantage to evaluate its efficacy in animals and to be developed in the future. In addition, the chemical structure of RS-1748 is also claimed in the patent published from Mitsubishi Tanabe Pharma Corp.11)

The analysis of bronchial biopsies from asthmatics strongly suggests the involvement of the CCR4 system in
asthma. Many animal studies have also attempted to elucidate the relation between asthma and CCR4; however, the results are not consistent so far. For example, although anti-CCL17 and anti-CCL22 antibodies suppressed ovalbumin-induced pulmonary inflammation and airway hyperreactivity in mice, CCR4-deficient mice did not show similar phenotypes. The same group also found that airway hyperreactivity in ovalbumin-challenged groups was attenuated when using Aspergillus antigens instead of ovalbumin. To grasp the above results correctly and to validate proof-of-concept of CCR4 antagonists, the expression pattern of CCR4 would be of concern. Indeed, CD4+CD25+ regulatory T cells were demonstrated to strongly express CCR4, which might function to attenuate activated T cells at inflammatory sites. The diversity of chemokines and their receptors, compensation of other chemokine systems during long-term depletion of CCR4 and different roles in different CCR4-expressing cells should be further investigated in detail.

In conclusion, we found a newly-synthesized CCR4 antagonist, RS-1748, which inhibits ovalbumin-induced infiltration of eosinophils into bronchoalveolar lavage fluid. The essential structure of RS-1748 derivatives including its structure–activity relationship still remains to be elucidated; however, this compound would be useful toward evaluating the therapeutic potential of CCR4 antagonists in allergic diseases.

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REFERENCES