A Locally Active Antiinflammatory Macrolide (MLD987) for Inhalation Therapy of Asthma

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One of the characteristic features of asthma is a persistent pulmonary inflammation, with increased numbers of eosinophils and activated T-lymphocytes in the airways. T-helper cells of the Th2 phenotype play a pivotal role in the pathogenesis of asthma, and they are believed to orchestrate the asthmatic response by releasing a wide repertoire of cytokines. Herein, we describe the design, synthesis, and evaluation in models of allergic asthma of a locally active T-cell modulator, MLD987 (**1**). Compound **1** is a potent immunosuppressant that inhibits the activation, proliferation, and release of cytokines from T-cells with IC_{50} values in the low nanomolar range. In a Brown−Norway rat model of allergic asthma, **1**, when given into the airways by intratracheal administration (ED_{50} = 1 mg/kg) or by inhalation (ED_{50} = 0.4 mg/kg), potently reduced the influx of leukocytes into bronchoalveolar lavage fluid samples obtained from antigen-challenged animals. In contrast, **1** had an appreciably weaker activity in this model when given orally or intravenously. Pharmacokinetic evaluation in rat and rhesus monkey showed that **1** had both a low oral (2−4%) and a low pulmonary (7%, monkey) bioavailability. These findings are consistent with a local site of action of the compound and rule out that its antiinflammatory activity in the lung was caused by systemically absorbed material, which had been swallowed during inhalation or which had entered the circulation via the airways. Local administration and the metabolically soft structure of **1**, which favors rapid systemic metabolism to less immunosuppressive metabolite **2**, are the main reasons for the low exposure and weak systemic activity of the compound. Administration of a locally active compound such as **1**, by inhalation, should reduce systemic side effects. Our results indicate that **1** has the potential to serve as an alternative to inhaled glucocorticosteroids for the long-term therapy of asthma of all grades of severity.

Introduction

Asthma is a chronic inflammatory disease of the airways, which are hyperreactive and constrict easily to a variety of diverse stimuli. Typically, the disease is characterized by a pulmonary inflammatory cell infiltrate, consisting mainly of eosinophils, neutrophils, and mononuclear cells, particularly activated T-lymphocytes.[1,2] According to their cytokine release profile, the lymphocytes display a Th2 phenotype.[3] The current view is that T-cells are key effector cells in the inflammatory pathology associated with asthmatic airways. It is believed that Th2-derived cytokines orchestrate the asthmatic response and play a crucial role in the initiation and the maintenance of the inflammatory process associated with the disease. For instance, IL-4 is an essential cofactor for immunoglobulin IgE production by B-lymphocytes, while IL-5 together with IL-3 attracts, activates, and prolongs the survival time of eosinophils.[4,5] While IgE plays a central role in the allergic reaction associated with asthma, eosinophil degranulation products can cause mucosal damage, mucus hypersecretion, and bronchial hyperresponsiveness. Thus, selectively modulating T-cell activity locally in the lung might represent a valid approach to the treatment of chronic asthma. The hypothesis is supported by the fact that glucocorticosteroids, the current mainstay antiinflammatory therapy for moderate to severe asthma, are potent inhibitors of T-cell activation.[6] Most importantly, however, the selective T-cell inhibitor cyclosporin A (CsA) (Chart 1) when given orally has been shown to have beneficial effects in the allergen-induced late asthmatic reaction both in man and in patients with severe, steroid-dependent asthma.[7,8,9]

Despite the fact that most asthmatic patients are able to control their disease by using inhaled steroids, there is still room for improvements of current therapies. For example, chronic inhaled glucocorticosteroid treatment can cause local side effects such as oropharyngeal candidiasis and hoarseness. A bigger concern, particularly with high doses of inhaled steroids, is the potential for systemic side effects such as suppression of the hypothalamic−pituitary−adrenal axis, effects on bone metabolism, cataracts, and growth retardation in children.[10] Moreover, a significant number of patients with severe asthma are dependent on oral glucocorticosteroids and, in the worst case, respond only poorly to glucocorticosteroid treatment.[10,11] It is possible that steroid resistant asthma is associated with a relative unresponsiveness of these patients' T-lymphocytes to

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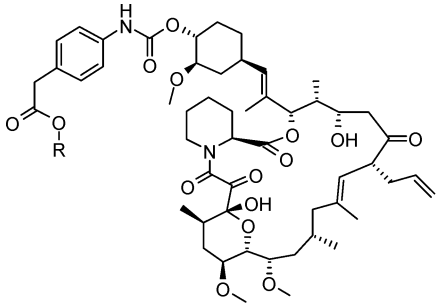
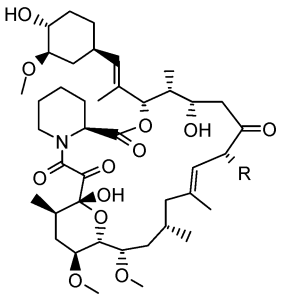
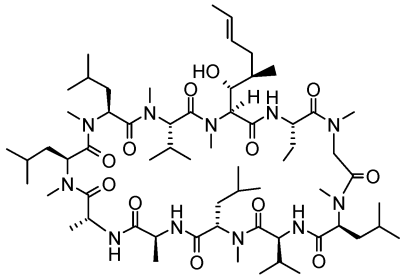
Chart 1



1, MLD987: R=methyl
2: R=H



FK506, R=allyl
ascomycin, R=ethyl



cyclosporin A

the inhibitory effects of therapeutic concentrations of glucocorticosteroids in vitro. In marked contrast, CsA inhibited T-cells from both steroid sensitive and steroid resistant patients to an equivalent extent.[12] Although the clinical relevance of this in vitro finding is unclear, there is clearly a major medical need for new therapies for patients who respond poorly to steroids.

On the basis of the above, we initiated a research program with the objective of finding potent and selective T-cell inhibitors for the treatment of chronic asthma. Our chemical starting points were ascomycin, a 23-membered macrolide originally isolated from a soil sample more than 30 years ago, and its close analogue FK506[13,14] (Chart 1). Both compounds have the same effector mechanism as CsA; thus, when bound to the intracellular binding protein FKBP12, they block the signal transduction in T-cells by inhibiting the phosphatase calcineurin A. As a consequence, the transcription factor NF-AT is unable to translocate into the nucleus resulting in a failure to activate genes necessary for T-cell activation.[15] Unfortunately, long-term systemic exposure to calcineurin inhibitors is associated with side effects such as impaired renal function, increased blood pressure, neurotoxicity, and hypertrichosis.[16] For severe asthma, which can be incapacitating and life-threatening, systemic immunosuppression is a risk that can be accepted if a substantial clinical benefit is obtained. However, for the majority of asthmatics, such therapy is unacceptable. As a consequence, and guided by the enormous therapeutic success of inhaled steroids with respect to efficacy and tolerability, our strategy was from the onset to search only for ascomycin derivatives that could be administered via inhalation.[17] Theoretically, such compounds should have a better therapeutic index per se since systemic exposure, and hence the related side effects, would be minimized due to the mode of administration. Indeed, in a recent clinical study, CsA was given by inhalation and was shown to be well-tolerated and only low systemic concentrations of the drug were detected.[18] This finding is in line with data generated in animal asthma models where CsA was 10-fold more active when given locally to the airways than by the oral route.[19]

Despite these encouraging findings, long-term administration of a selective T-cell inhibitor by inhalation does not per se guarantee to obviate systemic immunosuppression since inhalation devices do not prevent the majority of the inhaled drug from being deposited in the upper airways and subsequently swallowed.[10] As is clear from the inhaled steroids, it is this proportion of the inhaled drug together with the fraction that is absorbed via the lung that may cause systemic side effects. To further minimize the risk of systemic exposure, we decided, in addition to a local mode of administration, to incorporate a metabolically labile (soft) structural motif into our molecules. This strategy has been applied successfully in the past with inhaled glucocorticosteroids and was recently described also for CsA.[20] Herein, we describe the design, the synthesis, and the pharmacological profile of **1** (Chart 1), a locally active drug with potent antiinflammatory activity in a model of experimental asthma.[21]

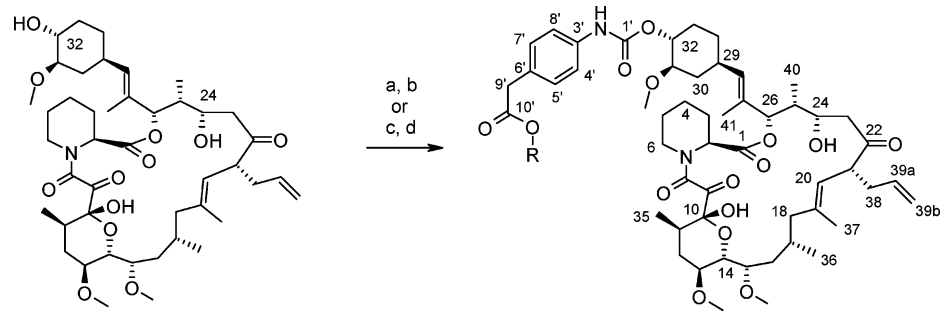
Design

The soft drug approach or retrometabolic drug design approach was mainly developed by Bodor et al.[22] The basic idea is to improve the therapeutic index of a drug by facilitating its metabolism to a less bioactive fragment after it exerts its therapeutic effect. Consequently, we set out to design a potent immunosuppressive ascomycin analogue, which, when given by inhalation, would display strong local antiinflammatory activity in the lung but, when partially swallowed during the inhalation manouver or absorbed from the airways, would be rapidly transformed systemically to a less immunosuppressive metabolite. Compound **1**, which is easily synthesized from the ascomycin analogue FK506, is such a compound since it contains a soft, metabolically labile ester group that is predicted to be hydrolyzed enzymatically to the corresponding, less immunosuppressive carboxylic acid **2**. We decided to attach the soft ester group to the cyclohexyl ring of FK506 via a phenyl carbamate linker since previous work from us and many other groups had shown that the biological activity of ascomycin derivatives was least affected by changes at this position.

Synthesis

The FK506 macrolide contains two reactive hydroxyl groups, namely, at the O−C(24) and the O−C(32) positions (Scheme 1). These two hydroxyl functionalities display different reactivities, the O−C(24) being sterically more hindered and less reactive.[23,24] Consequently, we succeeded in preparing **1** in a "one-pot" process, avoiding the use of protecting groups. FK506 was activated with 1/3 equivalent of triphosgene and an excess of 4-(dimethylamino)pyridine in dichloromethane

Scheme 1[a]



[a] Reagents and conditions: (a) Triphosgene, DMAP, CH_2Cl_2, −78°C. (b) Compound **1**: (4-amino-phenyl)acetic acid methyl ester, −78 to 20°C, 60−70%; compound **2**: (4-amino-phenyl)acetic acid trimethylsilyl ester, −78 to 20°C, then NH_4Cl, 26%. (c) 4-Nitrophenyl chloroformate, DMAP, CH_2Cl_2, −40°C. (d) Compound **1**: (4-amino-phenyl)acetic acid methyl ester, −40 to 0°C, 60−70%.

Table 1. In Vitro Pharmacology of **1**, Metabolite **2**, and Reference Compounds FK506 and Budesonide

	IC_{50} (nM)[a]						K_i (nM)[a]
compound	IL-2 RGA[b]	MLR[c]	T-cell proliferation[d]	IL-5 production[e]	IFN-γ production[e]	MBA-12 binding[f]	calcineurin inhibition[g]
1	0.18 ± 0.04	1.62 ± 0.40	20.5 (10.8/30.2)	0.02 ± 0.01	0.03 ± 0.02	1.45 ± 0.10	39 ± 5
2	6.18 ± 1.30	9.81 ± 1.37	23.6 (13.9/33.3)	0.69 ± 0.13	2.13 ± 1.84	1.88 ± 0.29	185 ± 31
FK506	0.19 ± 0.02	0.29 ± 0.04	0.18 (0.08/0.27)	0.06 ± 0.02	0.06 ± 0.03	0.95 (1.1/0.8)	35 ± 4
budesonide	ND	ND	ND	0.12 ± 0.03	0.32 ± 0.28	ND	ND

[a] Data are expressed as mean IC_{50} or K_i values ± SEM ($n = 3-6$) or as mean IC_{50} values of two experiments. [b] IL-2 reporter gene assay with the human T-cell line Jurkat stimulated by phytohemagglutinine and phorbol myristate acetate. [c] Murine MLR. [d] PPD-induced proliferation of HPBMNC isolated from blood. [e] Anti-CD3 monoclonal antibody stimulated and cultured CD4 positive cells purified from human HPBMNC. [f] Macrophilin-12 (FKBP-12) binding assay. [g] Calcineurin phosphatase enzyme activity.

at −78 °C (Scheme 1). The intermediate chloroformate was then treated in situ with an excess of (4-amino-phenyl)acetic acid methyl ester. As expected, regioselective acylation at the O−C(32) hydroxyl yielded **1**, together with a small amount of the 32,24-bis-adduct (3−4%). Remarkably, no monoacylation at the O−C(24) hydroxyl was observed. Alternatively, and in order to replace the potentially toxic triphosgene, **1** was prepared by using 4-nitrophenyl chloroformate as the activation reagent. The reaction process was similar to the triphosgene procedure, the only difference being that activation of FK506 with 4-nitrophenyl chlorofomate and the subsequent coupling with the amine were done at a higher temperature (Scheme 1).

The carboxylic acid metabolite **2** was synthesized in a fashion similar to **1** by in situ reaction of the intermediate chloroformate with trimethylsilyl-protected (4-amino-phenyl)acetic acid.[25] Aqueous workup and purification over silica gel gave pure compound **2**. It bears emphasis that attempts to transform **1** to its corresponding acid **2** by chemical hydrolysis failed and major decomposition was observed.

Results and Discussion

In the following sections, we describe in vitro data, which show that **1** is a potent T-cell inhibitor with cytokine modulating activity, in vivo data showing the local antiinflammatory effects of **1** in an animal model of asthma when applied directly into the airways, and pharmacokinetic data obtained from rat and monkey demonstrating the metabolically labile, soft structure of **1**, hence supporting the drug design concept.

The in vitro profiles of **1**, its metabolite **2**, and the reference compounds, FK506 and the glucocorticosteroid, budesonide, are presented in Table 1. Under experimental conditions where the ester group was stable, **1** showed potent immunosuppressive properties. For example, in the IL-2 reporter gene assay (IL-2 RGA) and with respect to calcineurin inhibition, **1** was equipotent to FK506, whereas in the murine mixed lymphocyte reaction (MLR), it was only five times less active. Importantly, the metabolite **2** was appreciably less active than either the FK506 or the parent compound in these assays. Because the carboxylic acid metabolite **2** was 5-fold less potent in inhibiting calcineurin phosphatase activity, which is the main molecular target of ascomycin derivatives, the lower potency of **2** is not due to reduced cell penetration but rather to the intrinsically weaker immunosuppressive activity of the compound. The ability of ascomycin derivatives to bind to FK506 binding protein, macrophilin-12 (FKBP-12), is a prerequisite for their immunosuppressive activity but does not per se predict the potency of the compounds. Both **1** and its corresponding acid **2** were bound to macrophilin-12 with almost the same affinity as FK506. Surprisingly, **1** and **2** were equally active in inhibiting PPD (purified protein derivative of *M. tuberculosis*)-stimulated proliferation of human peripheral blood mononuclear cells (HPBMNC, mainly T-cells) isolated from human blood; that is, both compounds were more than 100-fold weaker than FK506 in this assay. Because the extent of cellular proliferation was measured after 5 days and the assay medium was enriched with 20% fetal calf serum, this finding could indicate a breakdown of **1** to its metabolite **2** under the experimental conditions. In addition, blood mononuclear cells are known to express high esterase activity.[26] Furthermore, **1** showed strong cytokine-modulating activity and potently inhibited the produc-

Table 2. Comparison of **1** with Its Major Circulating Metabolite **2** and Reference Compounds FK506 and Budesonide in the BN Rat Model of OA-Induced Pulmonary Eosinophilia

route of administration	treatment schedule[b]	ED_{50} (mg/kg)[a]			
		1	**2**	FK506	budesonide
intratracheal	−1 h, +24 h	1	3	0.3	0.5
	−6 h, +24 h	1	3	0.3	ND[c]
oral	−1 h, +24 h	>10	ND	10	0.2
	−6 h, +24 h	30	ND	3	ND
intravenous	−1 h, +24 h	1 mg/kg, no effect	ND	0.3 mg/kg, >−50%[d]	ND
	−6 h, +24 h	1 mg/kg, no effect	ND	0.3 mg/kg, >−50%	ND

[a] Dose that inhibits by 50% the allergen OA-induced eosinophil influx into the airway lumen of actively sensitized rats (n = 5−20). Eosinophils are counted in the BALF. [b] The second dose given 24 h after allergen challenge has no effect by its own but improves the reproducibility of the experiment (data not shown). [c] ND, not determined. [d] Inhibition of eosinophil influx in BALF as % of control.

tion of IL-5 and IFN-γ from HPBMNC whereas **2** was significantly less active in these assays. Overall, the data demonstrate that **1** displays a strong immunosuppressive activity in a variety of in vitro assays whereas the corresponding carboxylic acid metabolite **2** was between 5 and 60-fold less active.

Infiltration by eosinophils of the mucosa and submucosa of the airways is a distinctive feature of asthma. In allergic asthma, an influx of eosinophils into the airway lumen occurs following inhalation of allergen, an effect that can be quantified by measurement of eosinophil numbers and activation state, as indicated by the presence of elevated levels of eosinophil peroxidase (EPO), in bronchoalveolar lavage fluid (BALF) samples, bronchial biopsies, and blood.[27] Analogous to the effects in asthmatics, an accumulation of inflammatory cells (neutrophils, eosinophils, and mononuclear cells) in the airways occurs when actively sensitized Brown−Norway (BN) rats inhale modest amounts of allergen.[28] Table 2 summarizes the effects of **1**, its metabolite **2**, and the reference compounds FK506 and budesonide after different routes of administration on ovalbumin (OA)-induced eosinophil accumulation in BALF from actively sensitized BN rats. When given locally into the trachea, **1** potently inhibited eosinophil influx into BALF from BN rats (ED_{50} = 1 mg/kg). The compound was equipotent when given either 1 h prior to or 6 h prior to antigen challenge (the second dose given 24 h after allergen challenge has no effect per se but improves the reproducibility of the experiment; data not shown). In contrast, the effect of **1** on eosinophil influx was more than 10-fold weaker after oral application (ED_{50} > 10 mg/kg, −1 h, +24 h; ED_{50} = 30 mg/kg, −6 h, +24 h) indicating that the inhibitory activity seen after intratracheal application was not due to the compound swallowed by the animal. By the intravenous route (−1 h, +24 h or −6 h, +24 h), **1** given at the approximate ED_{50} generated by the intratracheal route (1 mg/kg) did not reduce eosinophil numbers in BALF of OA-challenged rats. Thus, the effects on eosinophils seen after intratracheal administration are unlikely to be the result of systemic exposure to compound from the lung. Altogether, the data are consistent with a local antiinflammatory action of **1**. In contrast, when FK506 was given intravenously at its approximate ED_{50} by the intratracheal route (0.32 mg/kg), eosinophil numbers in BALF were inhibited by >50% demonstrating its strong systemic activity. When given orally, FK506 was also much less potent (ED_{50} = 10 mg/kg, −1 h, +24 h) although a longer pretreatment period to the allergen challenge seemed to increase its potency (ED_{50} = 3 mg/kg, −6 h, +24 h). Thus, when given intratracheally, both **1** and FK506 suppress eosinophil accumulation at a 10−30-fold lower dose than when given orally. Whereas the ratio of the effective doses of intratracheal vs oral administration (an indicator for potential bioactivity of the swallowed portion) was similar for both **1** and FK506, the ratio of the effective doses of intratracheal vs intravenous administration (an indicator for potential bioactivity of the pulmonary absorbed portion) was more in favor of the metabolically soft compound, **1**. Metabolite **2** was also tested in this model and, consistent with its weaker activity in the in vitro assays, was 3-fold less active than **1** when given by the intratracheal route. As expected, intratracheal administration of budesonide resulted in potent inhibition of eosinophil influx (ED_{50} = 0.5 mg/kg). Surprisingly, however, and in contrast to **1** and FK506, budesonide was more potent when given by the oral route (ED_{50} = 0.2 mg/kg).

As a next step, we set out to demonstrate the antiinflammatory properties of **1** when given by a clinically relevant mode of administration, i.e., as a micronized dry powder by inhalation. Inhalation of **1** (0.2−7 mg/kg; dose delivered to the animal over a 60 min period; it should be noted that the dose actually being deposited in the lung was unlikely to be more than 5% of the dose delivered to the animal) 6 h prior to and 24 h after antigen exposure induced a dose-dependent reduction in the number of leukocytes recovered in the BALF from actively sensitized BN rats following antigen challenge (Figure 1). The maximal inhibitory effect of **1** on eosinophil numbers was about 60%, there being no inhibition at 0.2 mg/kg and maximal, although incomplete, inhibition at 0.7 mg/kg and above (ED_{50} ∼ 0.4 mg/kg). The effect on eosinophil numbers was paralleled by a reduction in the level of EPO in BALF and, to a lesser extent, in lung (data not shown). Significantly, the numbers of T-cells recovered in BALF were also reduced at low doses of **1** consistent with the proposed mechanism of action. Compound **1** also inhibited dose dependently the quantity of protein recovered in BALF from actively sensitized BN rats following antigen challenge consistent with a reduction of protein extravasation in the lung. Macrophage and neutrophil influx as well as total cell influx were also inhibited.

Finally, we carried out a series of metabolism and disposition studies in rats and monkeys. An ADME study in rat showed that the absolute oral bioavailability of **1** was low (ca. 2%), that many metabolites were formed, and, importantly, that the major metabolite in plasma was indeed the predicted carboxylic acid **2** (AUC_{0-24h} ratio of metabolite **2** to **1**: 0.22 after intra-

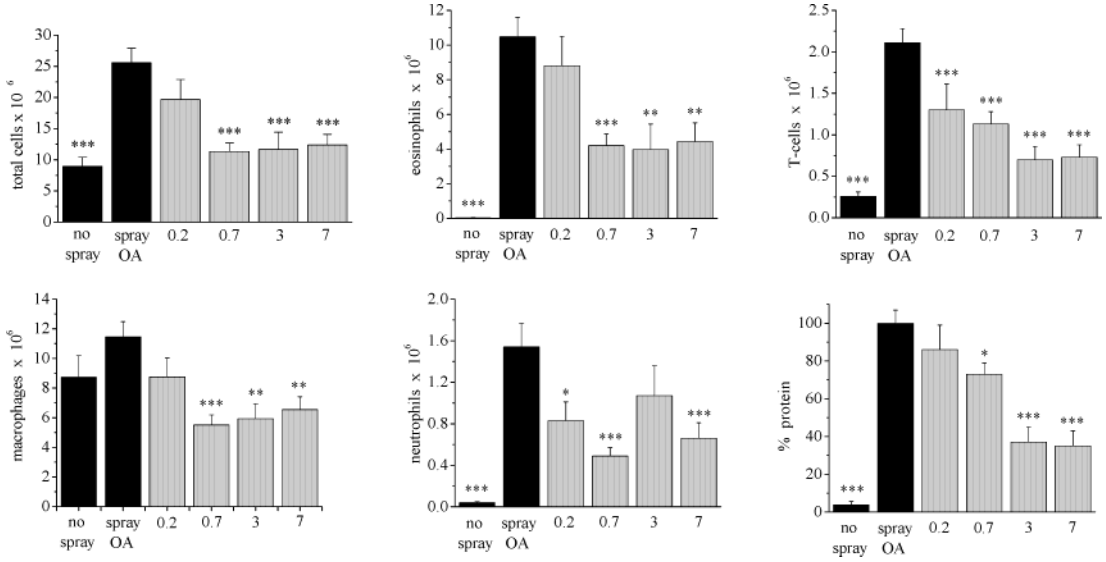


Figure 1. Compound **1** at doses of 0.2−7 mg/kg was delivered to the animal over 60 min by inhalation 6 h prior to and 24 h after antigen exposure. No spray: vehicle control (air exposed). Spray OA: OA only treated animals. Results are expressed as means ± SEM numbers of leukocytes recovered in BALF. The protein content in BALF is expressed as % of values obtained following vehicle treatment and OA exposure. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate significant differences by comparison with vehicle (air exposed) and OA (spray OA) treated animals (n = 9−39).

Table 3. Comparison of Mean Blood and Tissue Levels of **1** and Its Major Circulating Metabolite **2** in Rats (n = 6) at 6 h after an Intratracheal Application of **1** (10 mg/kg)

tissue	**1**	**2**
	concentration (μg/mL or μg/g)	
blood	0.131	0.014
lung	955.4	11.9
	ratios	
lung/blood	7293	850
1/2 in lung	80.3	
1/2 in blood	9.4	

venous, 0.27 after oral dosing). Because **1** was to be administered by inhalation, we also investigated the distribution of the compound following a single intratracheal administration. As can be seen in Table 3, **1** when given at 10 mg/kg intratracheally, was much more abundant in lung tissue than in blood at 6 h postdosing (ca. 0.01% of the concentration in lung). Thus, **1** showed a favorable distribution profile in this model with a very low systemic exposure, as compared with the local exposure in the lung. In addition, analysis of lung tissue revealed only minor amounts of metabolite **2** (ca. 1% of **1**) suggesting that local enzymatic hydrolysis of **1** to the less immunosuppressive metabolite **2** was slow. This finding was strengthened by a series of in vitro experiments (data not shown) showing that a rat lung acellular fraction (S9) was much less active in catalyzing the ester cleavage of **1** into metabolite **2** than was liver S9. Similarly, and under identical experimental conditions, human lung S9 was more than 15-fold less active than human liver S9. Accordingly, in the blood, the concentration of metabolite **2** amounted to ca. 10% of that of its parent compound, **1**. Interestingly, in human whole blood at 37 °C, hydrolysis of **1** to its main metabolite **2** was slow with an estimated half-life of 15−40 h. Taken together, these data indicate that deesterification of **1** into **2** is indeed the primary metabolic pathway and that it occurs mainly in the liver and not in blood or lung. Similar pharmacokinetic results were obtained in rhesus monkey. Again, **1** was administered by the oral, the intravenous, and the intratracheal routes. The key findings are summarized in Table 4. The absolute oral bioavailability of **1** in rhesus monkey was again very low (ca. 4%). Similarly, the pulmonary bioavailability after intratracheal application was also low (ca. 7%) and thus very favorable for a drug, which is to be administered by inhalation and expected to have a restricted local action in the lung. For comparison, in man, all glucocorticosteroids, when administered by the inhaled route, are well-absorbed from the lung. This pulmonary absorption is complete and therefore represents approximately 20% of the administered dose.[29] As in the rat, the carboxylic acid **2** was a significant metabolite in the rhesus monkey. Irrespectively, whether **1** was given intravenously, orally, or intratracheally, the AUC of the less immunosuppressive metabolite **2** amounted always to 25−30% of that of **1**. Importantly, after both oral or intravenous application, the systemic exposure to **1** was approximately 3-fold lower than that to FK506 (data not shown) when given at a same dose. In addition, in the monkey, the terminal half-life of the soft compound **1** (8.3 ± 2.1 h, n = 4) compared favorably with that of the metabolically more stable FK506 (14.8 ± 0.8 h, n = 4). Overall, the disposition and pharmacokinetic profile of **1** in rat and monkey are particularly favorable for an inhaled drug. Both low oral and low pulmonary bioavailability are excellent pharmacokinetic characteristics to guarantee strong local activity in the lung and very limited, unwanted systemic exposure by either drug swallowed during inhalation or by compound directly absorbed from the lung. Furthermore, in both species, the limited amounts of **1** reaching the systemic circulation were metabolized to a significant extent to the less immunosuppressive carboxylic acid **2**.

Table 4. Pharmacokinetic Characteristics of **1** after a Single Intravenous (1 mg/kg), Oral (10 mg/kg), and Intratracheal (0.1 mg/kg) Administration in the Rhesus Monkey

	1[a,b]			**2**[a,b]		
route of administration	c_{max}/dose[c] (ng/mL)	AUC_{inf}/dose[d] (ng h/mL)	F[e] (%)	c_{max}/dose (ng/mL)	AUC_{inf}/dose (ng h/mL)	relative amount of **2** (%)[f]
intravenous	3660 ± 470	2530 ± 460		75 ± 21	770	30
oral	6.25 ± 2.70	108 ± 52	4.3	1.01 ± 0.11	26.6 ± 5.6	25
intratracheal	58.9 ± 80.8	171 ± 43[g]	6.8	48.8 ± 29.2	49.6 ± 31.6[g]	29

[a] Results are expressed as means ± SD mean ($n = 4$). [b] Measurements in blood. [c] c_{max}/dose is the dose-normalized peak concentration in blood. [d] AUC_{inf}/dose is the dose-normalized area under the concentration−time curve extrapolated to infinity. [e] Absolute oral or pulmonary bioavailability in % calculated from the mean dose-normalized AUC after intravenous and oral or intratracheal administration, respectively. AUC_{inf}/dose and AUC_{0-48h}/dose were used for the calculation of the oral and pulmonary bioavailability, respectively. [f] Calculated from AUCs of **1** and metabolite **2**. [g] Value given is AUC_{0-48h}/dose. This is the dose-normalized area under the concentration−time curve between 0 and 48 h.

Conclusion

The goal of our research program was to find a potent, locally active T-cell modulator as an alternative treatment for allergic asthma. We have described the principle, the design, the synthesis, and the pharmacological profile of the novel immunosuppressive macrolide, **1**. The compound showed powerful immunosuppressive effects in vitro and, consistent with this, potent and long-acting antiinflammatory effects were seen in a rat model of allergic asthma when given directly into the airways. Because similar doses given intravenously or orally were without effect on eosinophil influx, the activity of **1** reflected a direct action in the airways. Pharmacokinetic investigations in rat and monkey showed both a low absolute oral and a low absolute pulmonary (only monkey investigated) bioavailability of **1** in these species. This finding accords with the pharmacological results and explains the low effect bioavailability of **1** after systemic administration. Furthermore, significant amounts of the less immunosuppressive carboxylic acid metabolite **2** were formed once **1** was absorbed systemically. Thus, the soft drug principle of the approach was confirmed. However, it remains to be shown to which extent the soft drug principle contributed to the restricted local action of **1** in comparison to the much higher concentrations of the drug in lung vs blood after local administration. Nevertheless, administration of a locally active compound like **1**, by inhalation, should reduce systemic side effects related both to swallowed compound and to compound absorbed directly from the lung. The profile of **1** is unique and should result in an improved therapeutic index as compared to other selective T-cell inhibitors such as FK506 and CsA. Our results demonstrate that compounds such as **1** have the potential to be alternatives to inhaled glucocorticosteroids as therapies for the long-term treatment of asthma of all grades of severity.

Experimental Section

Chemistry. Reactions were performed under argon. All commercially available reagents were applied without further purification. Middle pressure liquid chromatography (MPLC) was carried out using silica gel (Merck; Kieselgel 60 F254, 0.040−0.063 mm). Melting points were measured with a Buechi B-540 and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 (500 MHz) instrument, using residual solvent protons as references; because of complicated overlapping m, only relevant ^{1}H NMR data are reported. Fast atom bombardment (FAB) mass spectra were recorded on a VG70-SE in the presence of lithium in the FAB matrix. The new compounds synthesized were characterized by MS and NMR, and purity was determined by two diverse RP-HPLC systems using Hewlett-Packard, model 1050, with a NUCLEOSIL column (100−5 C18 AB, 5 μm, detection at 210 nm). RP-HPLC (no. 1): Isocratic method using various % of acetonitrile in water/acetonitrile/2-methyl-propan-2-ol/phosphoric acid (500:400:100:0.2). RP-HPLC (no. 2): Isocratic method using various % of acetonitrile in water with 0.1% TFA.

17-Allyl-1,14-dihydroxy-12-{2-[3-methoxy-4-(*p*-methoxycarbonyl-methylphenylene-carbamoyloxy)cyclohexyl]-1-methylvinyl}-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-aza-tricyclo[22.3.1.0^{4,9}]octacos-18-ene-2,3,10,16-tetraone (1). To a stirred solution of FK506 (10.0 g, 12.4 mmol) and 4-dimethylamino pyridine (7.5 g, 61.4 mmol) in dichloromethane (60 mL) was added dropwise a solution of triphosgene (1.36 g, 4.6 mmol) in dichloromethane (40 mL) at −77 °C. After 1 h, a solution of (4-amino-phenyl)acetic acid methyl ester (3.1 g, 18.8 mmol) in dichloromethane (5 mL) was added. The cooling bath was removed, and the suspension was stirred for 1 h at ambient temperature. Ethyl-acetate and a saturated aqueous solution of NaCl were added. The organic phase was washed with 1 N HCl and evaporated to dryness. Purification of the crude product by MPLC chromatography (acetone−water gradient, 4 bar) gave 7.6 g (62%) of pure, amorphous product; mp 105−109 °C. Mixture of two conformers (3:1). Major conformer: ^{1}H NMR ($CDCl_3$): δ 7.36 [br d, J = 7.3 Hz, H−C(8′), H−C(4′)], 7.22 [d, J = 7.3 Hz, H−C(7′), H−C(5′)], 6.60 (s, NH), 5.72 [m, H−C(39)], 5.34 [br s, H−C(26)], 4.68 [dt, J = 9.2 Hz, J = 4.3 Hz, H−C(32)], 4.44 [br d, J = 13.4 Hz, H−C (6 eq)], 4.23 (s, 10-OH), 3.69 [s, 3H, H−C(12′)], 3.59 [s, 2H, H−C(9′)], 3.25 [m, H−C(31)], 3.02 [br. t, J = 13.4 Hz, H−C (6 eq)], 2.80 [dd, J = 16.5 Hz, J = 2.4 Hz, H−C(23a)], 1.66 [s, 3H, H−C(41)], 1.64 [s, 3H, H−C(37)], 1.01 [d, J = 6.7 Hz, 3H, H−C(35)], 0.95 [d, J = 6.7 Hz, 3H, H−C(36)], 0.87 [d, J = 6.7 Hz, 3H, H−C(40)]. ^{13}C NMR ($CDCl_3$): δ 212.9 C(22), 196.2 C(9), 172.1 C(10′), 169.0 C(1), 164.6 C(8), 153.0 C(1′), 139.0 C(19), 137.0 C(3′), 135.5 C(39), 132.8 C(27), 129.8 C(5′)/C(7′), 128.9 C(28), 128.8 C(6′), 122.3 C(20), 118.8 C(8′)/C(4′), 116.7 C(39a), 97.0 C(10), 80.7 C(31), 77.5 C(26)/C(32), 75.1 C(15), 73.6 C(13), 72.8 C(14), 70.1 C(24), 57.2/57.0/2 × OMe, 56.6 C(2), 56.3 OMe, 52.7 C(21), 52.0 C(12′), 48.4 C(18), 42.7 C(23), 40.5 C(9′), 39.6 C(25), 39.2 C(6), 36.3 C(30), 35.1 C(38), 34.6 C(29)/C(11), 32.8 C(12), 32.7 C(16), 30.5 C(34), 30.0 C(33), 27.7 C(3), 26.2 C(17), 24.5 C(5), 21.2 C(4), 20.5 C(36), 16.2 C(37), 16.0 C(35), 14.3 C(41), 9.3 C(40). MS (m/z) $[M + Li]^+$ 1001. RP-HPLC, 97.4% purity. Anal. ($C_{54}H_{78}N_2O_{15}$) H, N; C: calcd, 65.17; found, 64.61.

(4-{4-[2-(17-Allyl-1,14-dihydroxy-23,25-dimethoxy-13,19,21,27-tetramethyl-2,3,10,16-tetraoxo-11,28-dioxa-4-aza-tricyclo[22.3.1.0*4,9*]octacos-18-en-12-yl)propenyl]-2-methoxy-cyclohexyloxycarbonylamino}phenyl)acetic Acid (2). To a stirred solution of FK506 (5.0 g, 6.2 mmol) and 4-dimethylamino pyridine (3.8 g, 31.1 mmol) in dichloromethane (40 mL) was added dropwise a solution of triphosgene (0.61 g, 2.1 mmol) in dichloromethane (7 mL) at −77 °C. After 1 h, a solution of (4-amino-phenyl)acetic acid trimethylsilyl ester (2.8 g, 12.5 mmol) in dichloromethane (3 mL) was added. After 30 min, the cooling bath was removed and the suspension was stirred for 2 h at ambient temperature.

The reaction mixture was poured onto 1 N HCl (30 mL), and the product was extracted with ethyl-acetate. The organic phase was washed with 1 N HCl and brine and evaporated to dryness. Purification of the crude product by flash chromatography (ethyl-acetate−hexane−AcOH; 18:10:0.2) and subsequent crystallization from ethanol−water gave 1.6 g (26%) of pure product; mp 135−138 °C. Mixture of two conformers (55:45). ^{1}H NMR (DMSO-d_6): δ 12.20 (br s, COOH), 9.58 (s, H−N1′), 7.39 [br d, J = 8.2 Hz, H−C(8′), H−C(4′)], 7.14 [d, J = 8.2 Hz, H−C(7′), H−C(5′)], 7.05/6.65 (s, 10-OH), 5.66 [m, H−C(39a)], 5.24 (d, J = 4.3 Hz)/5.07 (d, J = 5.6 Hz), H−C(26), 5.15 [d, J = 8.6 Hz, H−C(28)], 5.05 (d, J = 4.7 Hz)/4.44 (br. s), H−C(2), 5.02−4.90 [m, 2H, H−C(39b)], 4.79 (br. s, 24-OH), 4.78 (d, J = 9.5 Hz)/4.67 (d, J = 9.9 Hz), H−C(20), 4.54 [m, H−C(32)], 4.22 [br d, J = 13 Hz, H−C (6 eq)], 3.87 [br s, H−C(24)], 3.47 [s, 2H, H−C(9′)], 2.78 [br t, J = 13 Hz, H−C (6 ax)], 1.65 (s)/1.63 (s), 3H, H−C(37), 1.60 (s)/1.54 (s), 3H, H−C(41). ^{13}C NMR (DMSO-d_6): δ 210.5/209.4 C(22), 198.8 C(9), 173.5 C(10′), 169.4/169.2 (C1), 166.3 (C8), 153.6 (C1′), 139.5/138.3 C(19), 138.2 C(3′), 136.73/136.7 C(39a), 132.9 C(27), 132.0/130.7 C(28), 130.1 C(5′) and C(7′), 129.4 C(6′), 123.4/123.0 C(20), 118.6 C(4′) and C(8′), 116.6/116.4 C(39b), 98.5/98.3 C(10), 80.31 C(31), 80.3/78.1 C(26), 76.8/75.5 C(15), 76.0/75.9 C(32), 73.9 /73.7 C(13), 72.6 C(14), 68.9/68.4 C(24), 57.35, 57.29, 56.72, 56.14, 55.79 3 × OMe, 56.4/52.1 C(2), 52.6/52.5 C(21), 48.2/47.5 C(18), 47.1/44.9 C(23), 44.0/38.9 C(6), 40.5 (C9′), 40.0 C(25), 36.3/36.2 C(30), 35.9 C(38), 35.2/34.4 C(11), 34.8/33.8 C(16), 34.3 C(29), 32.3/32.0 C(12), 30.8 C(34), 30.3 C(33), 27.7/27.6 C(3), 26.4 C(17), 25.1/24.4 C(5), 21.6/21.1 C(4), 20.1/19.8 C(36), 16.7/16.4 C(37), 16.2/15.9 C(35), 13.6/13.3 C(41), 10.5/10.1 C(40). MS (m/z) [M + Li]$^+$ 987. RP-HPLC, 98.6% purity.

Analytics. Blood samples and lung were deep frozen at about −20 °C pending analysis. All samples were analyzed using a specific LC/MS method with internal standardization. Briefly, to an aliquot of 0.25 mL of blood, the labeled internal standard ($^{13}C^2H_3$) **1** was added and both the parent compound and the metabolite **2** were extracted with *tert*-butyl methyl ether. After evaporation, the residue was reconstituted in H_2O/acetonitrile (50/50, v/v) before separation of the analyte from coextracted substances on a reversed phase C18 column at 75 °C. During 10.5 min of runtime, an ammonium acetate/methanol gradient was applied with a flow rate of 1 mL/min. The lung tissue was homogenized at a final weight of 0.1 g/mL H_2O by means of 20 s ultrasonication and further diluted, if appropriate. To an aliquot of 0.25 mL of the homogenate, the internal standard ($^{13}C^2H_3$) **1** was added before extraction and further processing as described for blood.

Compounds **1** and **2** were determined simultaneously with a mass spectrometer (TSQ700, Finnigan) in single ion monitoring (SIM) as negative ions after atmospheric pressure chemical ionization (APCI). For detection, the selected m/z for **1**, **2**, and the internal standard were 994.5, 803.5, and 998.5, respectively.

Biological Methods. IL-2 RGA: The reporter gene assay was performed as described previously.[30] MLR: The mixed lymphocyte reaction has been described before.[31] MBA: The binding to FKBP-12 is described elsewhere.[32] The method for measuring IL-5 and INF-γ release from human HPBMNC was described previously.[33] Human T-cell proliferation was measured using a known procedure.[34] Calcineurin inhibition was measured according to a previously described method.[35] BN rat model of airway inflammation: The experiments were done as described previously.[36]

Supporting Information Available: Elemental analyses of compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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