Pharmacological Profile of the Novel Anti-inflammatory Corticosteroid NS-126, a Therapeutic Agent for Allergic Rhinitis

Naoki Inoue1,*, Asami Hashino1, Kiyoto Kageyama1, Xin Zhang2, Takahiro Sasagawa1, Naoko Kawakita1, Yosuke Takahashi1, Katsumi Yoshida1, Mikiko Hashimoto1, Kazuya Mori1, and Takashi Kyoi1

1Discovery Research Laboratories, 2Business Development & Strategic Planning Department, Nippon Shinyaku Co., Ltd., Kyoto 601-8550, Japan

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Abstract. NS-126 (9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione 21-cyclohexanecarboxylate 17-cyclopropanecarboxylate) is a novel, highly lipophilic anti-inflammatory corticosteroid. We compared NS-126 and the widely used intranasal corticosteroid fluticasone propionate (FP) in a guinea-pig model of allergic rhinitis and a rat model of airway eosinophilia. In the allergic rhinitis model, NS-126 and FP reduced sneezing and nasal obstruction to similar extents. In the airway eosinophilia model, both compounds inhibited the infiltration of eosinophils into the bronchoalveolar lavage fluid, but the effect of NS-126 was longer-lasting than that of FP. In vitro, NS-126 showed lower affinity than FP for the glucocorticoid receptor and was a weaker inhibitor of Th2 cytokine and chemokine production and mast-cell secretory responses. We also investigated DX-17-CPC, a metabolite of NS-126 generated in nasal tissue by carboxylesterase-catalyzed hydrolysis at the 17-position. DX-17-CPC showed greater affinity than NS-126 for the glucocorticoid receptor and was a stronger inhibitor of Th2 cytokine and chemokine production and mast-cell secretory responses. The long duration of the anti-allergic effects of NS-126 may be explained by its high lipophilicity, while the strength of its anti-allergic effects may be explained by the generation of the active metabolite DX-17-CPC. NS-126 is a long-acting intranasal corticosteroid and a promising therapeutic agent for allergic rhinitis.

Keywords: NS-126, dexamethasone cipencilate, DX-17-CPC, lipophilicity, allergic rhinitis

Introduction

The anti-allergic effects of corticosteroids are mediated by the glucocorticoid receptor (GR), a cytosolic receptor that is activated by glucocorticoid binding. The ligand-receptor complex is translocated to the nucleus, where it binds to a glucocorticoid response element in a target gene promoter and regulates gene transcription to suppress inflammatory responses (1 – 3).

NS-126 (9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione 21-cyclohexanecarboxylate 17-cyclopropanecarboxylate; dexamethasone cipencilate) is a novel synthetic anti-inflammatory corticosteroid in which the 17- and 21-hydroxyl groups of dexamethasone are esterified with cycloalkanecarboxylic acids (Fig. 1). The purpose of esterification is to achieve a high tissue retention, and hence a long-lasting anti-inflammatory effect. NS-126 has been approved for sale in Japan for the treatment of allergic rhinitis.
Allergic rhinitis is characterized by inflammation mediated by immunoglobulin E (IgE) after exposure to allergen (4). An aeroallergen enters the body through inhalation, after which it is taken up by antigen-presenting cells (including macrophages and dendritic cells), processed, and presented to helper T lymphocytes (5). If this series of events occurs in humans with an atopic background, Th2-type cytokines such interleukin-4 (IL-4) and IL-13 will be released and will interact with B lymphocytes to induce the synthesis of allergen-specific IgE antibodies (6, 7), which bind to specific high-affinity receptors on mast cells and basophils. This process is called sensitization. On nasal exposure to the same antigen, the antigen bridges two adjacent IgE molecules attached to the surface of mast cells in the nasal mucosa. This causes degranulation of the mast cells with rapid release of preformed chemical mediators (e.g., histamine), enzymes (e.g., tryptase and chymases), and cytokines [e.g., IL-4, IL-5, IL-8, IL-13, and tumor necrosis factor (TNF)-α] (8, 9). In addition, activated mast cells generate de novo several inflammatory mediators such as prostaglandins, cysteinyl leukotrienes, and platelet-activating factors (10). These acute-phase mediators, histamine in particular, cause vasodilation, increase vascular permeability and mucus secretion, and stimulate sensory nerves. All these processes result in symptoms such as nasal itching, sneezing, rhinorrhea, and nasal congestion (8, 10). These acute-phase responses typically subside within 30 – 60 min. Late-phase responses occur over a period of 30 min to 24 h, with an influx of inflammatory cells such as eosinophils, basophils, and lymphocytes (11). Late-phase responses result in the release of more pro-inflammatory mediators, including histamine, cationic proteins, kinins, and cysteinyl leukotrienes (7, 9). The symptoms of the late-phase response are similar to those of the acute-phase response except that there is less sneezing and itching and more-pronounced nasal congestion and mucus production. The symptoms of allergic rhinitis, including nasal congestion, are effectively relieved by intranasal corticosteroids (12 – 16).

In the present study, we investigated the pharmacological effects of NS-126 and DX-17-CPC, a metabolite of NS-126 generated by carboxylesterase-catalyzed hydrolysis at the 17-position and compared them with those of the widely used intranasal corticosteroid fluticasone propionate (FP) and the anti-inflammatory agent dexamethasone. NS-126 showed anti-allergic effects that were similar to those of FP but were longer-lasting. In addition, the hydrolytic metabolite of NS-126 was active in vitro and may contribute to the activity of NS-126 in vivo. NS-126 is a promising long-lasting alternative therapeutic agent for the treatment of allergic rhinitis.

Materials and Methods

Materials

NS-126 and DX-17-CPC were synthesized by SS Pharmaceutical (Narita). FP (fluticasone propionate) was purchased from KNC Laboratories (Kobe) and dexamethasone from Sigma-Aldrich (St. Louis, MO, USA). For in vitro experiments, compounds were dissolved in dimethyl sulfoxide (DMSO). For use in the allergic rhinitis model, compounds were suspended in 0.2% PEG-60 hydrogenated castor oil (HCO-60; Nikko Chemicals, Tokyo) in physiological saline. For use in the airway eosinophilia model, compounds were triturated with lactose (Pharmatose 450M; DMV International, Veghel, The Netherlands). Ovalbumin (OVA, Grade V), Norit SX-2 activated carbon, anti-2,4-dinitrophenol (DNP) IgE, DNP-conjugated human serum albumin (DNP-HSA), p-nitrophenyl-N-acetyl-β-D-glucosaminide, IL-1β, TNF-α, and IL-4 were purchased from Sigma-Aldrich. Alum adjuvant (Inject Alum) was from Pierce (Rockford, IL, USA); RPMI-1640 culture medium, Eagle’s Minimum Essential Medium (EMEM), fetal calf serum (FCS), penicillin, and streptomycin were from Invitrogen (Carlsbad, CA, USA); recombinant human glucocorticoid receptor (hGR) was from PanVera (Madison, WI, USA); and [3 H]dexamethasone was from PerkinElmer Life and Analytical Sciences (Boston, MA, USA).

Animals

Male Balb/c mice (five or six weeks of age) and Hartley guinea pigs (five weeks of age) were purchased from Japan SLC (Hamamatsu). Brown Norway rats (six weeks of age) were purchased from Charles River Japan (Yokohama). The animals were used after quarantine and acclimation for a week. Mice were housed two per cage; rats, eight or nine per cage; and guinea pigs, three per cage under a 12-h light-dark cycle (lights on, 8:00 – 20:00) at 20°C – 26°C, a relative humidity of 35% – 75%, and a ventilation frequency of at least 15 times/h. They were allowed free access to pellet chow (mice and rats: F-2; Funabashi Farm, Funabashi and guinea pigs: Labo RG-RO; Nihon Nosan, Yokohama) and tap water. The study was conducted in compliance with the Law for the Humane Treatment and Management of Animals (Law No. 105, 1973).

Allergic rhinitis model

Sensitization: On days 0, 14, and 28, guinea pigs received intraperitoneal injection of 1 ml of saline containing 10 μg/ml OVA adsorbed to alum adjuvant. From day 33 to day 37 and from day 40 to day 44, 20 μl of 0.1 mg/ml OVA solution per day were dripped into each
nostril.

**Nasal symptoms:** NS-126 and FP were suspended in 0.2% HCO-60/saline at concentrations of 5 and 10 mg/ml, and 40 μl of suspension (containing 200 or 400 μg of test compound) was dripped into each nostril every day for four days (day 47 to day 50). Twenty-four hours after the last administration, topical antigen challenge was performed by dripping 20 μl of 10 mg/ml OVA solution into each nostril. Nasal symptoms were assessed by counting the number of sneezes for 30 min after antigen challenge.

**Nasal obstruction:** NS-126 and FP were suspended in 0.2% HCO-60/saline at concentrations of 0.1, 1, and 10 mg/ml and 40 μl of suspension (containing 4, 40, or 400 μg of test compound) was dripped into each nostril for four days (day 47 to day 50). Twenty-four hours after the last administration, topical antigen challenge was performed by dripping 20 μl of 10 mg/ml OVA solution into each nostril. Late-phase nasal obstruction was assessed by measuring the specific airway resistance (sRaw) at 3, 4, 5, 6, and 7 h after antigen challenge with a two-chambered, double-flow plethysmograph system (Pulmos-1; M.I.P.S., Osaka), and the area under the curve of sRaw between 3 and 7 h (AUC\textsubscript{3–7h}) was calculated by Excel software (Microsoft, Redmond, WA, USA).

**Airway eosinophilia model**

Brown Norway rats were sensitized with OVA by intraperitoneal injection of 1 ml of saline containing 10 μg/ml OVA adsorbed to alum once a day for three consecutive days. Three weeks after the first injection, the rats were challenged with antigen by exposing them to an aerosol of 1% OVA in saline for 15 min. The aerosol was generated with an ultrasonic nebulizer (NE-U12; Omron, Tokyo). Airway inflammation was assessed by examining the accumulation of eosinophils in the bronchoalveolar lavage fluid (BALF). Twenty-four hours after antigen challenge, the rats were sacrificed by intraperitoneal injection of 1 ml of saline containing 5 μg of pentobarbital. The lungs of the airway was washed three times with 5 ml of saline containing 5 units/ml heparin. The washings (total volume, 15 ml) were combined, transferred to a plastic tube, cooled on ice, and centrifuged at 200 × g and 4°C for 10 min. Cell pellets were resuspended in 1 ml of the same solution and the cells were counted with a Sysmex K2000 cell counter (Sysmex, Kobe). To determine the relative numbers of different cell types in the BALF, a smear was prepared with a cytocentrifuge (Cytopsin 3; Shandon, Cheshire, UK) and stained with a Diff-Quik staining kit (Sysmex). Differential counts were made on the smear on the basis of standard morphologic criteria of at least 100 cells. From the total cell count and the differential count, the number of eosinophils was calculated by Excel. NS-126 and FP were triturated 3,000-fold with lactose and administered 24, 48, or 72 h before antigen challenge by intratracheal injection of 3.3 μg/lung. For intratracheal injection, rats were anesthetized with 50 mg/kg pentobarbital and the trachea was exposed through a 1-cm incision. The triturated test compound (10 mg of powder) was placed in a cut-off pipet tip, which was then fitted to a 22G Terumo non-bevel needle. The needle was inserted into the trachea and a 5-ml disposable syringe fitted to the pipet tip. The powder was injected into the trachea by expelling 3 ml of air through the syringe.

**hGR binding assay**

The binding affinity of each test compound for hGR was determined by a displacement method. hGR was dissolved in binding buffer [50 mM Tris-HCl, pH 7.4, containing 5 mM dithiothreitol, 0.1 mM disodium ethylenediaminetetraacetic acid (EDTA) and 10 mM sodium molybdate]. Recombinant hGR (44.2 μg) was incubated with \(^{3}\text{H}\)dexamethasone (0.39, 0.78, 1.51, 3.05, 6.15, 12.38, and 25.33 nM) in a total volume of 500 μL of binding buffer at 4°C for 16 h. For the saturation binding assay, hGR was incubated with various concentrations of \(^{3}\text{H}\)dexamethasone. For the displacement binding assay, hGR was incubated with 5 nM \(^{3}\text{H}\)dexamethasone in the presence of various concentrations of test compound. After incubation, dissociation suspension (50 mM Tris-HCl, pH 7.4, containing 1% Norit SX-2 and Dextran 70) was added to remove free ligand and incubation was continued at 4°C for a further 30 min. The suspension was then centrifuged at 1,600 × g and 4°C for 15 min, and the radioactivity of the supernatant containing the receptor-bound ligand was measured in a Tri-Carb 2500TR liquid scintillation counter (PerkinElmer Life and Analytical Sciences). Nonspecific binding was determined as the binding observed in the presence of excess (0.1 mM) cold dexamethasone. The specific binding was determined by subtraction of the nonspecific from the total binding and Scatchard analysis was used to calculate the dissociation constant (\(K_{D} = 3.13\) nM) and maximal number of binding sites (\(B_{max} = 1,940\) fmol/mg protein). The ligand concentration that gave 50% inhibition of specific binding (IC\textsubscript{50}) was estimated by linear regression. The inhibition constant (K\textsubscript{i}) for each compound was calculated from the equation K\textsubscript{i} = IC\textsubscript{50} / (1 + [L] / K\textsubscript{D}), where [L] is the concentration of \(^{3}\text{H}\)dexamethasone.

**Measurement of Th2 cytokine production by splenocytes**

On day 0 and day 14, two Balb/c mice were sensitized by intraperitoneal injection of 10 μg of OVA with alum. On day 18, the mice were sacrificed, their spleens were removed, and splenocytes were isolated, suspended in RPMI-1640 medium containing 10% FCS, penicillin
(100 units/ml) and streptomycin (100 μg/ml), and seeded in 96-well culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 1 × 10^6 cells in 160 μl. After the addition of OVA (20 μl; final concentration, 10 μg/ml) and several concentrations of test compound (added as 20 μl of 1% DMSO solution; final DMSO concentration, 0.1%) to give a total volume of 200 μl, the splenocytes were incubated in a humidified atmosphere containing 5% CO₂ at 37°C. After 72 h, the culture medium was collected and centrifuged at 1,500 × g at 4°C for 5 min. IL-5 and IL-13 in the supernatants were determined with Quantikine enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN, USA).

Assessment of secretory responses of RBL-2H3 cells to IgE cross-linking

Degranulation: RBL-2H3 cells, an adherent rat basophilic leukemia cell line (Health Science Research Resources Bank, Osaka), were used as a mast-cell model, and degranulation was assessed by measuring the release of β-hexosaminidase essentially as previously described (17). RBL-2H3 cells in EMEM containing 10% FCS were seeded into 24-well culture plates at a density of 2 × 10^5 cells in 1 ml and incubated overnight at 37°C in a humidified atmosphere containing 5% CO₂ with excess monoclonal anti-DNP IgE (50 ng/ml) to sensitize the cells. A solution of test compound in DMSO was then added to each well to give a range of concentrations up to 1 μM (final DMSO concentration, 0.1%) to sensitize the cells. A solution of test compound was then added to each well (final DMSO concentration, 0.1%) and incubated at 37°C for 10 min. After incubation, the cells were stimulated for 10 min with the antigen DNP-HSA (final concentration, 100 ng/ml) at 37°C in a humidified atmosphere containing 5% CO₂. The cells were then washed twice with phosphate-buffered saline to remove excess IgE and then incubated in 240 μl of EMEM containing 10% FCS at 37°C for 6 h. After stimulation, the medium was collected and centrifuged at 1,500 × g at 4°C for 5 min, and the supernatant was used for the determination of prostaglandin D₂ (PGD₂) with an enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA).

TNF-α production: RBL-2H3 cells in EMEM containing 10% FCS were seeded into 24-well culture plates at a density of 3 × 10^5 cells in 1 ml and sensitized by overnight incubation with monoclonal anti-DNP IgE (50 ng/ml) at 37°C in a humidified atmosphere containing 5% CO₂. The cells were then washed twice with phosphate-buffered saline to remove excess IgE and incubated in 240 μl of EMEM containing 10% FCS at 37°C. After 10 min, a solution of test compound in DMSO was added to each well to give a range of concentrations up to 1 μM (final DMSO concentration, 0.1%) and incubated at 37°C for 6 h. The cells were then stimulated with DNP-HSA (final concentration, 100 ng/ml) at 37°C for 6 h. After stimulation, the medium was collected and centrifuged at 1,500 × g at 4°C for 5 min, and the supernatant was used for the determination of TNF-α with an EIA kit (R&D Systems).

Measurement of chemokine production by human epithelial cells

RANTES (regulated upon activation, normal T-cell expressed, and secreted: CCL5), eotaxin-3 (CCL-26), MCP-1 (monocyte chemotactic protein-1, CCL-2), and MCP-4 (CCL-13): A549 cells (American Type Culture Collection, Manassas, VA, USA), a human type II alveolar epithelial cell line, were seeded in 24-well culture plates at a density of 1 × 10^5 cells in 1 ml of RPMI-1640 medium supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 24 h, the medium was replaced by 0.8 ml of RPMI-1640 medium supplemented with 1% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml). A solution of test compound was then added to each well (final DMSO concentration, 0.1%) and incubation was continued for 30 min. RANTES was determined after stimulation of the cells with 0.1 ml of 100 ng/ml IL-1β (final concentration, 10 ng/ml) at 37°C for 6 h. Eotaxin-3 and MCP-4 were determined in the same medium after

Prostaglandin D₂ synthesis: RBL-2H3 cells in EMEM containing 10% FCS were seeded into 24-well culture plates at a density of 3 × 10^5 cells in 1 ml and sensitized by overnight incubation with excess monoclonal anti-DNP IgE antibody (50 ng/ml) at 37°C in a humidified atmosphere containing 5% CO₂. The cells were then washed twice with phosphate-buffered saline to remove excess IgE and then incubated in 240 μl of EMEM containing 10% FCS at 37°C. After 10 min, a solution of test compound was added to each well (final DMSO concentration, 0.1%) and incubation was continued at 37°C for 2 h. The cells were then stimulated with DNP-HSA (final concentration, 100 ng/ml) at 37°C for 1 h. After stimulation, the medium was collected and centrifuged at 1,500 × g at 4°C for 5 min, and the supernatant was used for the determination of prostaglandin D₂ (PGD₂) with an enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA).
stimulation of the cells with 0.1 ml of 300 ng/ml IL-4 (final concentration, 30 ng/ml) and 100 ng/ml IL-1β (final concentration, 10 ng/ml) at 37°C for 48 h. MCP-1 was determined after stimulation of the cells with 0.1 ml of 100 ng/ml TNF-α (final concentration, 10 ng/ml) at 37°C for 48 h. After incubation, samples of the medium were withdrawn for determination of chemokines with Quantikine ELISA kits (R&D Systems).

**IL-8 (CXCL-8):** NCI-H292 cells (American Type Culture Collection), a human airway epithelial cell line, were seeded in 24-well culture plates at a density of 2 × 10⁵ cells in 1 ml of RPMI-1640 medium supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 24 h, the medium was replaced by 0.8 ml of RPMI-1640 medium supplemented with 1% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml). A solution of test compound was then added to each well (final DMSO concentration, 0.1%) and incubation was continued for 30 min. IL-8 was determined after stimulation of the cells with 0.1 ml of TNF-α (final concentration, 3 ng/ml) at 37°C for 6 h. After incubation, samples of the medium were taken for determination of IL-8 with an ELISA kit (GE Healthcare, St. Giles, UK).

**Assessment of NS-126 metabolism in guinea-pig nasal mucosal homogenate**

Nasal epithelial tissue obtained from guinea pigs was homogenized in 100 mM potassium phosphate buffer, pH 7.4, containing 1.5 mM EDTA. The homogenate was centrifuged at 240 × g and 4°C for 1 min to remove debris, and the supernatant was used to assess NS-126 metabolism. The protein concentration in the debris-free homogenate was determined with the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA) with bovine serum albumin as the standard protein. The homogenate was diluted to 0.2 mg protein/ml with 100 mM potassium phosphate buffer, pH 7.4, containing 1.5 mM EDTA. NS-126 was incubated in 0.5 ml of reaction mixture containing nasal mucosal homogenate (0.1 mg protein/ml), 1 mM NADPH, 100 mM potassium phosphate (pH 7.4), and 1.5 mM EDTA. After preincubation at 37°C for 5 min, the reaction was initiated by the addition of 10 μl of NS-126 in 50% acetonitrile to give a final concentration of 0.4 μM. After incubation at 37°C for 30 or 60 min, the reaction was stopped by the addition of 50 μl of acetic acid. After the addition of 50 μl each of 1% formic acid, internal standard solution (1 μg/ml methyltestosterone in methanol), and 50% acetonitrile, the reaction mixture was applied to an Oasis HLB μ-Elution 96-well plate (Waters, Milford, MA, USA) conditioned with 0.3 ml of methanol and 0.3 ml of water. Each well was washed with 0.5 ml of water and 0.2 ml of 25% methanol, and adsorbed material was eluted with 50 μl of acetonitrile containing 1% formic acid. A 10-μl sample of the eluate was analyzed by high-performance liquid chromatography / tandem mass spectrometry. An API-3200 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, San Francisco, CA, USA) fitted with a Turbo Ion Spray interface was used in conjunction with an LC-20AD pump (Shimadzu, Kyoto) and an SIL-20AC autosampler (Shimadzu). The high-performance liquid chromatography column was a Unison UK-Siulia UKS04 (100 × 4.6 mm, 3 μm; Imtakt, Kyoto) operated at a flow rate of 1.0 ml/min and developed with a gradient of mobile phases A and B, where mobile phase A was 1% formic acid and mobile phase B was 1% formic acid in acetonitrile. DX-17-CPC (m/z transition 461 → 355) and the internal standard methyltestosterone (m/z transition 303 → 267) were monitored by quantitative mass-spectrometric multiple reaction monitoring in positive-ion mode. The measured DX-17-CPC concentrations in the samples were used to calculate its rate of formation in the assay.

**Statistical analyses**

The statistical significance of differences between control and treated groups was analyzed by the Wilcoxon rank-sum test for the number of sneezes and Dunnett’s multiple comparison test for AUC_3–7h and the number of eosinophils. Values of P < 0.05 were regarded as statistically significant. IC₅₀ values were calculated by nonlinear regression. Statistical analyses were carried out with SAS software (version 8.2; SAS Institute, Cary, NC, USA).

**Results**

**Allergic rhinitis model**

**Nasal symptoms:** NS-126 and FP were administered to OVA-sensitized guinea pigs for four consecutive days. Twenty-four hours after the last dose, guinea pigs were challenged with 1% OVA and the number of sneezes was counted for 30 min after challenge (Fig. 2). In the control group, the median number of sneezes was 17.5. At 400 μg/site, NS-126 and FP significantly reduced the median number of sneezes to 3.5 and 4.5, respectively.

**Nasal obstruction:** sRaw was measured from 3 to 7 h after challenge and was used to calculate AUC_3–7h as a measure of late-phase nasal obstruction (Fig. 3). The AUC_3–7h of the basal (unchallenged) group was 12.4 ± 0.3 cmH₂O × ml / (ml/s) × h. Antigen challenge significantly increased the resistance of the nasal airway (AUC_3–7h for the control group, 27.4 ± 2.4 cmH₂O × ml / (ml/s) × h). NS-126 and FP produced a dose-dependent reduction in
nasal airway resistance, with NS-126 producing a significant reduction at 40 and 400 µg/site and FP at 400 µg/site (Fig. 3). Both compounds produced similar reductions at 400 µg/site.

Airway eosinophilia model

The powder formulations of NS-126 and FP were administered intratracheally 24, 48, or 72 h before OVA challenge. Twenty-four hours after challenge, animals were sacrificed, BALF was harvested, and the white blood cells in the BALF were counted; then the eosinophils were counted to determine the percentage of eosinophils (Fig. 4). The average numbers of eosinophils for the 24-, 48-, and 72-h pre-treatment controls were (5.7 ± 0.85) × 10⁶, (5.7 ± 0.84) × 10⁶, and (7.0 ± 0.97) × 10⁶, respectively, per ml. NS-126 significantly inhibited eosinophil infiltration into the BALF at all pre-treatment times. FP significantly inhibited eosinophil infiltration only at the 24-h pre-treatment time, to about the same extent as NS-126. At the 48- and 72-h pre-treatment times, however, FP did not significantly inhibit eosinophil infiltration. Hence, the effect of NS-126 was longer-lasting than that of FP.

Affinity of binding to hGR

The Kᵢ value for the binding of NS-126 to the hGR was about 3.5-fold higher than those of FP and Dex (Table 1), whereas the Kᵢ value for DX-17-CPC, a metabolite of NS-126 generated by carboxylesterase-catalyzed hydrolysis at the 17-position, was about the same as those of FP and Dex.

![Fig. 2. Effect of NS-126 and FP on the number of sneezes after ovalbumin challenge in sensitized guinea pigs (n = 8). The short, horizontal line dividing each set of data points represents the median value. **P < 0.01, significantly different from the control by the Wilcoxon rank-sum test.](image)

![Fig. 3. Effect of NS-126 and FP on ovalbumin-induced increase in nasal airway resistance (AUC₃₋₇h) in sensitized guinea pigs (n = 8). Each bar represents the mean ± S.E.M. **P < 0.01, significantly different from the basal group by the Aspin-Welch test. *P < 0.05, **P < 0.01, significantly different from the control by Dunnett’s multiple comparison test.](image)

![Fig. 4. Effect of NS-126 and FP on ovalbumin-induced influx of eosinophils in sensitized Brown Norway rats (n = 8 or 9). Each bar represents the mean ± S.E.M. *P < 0.05, **P < 0.01, significantly different from the control by Dunnett’s multiple comparison test.](image)

<table>
<thead>
<tr>
<th>Table 1. Binding affinities of test compounds for the hGR</th>
<th>Kᵢ (nM)</th>
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<tbody>
<tr>
<td>NS-126</td>
<td>2.5</td>
</tr>
<tr>
<td>DX-17-CPC</td>
<td>0.72</td>
</tr>
<tr>
<td>FP</td>
<td>0.53</td>
</tr>
<tr>
<td>Dex</td>
<td>0.83</td>
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</table>

Compounds were incubated with hGR and [3H]dexamethasone for 16 h at 4°C as described in Materials and Methods. Kᵢ values are expressed as the mean of three experiments.
Pharmacology of the Corticosteroid NS-126

Inhibition of Th2 cytokine production from mouse splenocytes

After incubation of sensitized mouse splenocytes with OVA for 72 h, the concentrations of IL-5 and IL-13 in the culture medium were 343.3 ng/ml and 1,380 pg/ml, respectively. The production of both cytokines was inhibited by all test compounds in a concentration-dependent manner, and the rank order of the potency of the test compounds for both cytokines was FP > DX-17-CPC > NS-126 > Dex (Table 2).

Inhibition of degranulation, PGD2 synthesis, and TNF-α production in rat basophilic leukemia cells

After stimulation of sensitized RBL-2H3 cells with DNP-HSA antigen, the cells secreted β-hexosaminidase (a marker of degranulation) into the culture medium, synthesized PGD2, and produced TNF-α. All compounds tested suppressed all of these secretory responses in a concentration-dependent manner (Fig. 5). In the suppression of β-hexosaminidase secretion, NS-126 showed the weakest effect among the four compounds, while its hydrolytic metabolite DX-17-CPC showed the strongest effect (Fig. 5A). Even at the highest concentration tested, 1 μM, all compounds suppressed β-hexosaminidase secretion to a maximum extent of <60%. In the inhibition of the synthesis of PGD2, NS-126 was effective at 100 nM, while DX-17-CPC was effective from 1 nM (Fig. 5B), so that DX-17-CPC was about 100 times more potent than NS-126 itself in suppressing this response. TNF-α production was suppressed with IC50 values of 5.6 nM (NS-126), 0.17 nM (DX-17-CPC), 0.036 nM (FP), and 0.38 nM (Dex) (Fig. 5C).

Table 2. IC50 values of test compounds for inhibition of IL-5 and IL-13 production from mouse splenocytes

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>IC50 (nM)</th>
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<tr>
<td></td>
<td>NS-126</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.13</td>
</tr>
<tr>
<td>IL-13</td>
<td>0.30</td>
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</table>

Splenocytes from sensitized Balb/c mice were incubated with 10 μg of OVA for 72 h as described in Materials and Methods. IC50 values are expressed as the mean of two experiments.

Fig. 5. Effect of NS-126, DX-17-CPC, FP and Dex on secretory responses of RBL-2H3 mast cells. In all experiments, RBL-2H3 cells were sensitized by overnight incubation with monoclonal anti-DNP IgE antibody. All incubations were carried out at 37°C. A: Degranulation. Test compound was added to sensitized cells and the cells were incubated for 6 h. The cells were then stimulated with DNP-HSA for 10 min and the release of β-hexosaminidase was measured. B: Prostaglandin D2 synthesis. Test compound was added to sensitized cells and the cells were incubated for 2 h. The cells were stimulated with DNP-HSA for 1 h and PGD2 in the medium was determined. C: TNF-α production. Test compound was added to sensitized cells and the cells were incubated for 1 h. The cells were stimulated with DNP-HSA for 6 h and TNF-α in the medium was determined. The TNF-α concentration of the control well was 93.5 pg/ml.
Inhibition of chemokine production from epithelial cells

Pro-inflammatory cytokines induced the production of chemokines by the epithelial cell lines A549 and NCI-H292. After cytokine stimulation, the concentrations of RANTES, eotaxin-3, MCP-1, MCP-4, and IL-8 in the culture medium were 137.5, 311.2, 239.5, 23.0, and 208.6 pg/ml, respectively. All compounds tested inhibited the production of these chemokines in a concentration-dependent manner with the IC\textsubscript{50} values shown in Table 3. The production of all chemokines was the most strongly inhibited by FP. Generally, DX-17-CPC inhibited chemokine production more strongly than Dex and NS-126, more weakly.

In vitro metabolism of NS-126 in guinea-pig nasal mucosal homogenate

After incubation of 400 nM NS-126 with debris-free guinea-pig nasal mucosal homogenate for 30 min, the concentration of NS-126 remaining was 183 ± 24 nM and that of DX-17-CPC formed was 204 ± 10 nM. At 60 min, the concentration of NS-126 was 179 ± 14 nM and that of DX-17-CPC was 219 ± 10 nM. The presence of esterase activity in the nasal mucosal homogenate was confirmed by measuring the rate of hydrolysis of \( p \)-nitrophenyl acetate, which was found to be 24.5 nmol·min\(^{-1} \)·mg protein\(^{-1} \). We also checked for cytochrome P-450 activity in the homogenate by assessing the hydroxylation of midazolam, but we did not detect any hydroxylated products (data not shown).

Discussion

NS-126 is a novel synthetic anti-inflammatory corticosteroid in which the 17- and 21-hydroxyl groups of dexamethasone are esterified with cycloalkanecarboxylic acids to promote tissue retention. In the present study, we compared NS-126 to the intranasal corticosteroid FP in a model of allergic rhinitis in guinea pigs and a model of airway eosinophilia in rats. Although their effects were almost equal in the allergic rhinitis model, the duration of the anti-inflammatory effect of NS-126 was greater than that of FP in the airway eosinophilia model. Corticosteroids with high lipophilicity have previously been shown to be absorbed more easily and retained for longer in the nasal mucosa than are those with low lipophilicity (18). We calculated the clogP values of NS-126 and modern synthetic corticosteroids by using PC Models version 4.94 (Daylight Chemical Information Systems, Aliso Viejo, CA, USA). The clogP value of NS-126 was 5.475, compared to 5.253, 4.256, 4.121, 4.258, 3.800, and 1.785, respectively, for ciclesonide, fluticasone furoate, mometasone furoate, beclomethasone dipropionate, FP, and Dex, so that NS-126 is a highly lipophilic corticosteroid. The long duration of its effect may be due to its lipophilic nature, which contributes to a long residency time in the nasal mucosa.

To improve our understanding of the pharmacological profile of NS-126, we compared it with FP and Dex in vitro. First, we investigated the binding of NS-126 to the GR. Though the \( K_i \) of NS-126 was several fold greater than that of FP and Dex, it was still low, in the nanomolar range. We next tested the effects of NS-126 at the cellular level. The pathogenesis of allergic rhinitis involves allergen-induced proliferation of Th\(_2\) lymphocytes with the release of their characteristic cytokines, including IL-3, IL-4, IL-5, IL-9, and IL-13. These cytokines promote IgE production, eosinophil activation, and mast-cell proliferation (6, 7, 19). In the present study, NS-126, as well as the other corticosteroids tested, potently inhibited the production of the Th\(_2\) cytokines IL-5, and IL-13 by mouse splenocytes.

Cross-linking of IgE on the surface of the mast cell on exposure to specific allergen activates the tyrosine kinases Fyn, Lyn, and Syk (20). This leads to degranulation of the mast cell, which is the critical initiating event of acute allergic symptoms, and to the activation of phospholipase A\(_2\), which releases arachidonic acid from the sn-2 position of membrane phospholipids (21).

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>IC\textsubscript{50} (nM)</th>
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<tbody>
<tr>
<td>RANTES</td>
<td>NS-126 8.8</td>
</tr>
<tr>
<td>Eotaxin-3</td>
<td>NS-126 3.5</td>
</tr>
<tr>
<td>MCP-1</td>
<td>NS-126 1.9</td>
</tr>
<tr>
<td>MCP-4</td>
<td>NS-126 0.87</td>
</tr>
<tr>
<td>IL-8</td>
<td>NS-126 2.1</td>
</tr>
</tbody>
</table>

For RANTES, eotaxin-3 plus MCP-4, and MCP-1 production, A549 cells were incubated with IL-1\( \beta \) for 6 h, IL-1\( \beta \) plus IL-4 for 48 h, and TNF-\( \alpha \) for 48 h, respectively. For IL-8 production, NCI-H292 cells were incubated with TNF-\( \alpha \) for 6 h. IC\textsubscript{50} values are expressed as the mean of three experiments.
Arachidonic acid can then be further metabolized by cyclooxygenases. In mast cells, PGD$_2$ is the predominant product of cyclooxygenase metabolism, and activation of CRTH$_2$, a cognate receptor for PGD$_2$, by PGD$_2$ is important for eosinophil infiltration (22). A few hours after cross-linking, cytokines such as TNF-$\alpha$, IL-4, IL-5, IL-6, and IL-13 may also be released from mast cells (21). Several studies have shown that corticosteroids suppress these mast-cell responses that follow IgE cross-linking (23 – 25). Consistent with these studies, we found that NS-126 and other corticosteroids suppressed these responses in rat RBL-2H3 mast cells. Epithelial cells are a source of inflammatory mediators, including the chemokines RANTES and IL-8, eotaxin-family chemokines, and MCP family chemokines. These are key chemokines that mediate eosinophil infiltration (26). NS-126 and other corticosteroids inhibited the production of these chemokines by human epithelial cell lines.

From these in vitro results, we conclude that NS-126 has corticosteroid activity similar to that of Dex but weaker than that of FP. Nevertheless, the efficacy of NS-126 in animal allergic rhinitis and airway eosinophilia models was almost equal to that of FP. We also found that DX-17-CPC, the major metabolite of NS-126 generated in the nasal mucosal homogenate, had stronger corticosteroid activity in vitro than NS-126 itself. There was an apparent discrepancy between the relative affinity of DX-17-CPC and NS-126 in the hGR binding assay and their relative potency in suppressing the secretory responses of mast cells. In the hGR binding assay, DX-17-CPC was about four times as potent as NS-126, whereas in the cellular assays, DX-17-CPC was up to 100 times as potent as NS-126. The reason for this discrepancy is not clear, but it may be attributable to differences between the cell-free hGR binding assay and the cell-based secretory assays. For example, the highly lipophilic NS-126 may bind better than DX-17-CPC to albumin in the culture medium, so that less free NS-126 is available to enter the cells. We emphasize that, despite the discrepancy, the rank order of the compounds was the same in both assay systems.

On the basis of our results, we propose the following in vivo mechanism of action for NS-126 (see Fig. 6). The highly lipophilic NS-126 is absorbed by the nasal mucosa and is retained there for a long time. In the nasal mucosa, NS-126 is gradually converted by carboxylesterase-catalyzed hydrolysis at the 17-position to the highly active metabolite DX-17-CPC. NS-126 and DX-17-CPC then coexist in the nasal mucosa and both can contribute to the observed anti-allergic effects by a GR-mediated pathway. (In guinea-pig nasal mucosa, we measured only NS-126 and DX-17-CPC. In a homogenate of human nasal mucosa, Dex was generated from DX-17-CPC, but only in amounts less than 1% of the DX-17-CPC generated from NS-126.)

In conclusion, the novel anti-inflammatory corticosteroid NS-126 showed both corticosteroid activity and long-lasting in vivo anti-allergic effects. Its high lipophilicity may contribute to the long duration of its activity, while its active hydrolytic metabolite DX-17-CPC may contribute to the strength of the observed anti-allergic effects. Although intranasal corticosteroids are the most effective treatment for allergic rhinitis, in Japan most such medications are taken two to four times a day. A long-lasting intranasal corticosteroid such as NS-126 would need to be taken less frequently and so can be expected to have better patient compliance. These pharmacological characteristics of NS-126 make it suitable as an important additional anti-allergic agent for the treatment of allergic rhinitis.

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**References**


