Anti-Inflammatory Effects of a Cyclosporine Receptor-Binding Compound, D-43787

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ABSTRACT

By virtue of its binding to cyclophilin, the cellular receptor for cyclosporine (CsA), we could identify a new compound D-43787 \( \text{N}^1-[\text{N}^1-2-(\text{S})-\text{indolin-2-(S)-carbonic acid}]-\text{N}^1-\text{lysin} \) exhibiting immunomodulating properties. It inhibited cell proliferation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA)/ionomycin and anti-CD3/CD28 with an IC\( _{50} \) of 0.3 \( \mu \text{M} \). The protein phosphatase calcineurin, which is the target of the CsA-cyclophilin complex, is not inhibited by D-43787. It inhibited T helper cell (Th) 2 cytokines interleukin (IL)-4, -5, and -13 more effectively than the Th1 cytokine interferon (IFN)-\( \gamma \) in human primary T cells. The IC\( _{50} \) for IL-5 and IL-13 in TPA/ionomycin-stimulated peripheral blood mononuclear cells (PBMC) is 0.7 \( \pm 0.1 \) and 0.5 \( \pm 0.1 \) \( \mu \text{M} \), respectively, whereas the IC\( _{50} \) for IFN-\( \gamma \) is 2.0 \( \pm 0.4 \) \( \mu \text{M} \). When PBMC were stimulated with anti-CD3/CD28, the IC\( _{50} \) for IL-4, -5, and -13 were 1.5 \( \pm 0.2 \), 1.8 \( \pm 0.2 \), and 1.9 \( \pm 0.4 \) \( \mu \text{M} \), respectively. IFN-\( \gamma \) was only partially inhibited under these conditions. This effect was even more pronounced in pure CD4\( ^+ \) T cells. Pretreatment of human monocytes with D-43787 inhibited lipopolysaccharide-induced proinflammatory cytokines IL-6 and TNF\( \alpha \) with an IC\( _{50} \) of 1.2 \( \pm 0.1 \) and 4.7 \( \pm 0.9 \) \( \mu \text{M} \), respectively. In vivo, D-43787 potently inhibited late-phase eosinophilia in actively sensitized and challenged guinea pigs (10 mg/kg, i.p.: 51%) and Brown-Norway rats (1 mg/kg, intrapulmonary: 66% 30 mg/kg, i.p.: 50%). In adjuvant-induced arthritis, D-43787 (10–40 mg/kg, b.i.d., i.p.) dose dependently reduced edema development on both hind paws. The potency of D-43787 was comparable with that of indomethacin and dexamethasone. In conclusion, we characterized a novel Th2 selective immunosuppressive drug with possible anti-asthmatic/anti-inflammatory effects. Its mode of action is distinct from that of CsA.

CsA has not only revolutionized the field of organ transplantation but also has been used as a molecular tool for dissecting intracellular signal transduction pathways involved in T-cell activation (Schreiber, 1991, 1992). CsA binds to its cytosolic receptor, cyclophilin. The resulting complex binds to and inhibits the phosphatase activity of the protein phosphatase calcineurin (Liu et al., 1991). A novel cyclophilin-binding compound, sanglifehrin, has been identified recently. Despite also possessing immunosuppressive activity, it shows a different mechanism of action (Zenke et al., 2001; Zhang and Liu, 2001). Similar observations have been made for FK-506 and rapamycin. Although they share the same receptor (FKBP), the FKBP-FK-506 and FKBP-rapamycin complexes bind to distinct cellular targets and interfere with T-cell activation at different sites (Fruman et al., 1994).

Besides its widespread use in transplantation medicine, CsA has also been employed for the treatment of other immunological disorders such as asthma or arthritis. Asthma is characterized by a complex inflammatory response including pulmonary eosinophilia, edema, mucus hypersecretion, airway remodeling, and airway hyper-reactivity (Holt et al., 1999). Inhaled allergen challenge provokes an immediate airway hypersensitivity reaction called early airway response, which is frequently followed by a delayed phase of airway inflammation several hours later, called late-phase airway response. During late-phase airway response, an influx of eosinophils, lymphocytes, and macrophages into the bronchial lumen occurs. After recovery from the late-phase airway response, there is an increase in acquired airway hyperreactivity to agents such as methacholine or histamine (Busse and Lemanske, 2001). CD4\( ^+ \) Th and CD8\( ^+ \) cytotoxic T cells are apparently essential for this chronic inflammatory phase of asthma. These lymphocytes infiltrating the airways of asthmatic

ABBREVIATIONS: AIA, adjuvans-induced arthritis; BAL, bronchoalveolar lavage; BN, brown Norway; CsA, cyclosporine; GC, glucocorticoid; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; OVA, ovalbumin; PBMC, peripheral blood mononuclear cells; Th, T helper cell; TNF, tumor necrosis factor; TPA, 12-O-tetradecanoylphorbol-13-acetate.
subjects are recruited to the lungs by antigen challenge (Romagnani, 2000). There is convincing evidence that allergen-specific CD4+ Th2 cells play a key role in allergic asthma. Th2-associated cytokines such as IL-4, -5, and -13 are known to be involved in IgE production, airway eosinophilia, and airway hyper-responsiveness (Yssel and Groux, 2000). As a consequence, the inhibition or modulation of allergen-specific Th2 cells and their cytokines has become an attractive target for novel therapeutic intervention strategies in allergy (Cohn and Ray, 2000). On the other hand, the central role of CD4+ T cells and the delicate balance between T-helper subpopulations have also been extensively studied in the pathogenesis of autoimmune diseases (Pearson and McDevitt, 1999). Proteoglycan-induced arthritis is a murine model for rheumatoid arthritis, which is characterized by a Th1 dominance at the onset of the disease (Hollo et al., 2000). There is evidence that experimental adjuvant-induced arthritis (AIA) can be divided into two phases: early (up to day 14 after adjuvant injection) and late (after day 14). The early phase is characterized by enhanced production of TNFα, IL-1β, and MIP-1α, whereas the late phase is dominated by elevated levels of IL-6 (Szekanecz et al., 2000).

GCs are the gold standard in the treatment of asthma and arthritis. The efficacy of GCs is ascribed to their multiple pharmacological actions, one of which is the suppression of inflammatory cytokine production (Barnes, 1998). GCs, however, possess a wide range of pharmacological actions not only on the immune system, but also on various tissues and organs leading to multiple side effects including hypertension, diabetes mellitus, osteoporosis, etc., which often limit their clinical usefulness. Although there is no doubt, that immunosuppressants such as FK506 and CsA are effective in treatment of asthma and arthritis, unacceptable side effects limit their broad application (Corrigan et al., 1996; Lock et al., 1996; Khan et al., 2000; Mori et al., 2000). These agents have a more restricted cell specificity compared with GCs, but they interfere with multiple T-cell functions, thereby, causing generalized immune suppression. An agent capable of selectively regulating cytokine synthesis with little effect on other major T-cell cytokines such as IL-2 would provide an ideal treatment for inflammation without severe side effects including general immune suppression.

In a general screening for compounds binding to the CsA receptor cyclophilin, D-43787 (Fig. 1) has been identified (M. Maurer, C. Griesinger, D. Reichert, and G. Quinkert, manuscript in preparation). Here, we report that this compound inhibits T-cell proliferation similar to CsA. In contrast to CsA, calcineurin is not inhibited by this compound. In contrast to CsA, calcineurin is not inhibited by this compound. By analyzing different cell systems, we found that D-43787 is able to inhibit selectively Th2 cytokines in T cells and proinflammatory cytokines in monocytes. These immunosuppressive and anti-inflammatory effects were further corroborated by demonstrating its potency in animal models of immunological inflammation e.g., asthma and arthritis. In conclusion, we have identified and characterized a novel immunosuppressive substance with anti-asthmatic/anti-inflammatory effects.

### Materials and Methods

#### Reagents

Oligonucleotides were synthesized by TIB Molbiol (Berlin, Germany). TPA, ionomycin, ovalbumin, indomethacin, pyrilamine maleate (mepyramine), cyclosporin A, and dexamethasone were purchased from Sigma (Deisenhofen, Germany). Aluminum hydroxide was obtained from Merck (Darmstadt, Germany). D-43787 was synthesized by the Department of Chemistry, ASTA Medica AG (Frankfurt/Main, Germany). The structure is shown in Fig. 1. Synthesis and chemical characterization of this compound will be described elsewhere.

#### PPIase and Calcineurin Activity

Human recombinant cyclophilin A fused with a hexa-histidine tag was expressed in E. coli and prepared by Ni-NTA affinity purification (QIAGEN, Hilden, Germany). PPIase activity was determined according to Fischer et al. (1989). Cyclophilin and the test substance 89 were preincubated for 30 min. Calcineurin activity was determined using Biomol green calcineurin assay kit (Biomol, Hamburg, Germany).

#### Preparation of Cells

PBMC were isolated from heparinized blood samples by density gradient centrifugation over Histopaque 1077 (Sigma), washed twice in Hanks' buffer (Invitrogen, Carlsbad, CA) and resuspended in RPMI 1640 (Invitrogen) supplemented with 10% FCS (Roche Molecular Biochemicals, Mannheim, Germany).

#### Cell Proliferation

Cells were cultured in triplicate samples in flat-bottomed, 96-well microtiter plates twice at 10^5 cells per well together with various compounds. The cultures were incubated at 37°C for 72 h. Proliferation was measured using the WST assay as described by the manufacturer (Roche Molecular Biochemicals). All data are shown as the mean of triplicate cultures. Percent inhibition was calculated as 

$$
\text{Percent inhibition} = \left(1 - \frac{\text{OD}_{\text{compound}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{vehicle}} - \text{OD}_{\text{blank}}}\right) \times 100
$$
Cell Culture

For cytokine production, PBMC were resuspended at 10⁶ cells/ml and incubated in 500-μl volumes in 24-well tissue culture plates. After preincubation with test substances for 30 min, cells were stimulated with 25 ng/ml TPA and 1 μM ionomycin for 6 h. After this, cells were sedimented by centrifugation, lysed, and stored at −80°C.

Cytokine Expression

RNA was prepared from frozen lysates using RNeasy (QIAGEN). One-tube RT-PCR was performed using TaqMan EZ RT-PCR Kit from Applied Biosystems (Weiterstadt, Germany). Expression of cytokines were determined in relation to β-actin by real time PCR using TaqMan assay on a ABI Prism 7700. Primers and probes are shown in Table 1. Quantity of mRNA was calculated using ΔΔt method. Actin was used as a housekeeping gene to normalize mRNA levels: Ct (Parameter)−Ct (Actin) = ΔCt. This value was set in relation to the vehicle control which represents mRNA levels of untreated stimulated cells: ΔCt (compound X) − ΔCt (vehicle) = ΔΔCt (compound X). The relative mRNA level for compound X was then calculated as 2−ΔΔCt × 100% based on the results of control experiments where the efficiency of the PCR reaction was approximately 100% (according Applied Biosystems User Bulletin 2; ABI PRISM 7700 Sequence Detection System, 1997).

Animal Models of Immunological Inflammation

Animals. An experimental model of bronchial asthma was established using male BN rats weighing 180 to 230 g or in Dunkinn–Hartley guinea pigs (250–350 g). For the arthritis studies, male Lewis rats weighing 150 to 200 g were employed. Animals were purchased from Charles River Laboratories (Kisslegg, Germany, and Budapest, Hungary, respectively). The animals were kept at constant environmental conditions (temperature, 22°C; humidity, 40–60%; light cycle, 7 AM–7 PM). They had free access to standardized food pellets (also purchased from Charles River Laboratories) and tap water. All animal studies were performed in accordance with the national animal protection rules and approved by the local governmental authority (Regierungspräsidium, Dresden, Germany).

Experimental Model of Allergic Asthma. To evaluate the effect of the compounds used in the present study on allergen-induced lung eosinophilia, guinea pigs were actively sensitized with i.p. injections of OVA and aluminum hydroxide on two consecutive days. Fourteen days after the second injection, the animals were exposed to an OVA aerosol for 30 s in an inhalation chamber. Two hours before the OVA challenge, compounds were given intraperitoneally or intrapulmonary (as a powder directly into the lungs). Animals were also treated with pyrilamine maleate (mepramine) to prevent the guinea pigs from anaphylactic shock during OVA challenge. Twenty-four hours later, the animals were sacrificed by an urethane overdose. Afterward, a bronchoalveolar lavage (BAL) was performed twice with 5 ml of buffered saline. The total cell number and the number of eosinophils from the pooled BAL samples were counted by using a hemocytometer (Technicon H1E; Bayer AG).

The reduction of eosinophils was calculated in relation to vehicle-treated control animals challenged with ovalbumin aerosol. In another series of experiments, 30 mg/kg of D-43787 and CsA, respectively, were given twice daily subcutaneously (s.c.) over 3 days after the first sensitizing injection. No drug treatment was given thereafter. Twenty-four hours after challenge, BAL was performed as described above. Cells were counted by a hemocytometer (Technicon H1E; Bayer AG).

BN rats were actively sensitized by subcutaneous injections of ovalbumin mixed with Al(OH)3 gel and i.p. Bordetella pertussis vaccine on days 1, 14, and 21. On day 28, the animals were used for experiments. Compounds were given intraperitoneally 2 h before challenge. Then, the rats were exposed to an ovalbumin-containing aerosol in a nose-only inhalation system for 60 min. Control animals were sensitized and exposed to ovalbumin aerosol. Animals were sacrificed 48 h later by an overdose of urethane and a BAL was performed three times by 4 ml of Hank’s balanced solution. The total cell number and the number of eosinophils from the pooled BAL samples were counted by using a hemocytometer (Technicon H1E; Bayer AG).

AIA in Lewis Rats. Basically, the method described by Newbould (1963) was used. In brief, arthritis was induced by intraplantar injection of 0.1 ml of Freund’s adjuvant (2.5 mg of dried, heat-inactivated Mycobacterium butyricum suspended in 1 ml of paraffin oil) into the right hind paw. D-43787, dexamethasone, and indomethacin were first homogenized in TWEEN 80 (one to two drops), then diluted in saline, and administered intraperitoneally (i.p.) in a volume of 0.5 ml/100 g b.w. The daily intraperitoneal doses were as follows: D-43787, twice a day 10.0 or 40.0 mg/kg; dexamethasone, once daily 0.7 mg/kg; indomethacin, once daily 3.0 mg/kg. Body weights were determined every day. The volumes of the right and left hind paws were measured by mercury plethysmometry on days 0, 3, 6, 10, 13, 17, 20, and 22. For statistical evaluation of paw edema and body weight changes, the differences (Δ) were calculated individually by subtracting the control value (recorded on day 0) from those recorded subsequently.

Statistics

Mean ± S.D. were calculated, and drug effects were analyzed as specified above. In asthma studies, Student’s t test for unpaired values was used. In the experiments on adjuvant arthritis, statistical significance was assessed by one-way or two-way analysis of variance followed by the Newman-Keuls assay for multiple comparisons. IC₅₀ values were calculated using the computer program PRISM 3.0 (GraphPad Software Inc., San Diego, CA). Mean ± S.D. of IC₅₀ values were calculated for at least three different donors.

Results

PPiase Activity of Human Cyclophilin A Is Inhibited by D-43787. Chemical libraries were screened to identify compounds that bind to human cyclophilin A (hCypA). After identification of a lead compound and its subsequent optimization, D-43787 was characterized more completely. To assess the effect of D-43787 on hCypA, its effect on the PPiase
activity was determined. This compound dose dependently inhibited the PP1ase activity of human cyclophilin A with an IC$_{50}$ of 10 µM. CsA inhibited the activity of hCypA with an IC$_{50}$ of 5 nM, which is in agreement with previous results (Fischer et al., 1989). Because CsA exerts its immunosuppressive effects by inhibition of calcineurin, we tested whether the complex of human cyclophilin A and D-43787 was able to inhibit the protein phosphatase activity of calcineurin. Concentrations up to 100 µM did not affect the phosphatase activity (data not shown).

**Dose Response Effect on Cell Proliferation.** To determine the immunosuppressive activity of D-43787, we first determined its capacity to inhibit mitogen-induced cell proliferation. As shown in Fig. 2, D-43787 dose dependently inhibited the proliferation of human PBMC stimulated by TPA/ionomycin and anti-CD3/anti-CD28 antibodies. The IC$_{50}$ is 0.3 µM for both stimulation conditions. CsA inhibited only proliferation induced by TPA/ionomycin with an IC$_{50}$ of 3 nM, whereas concentrations up to 1 µM were ineffective inhibiting anti-CD3/anti-CD28-induced proliferation (data not shown). Because PBMC are a heterogeneous cell population, we studied the antiproliferative effect of D-43787 on purified CD4$^+$ T cells and monocytes. The IC$_{50}$ values for the inhibition of proliferation of CD4$^+$ T cells were similar for unstimulated and stimulated cells (2.2 versus 2.1; Table 2). Monocytes were more sensitive to inhibition (Table 2). The IC$_{50}$ for unstimulated cells was 0.9 ± 0.2, whereas LPS stimulated monocytes were about 3-fold more sensitive (IC$_{50}$ = 0.4 ± 0.1). We excluded cytotoxicity by measuring the proliferation of various cell lines. No growth inhibition of the lung epithelial cell line A549, JURKAT T cells, and of human T-cell clones could be observed up to 20 µM (data not shown). Toxicity on monocytes was excluded by trypan blue staining.

**D-43787 Inhibits Th2 Cytokines.** We analyzed the effects of D-43787 on the induction of cytokines. PBMC were stimulated either with TPA/ionomycin or with anti-CD3/anti-CD28 antibodies. Induction of cytokines was measured by determining mRNA levels using real-time RT-PCR. Figure 3A shows the effect of D-43787 on different cytokines produced by T cells after mitogenic stimulation. Whereas CsA inhibited all cytokines completely, D-43787 predominantly inhibited the Th2 cytokines as IL-4, -5, and -13. Hardly any effect on Th1 cytokines IL-2 and IFN-γ was seen. Similar effects were observed after anti-CD3/anti-CD28 stimulation (Fig. 3B). Because PBMC are a mixed-cell population, we purified CD4$^+$ T cells and repeated the experiment with the TPA/ionomycin stimulation. The selectivity of inhibiting Th2 cytokines was even more prominent in this pure cell population than in PBMC (Fig. 4). To extend our findings of the selective inhibition of Th2 cytokines, we analyzed the effects of D-43787 on the induction of cytokines in different T-cell clones. These clones were stimulated by anti-CD3/anti-CD28 antibodies. Figure 5 shows representative results with two different clones, which were characterized as Th2. D-43787 inhibited in both Th2 cytokines IL-4 and -13 to a similar extent as CsA.

**Dose Response Effect on Th1/Th2 Cytokines.** To compare the inhibition of cytokines with the data on the cell proliferation, we determined the dose response curves of D-43787 at the mRNA expression and protein level. Figure 6A shows the effect of D-43787 on TPA/ionomycin-induced stimulation. Because the dose response curves for the mRNA and the protein were almost identical, only those for protein are shown. The IC$_{50}$ values for IL-5 and -13 were 0.7 ± 0.1 and 0.5 ± 0.1 µM, respectively. IL-4 was not detectable under these conditions. The IC$_{50}$ for suppression of IFN-γ (2.0 ± 0.4 µM) was higher than those for the Th2 cytokines. We also determined the dose response curves after stimulation with anti-CD3/anti-CD28 (Fig. 6B). The IC$_{50}$ values for IL-4, -5, and -13 were 1.5 ± 0.2, 1.8 ± 0.2, and 1.9 ± 0.4 µM, respectively. IFN-γ release was only partially inhibited under these conditions.

**Dose Response Effect on Proinflammatory Cytokines.** Because we also observed anti-proliferative action on monocytes, we determined the influence of D-43787 on proinflammatory cytokines induced by LPS (Fig. 7A). The mRNA levels of IL-6 and TNFα were determined using real-time RT-PCR. Surprisingly, D-43787 reduced the induction of each of these genes down to basal levels. The IC$_{50}$ values for IL-6 and TNFα were 1.2 ± 0.1 and 4.7 ± 0.9 µM, respectively. Under identical conditions dexamethasone inhibited these cytokines much more effectively (Fig. 7B). The IC$_{50}$ values for IL-6 and TNFα were 0.6 ± 0.2 and 0.9 ± 0.4 nM, respectively.

**Effect in an in Vivo Model of Asthma.** The results are summarized in Table 3.

**Intraperitoneal Administration.** D-43787 (10–30 mg/kg) significantly reduced the influx of eosinophils into the lung of actively sensitized and challenged guinea pigs and BN rats, respectively. CsA was also able to reduce eosinophil recruitment in the BAL fluid by 59 to 70% at a dose of 5
mg/kg i.p. (Table 3). Indomethacin was investigated only in BN rats, and it was without any effect at the i.p. dose of 5 mg/kg. Eosinophil accumulation in the BAL was almost completely abolished by dexamethasone i.p. at doses of 0.1 (BN rats) and 5 (guinea pigs) mg/kg (Table 3).

Subcutaneous Administration. In a separate series of experiments, guinea pigs were treated with 30 mg/kg D-43787 and 30 mg/kg CsA twice daily subcutaneously on the first three days of active sensitization. To avoid a possible interaction between drugs and sensitizing agents (OVA and Al(OH)₃), drugs were given subcutaneously. In guinea pigs treated with CsA, no airways developed eosinophilia. The number of eosinophils in the BAL fluid was reduced by 126% (number was reduced below the control value) (not shown). By contrast, animals treated with D-43787 were not protected against allergen challenge-induced late-phase eosinophilia in the lungs (data not shown).

Intrapulmonary Administration. When CsA, dexamethasone, and D-43787 were given intrapulmonary to guinea pigs, the development of airway eosinophilia was strongly inhibited (Table 3). Dexamethasone protected actively sensitized guinea pigs against pulmonary eosinophilia.
with an ED_{50} of 10 μg/kg. CsA and D-43787 (1 mg/kg) reduced the accumulation of eosinophils in the BAL fluid by 86 and 66%, respectively (Table 3).

Effects on Adjuvant Arthritis. As described in earlier studies (Bendele et al., 1999), we were also capable of demonstrating the biphasic course of AIA. The early phase extends up to the day 14 after adjuvant injection; the so-called late phase occurs thereafter (Fig. 8). Considerable edema developed on the right (injected) paw already by day 3. Further progressive enhancement of edema was detected between days 10 and 22. This biphasic course is characteristic for this form of experimental arthritis in rats. D-43787 dose dependently inhibited the right (injected) paw edema (Fig. 8). Up to day 10, the attenuation of swelling, as compared with the positive controls, was statistically significant even at the smaller dose level (10 mg/kg). Indomethacin (3 mg/kg, i.p.) and dexamethasone (0.1 mg/kg, i.p.) almost completely aboliished the early phase swelling of the injected paw (Fig. 8).

CsA (3 mg/kg, p.o.) also inhibited edema development on the injected paw (Fig. 8). The late-phase edema measured at days 20 and 22 was significantly inhibited by the high dose of D-43787 but not by the lower dose (Fig. 8). Indomethacin considerably diminished the development of both the early and the late-phase edema (Fig. 8). CsA and dexamethasone completely attenuated this phase of edema development (Fig. 8).

As shown in Fig. 9, the edema development on the noninjected (left) paw started on day 10 and continuously increased up to day 20. All the drugs considerably diminished the secondary inflammation, the development of edema on the noninjected left paw. D-43787 showed a dose-dependent effect whereby the lower dose was as effective as indomethacin and the effect of the higher dose was comparable with that of oral CsA, which attenuated edema development on the noninjected paw (Fig. 9). Dexamethasone induced an
even more robust action. Actually, dexamethasone completely abolished the paw edema; the paw volume of dexamethasone-treated rats was similar to that of the untreated negative control animals (Fig. 9).

The body weight gain was significantly modified by arthritis per se and the drug treatments as well (Fig. 10). As a usual manifestation of inflammation, the arthritic animals showed significant body weight loss i.e., the weight of the negative control group exceeded that of the positive control throughout the 3-week period. (Comparing the negative and positive controls by one-way analysis of variance $F_{4,142} = 49.54, p < 0.001$ and $F_{4,145} = 21.36, p < 0.001$, in the first and second decade, respectively.) The inflammation-induced body weight loss was diminished by both doses of D-43787 (Fig. 10). Indomethacin (Fig. 10) caused only a moderate weight gain, and it was only detectable in the second decade on days 20 and 22. At the end of the study, D-43787, CsA, and indomethacin diminished inflammation-induced body weight loss.

### Discussion

In our present study, we demonstrated that D-43787, originally identified as a cyclophilin-binding compound, inhibits the PPIase activity of human cyclophilin A without influencing calcineurin activity. Proliferation of human PBMC, CD4$^+$ T cells, and isolated monocytes were inhibited with an IC$_{50}$ between 0.4 and 2.2 M. D-43787 predominantly inhibits Th2 cytokines in stimulated human PBMCs and CD4$^+$ T cells. In contrast to CsA, D-43787 also inhibits the synthesis of proinflammatory cytokines such as IL-6 and TNF$\alpha$ in stimulated human monocytes. It has been found to be effective in animal models of immunological inflammation such as asthma and arthritis.

First the action of D-43787 on human T-cells in vitro was studied, and its activity was compared with that of CsA. D-43787 was selected by virtue of its binding to the CsA receptor cyclophilin (M. Maurer, C. Griesinger, D. Reichert, and G. Quintkert, manuscript in preparation). As demonstrated by the inhibition of the PPIase activity of cyclophilin, D-43787 binds to this receptor and inhibits its enzymatic

### Table 3

<table>
<thead>
<tr>
<th>Drug</th>
<th>Guinea Pigs, Intraperitoneal</th>
<th>Brown-Norway Rats, Intraperitoneal</th>
<th>Brown-Norway Rats, Intrapulmonary</th>
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<tbody>
<tr>
<td></td>
<td>Dose</td>
<td>Inhibition</td>
<td>Dose</td>
</tr>
<tr>
<td>D-43787</td>
<td>10</td>
<td>51 ± 7 (8)</td>
<td>1</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>5</td>
<td>70 ± 8 (6)</td>
<td>1</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>N.I.</td>
<td>N.I.</td>
<td>N.I.</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>5</td>
<td>91 ± 6 (6)</td>
<td>10$^*$</td>
</tr>
</tbody>
</table>

N.I., not investigated.

$^*$ ID$_{50}$ value in micrograms per kilogram.
activity as does CsA (Fischer et al., 1989). In contrast to CsA, D-43787 does not inhibit the phosphatase activity of calcineurin. It also differs from CsA with regard to inhibition of cell proliferation. Whereas CsA only inhibits TPA/ionomycin-induced T-cell proliferation, D-43787 inhibited the proliferation in human PBMCs and CD4+ T cells stimulated by TPA/ionomycin and the proliferation stimulated by anti-CD3/anti-CD28 at similar concentrations. The latter has been reported to be resistant to CsA (June et al., 1987). These results point to a different molecular mechanism for the action of D-43787. Corroborating this hypothesis, NMR studies revealed that D-43787 binds to a region on cyclophilin apart from the CsA-binding pocket (M. Maurer and C. Griesinger, personal communication). A lower IC50 for cell proliferation compared with the IC50 for PPIase activity also indicates that binding at the active site of cyclophilin is not relevant for the action of D-43787. However, we cannot exclude the possibility that interactions between D-43787 and another yet unidentified molecular target cause its observed immunological effects.

Analysis of T-cell derived cytokines has allowed us to conclude that D-43787 inhibits the induction of Th2 cytokines more effectively than the induction of Th1 cytokines. We investigated three different cell types: human PBMC, purified CD4+ T cells, and Th2 clones. In these cell types, the production of Th2 cytokines IL-4, -5, and -13 was effectively inhibited with an IC50 between 0.47 and 1.92 μM. The IC50 values for the inhibition of cytokines are similar to the IC50 values determined for proliferation indicating a common molecular mechanism. The production of the prototypic Th1 cytokine IFN-γ was less effectively inhibited in human PBMC and CD4+ T cells. CsA inhibited Th1 and Th2 cytokines equally with an IC50 of around 2 nM, as reported earlier (Quesniaux, 1993). To our knowledge, D-43787 is the first low-molecular weight compound inhibiting more than one Th2 cytokine selectively.

The preferential inhibition of Th2 cytokines by D-43787 in vitro prompted us to investigate whether this drug might be useful in a disease characterized by a Th1-Th2 imbalance. Recent work has shown that asthma is dominated by Th2 cells and that Th2 cell-derived cytokines play an important role in the pathogenesis of asthma (Busse and Lemanske, 2001). The primary inflammatory reaction of asthma consists of accumulation of CD4+ Th2 lymphocytes and eosinophils in the airway mucosa. Th2 lymphocytes probably orchestrate the airway inflammation through a series of cytokines (IL-4, -5, and -13) (Yssel and Groux, 2000). Many investigators have concluded that Th1 cells protect against asthma and allergy and can reverse the effects of Th2-driven inflammation (Koh et al., 2001). Taken together, it is likely that bronchial asthma is primarily a Th2-driven inflammation combined with an impaired Th1 function.

The effect of D-43787 was investigated on the late-phase eosinophilia using two species: guinea pigs and BN rats. It effectively inhibited the eosinophil recruitment when given either intraperitoneally or locally into the lungs. As expected, dexamethasone considerably reduced eosinophil infiltration in both species. Because dexamethasone is a so called old-fashioned GC, it is rarely used in experimental asthma studies. However, it has been shown that dexamethasone, similarly to our study, considerably reduced eosinophil infiltration in actively sensitized guinea pigs at the dose of 30 mg/kg p.o. (Asanuma et al., 2001). Besides clinical trials, there is some experimental evidence that oral and intrapulmonary administration of CsA prevented the allergen-induced late-asthmatic response in actively sensitized guinea pigs (Arima et al., 1994). We administered CsA (30 mg/kg, s.c.) 5 days after sensitization. This treatment resulted in a complete inhibition of eosinophil recruitment in BN rats. In contrast to CsA, D-43787 was not able to inhibit the development of active sensitization when it was repeatedly given immediately after sensitization agents (OVA and Al(OH)3), indicating that D-43787 has no influence on antigen recognition and presentation. The differences in effective doses for the various compounds possibly reflect their different affinity for their receptors. Dexamethasone and CsA bind with high affinity to the glucocorticoid receptor and cyclophilin, respectively. In contrast, D-43787 inhibited the PPIase activity of cyclophilin and cytokine induction in the micromolar range, indicating much lower affinity.

Chronic inflammatory immunological diseases, such as asthma and arthritis, are frequently associated with mixed Th2 and Th1 cell responses but also with mast cells and macrophages/macrophages. Because we found that D-43787 inhibited the proliferation of monocytes, we looked for its ability to inhibit the release of proinflammatory cytokines. The release of IL-6 and TNFα after LPS stimulation of human monocytes was inhibited with an IC50 of 1.2 ± 0.1 and 4.7 ± 0.9 μM, respectively. Although CsA is not able to inhibit these cytokines (data not shown; Quesniaux, 1993), the GC dexamethasone was active in the subnanomolar range.

It has become evident during the past years that IL-6 and TNFα, mainly produced by monocytes and macrophages, are the principal mediators of tissue destruction in many immunoinflammatory diseases such as RA and bronchial asthma. Subratty and Hooloman (1998) reported that detectable concentrations of IL-6 and TNFα were more common in patients with acute asthma attacks than in controls. Thus, suppression of IL-6 and TNFα production could be beneficial in the therapy of chronic inflammatory diseases (Hisadome et al., 2000). It is known that TNFα is released in allergic responses from both mast cells and macrophages via IgE-dependent mechanisms. Elevated levels have been demonstrated in the bronchoalveolar fluid of asthmatic subjects undergoing allergen challenge. In asthma, TNFα may function as a proinflammatory cytokine that causes the recruitment of neutrophils and eosinophils (Thomas, 2001). Local levels of TNFα are elevated in chronically inflamed joints in rats with AIA suggesting an important role of TNFα in chronic inflammation (Smith-Oliver et al., 1993).

Rheumatoid arthritis is a common, frequently severe, chronic inflammatory disease. It is characterized by chronic inflammation of the synovial joints resulting from hyperplasia of synovial fibroblasts and infiltration of lymphocytes, macrophages, and plasma cells. Freund’s AIA in male Lewis rats is a well-established experimental model of rheumatoid arthritis to evaluate the inherent anti-inflammatory and/or immunosuppressive/imunomodulatory properties of drugs. D-43787 dose dependently inhibited both the primary edema on the right paw and the secondary, generalized, inflammation-induced swelling of the left paw developing in the second phase (days 13–22) of the chronic disease. Our data obtained in vivo are in good agreement of data published in the literature. Dexamethasone mostly used in a dose range of 0.025 to 1.0 mg/kg/day, p.o. inhibited swelling in adjuvant arthritic rats (Bekemeier and...
Hirschelmann, 1986; Issekutz and Issekutz, 1991; Otterness et al., 1991; Franch et al., 1994; Tatsuo et al., 1994). It is well known that nonspecific inhibitors of cyclooxygenase such as indomethacin (2–5 mg/kg/day p.o.) (Winter and Nuss, 1966; Tatsuo et al., 1994; Iida and Saito, 1999) potently inhibit the edema in adjuvant arthritic rats. As mentioned before, immunomodulating agents might have certain therapeutic significance in the treatment of asthma or RA. Indeed, CaS markedly decreased hind paw edema and antagonized the inflammation-induced body weight loss in adjuvant arthritic rats (Jaffee et al., 1989, Blackham and Griffiths, 1991; Brauer et al., 1994; del Pozo and Zapf, 1994). As shown by Arima et al. (1994), oral and inhaled administration of CsA prevented the allergen-induced late-asthmatic response in actively sensitized guinea pigs.

In conclusion, D-43787 is a new immunomodulating agent with a unique pharmacodynamic profile: 1) it preferentially inhibits Th2 cytokines and 2) it also inhibits the production of macrophage/monocyte cytokine TNFα and IL-6. This profile may explain its ability to counteract the development of late-phase eosinophilia in actively sensitized and challenged animals and the development of paw edema in rat AIA. D-43787 is the first low-molecular weight substance that inhibits more than one Th2 cytokine selectively. The different pharmacodynamic profile and receptor binding indicates that D-43787 exerts its effects via a molecular mechanism different from CsA. Further investigations are needed to identify the molecular target of D-43787 complex to identify Th2-specific signaling pathways. On the basis of these results, D-43787 seems to be a promising candidate for the treatment of diseases accompanied by immunological inflammation.

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References


