JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 1609–1617

Received April 22, 2005
Accepted September 5, 2005
DOI 10.1211/jpp.57.12.0011
ISSN 0022-3573

The phosphodiesterase 4 inhibitor AWD 12-281 is active in a new guinea-pig model of allergic skin inflammation predictive of human skin penetration and suppresses both Th1 and Th2 cytokines in mice

Joachim Hoppmann, Wolfgang Bäumer, Christin Galetzka, Norbert Höfgen, Manfred Kietzmann and Chris Rundfeldt

Abstract

The selective phosphodiesterase 4 (PDE4) inhibitor AWD 12-281 is structurally optimized for topical administration. It has potent effects in models of lung inflammation if administered as a dry powder inhalation. It has also demonstrated its anti-inflammatory property in a mouse model of cutaneous inflammation after topical administration. The aim of this study was to evaluate whether AWD 12-281 may be capable of penetrating human skin. Therefore a new guinea-pig model of allergic skin inflammation had to be developed. In ovalbumin-sensitized guinea-pigs, intracutaneous administration of ovalbumin results in a rapid development of allergic skin wheals. Topically administered AWD 12-281 was capable of reducing the development of wheals, indicating that this compound can penetrate the stratum corneum of guinea-pig skin as a predictor of human skin penetration. A secondary aim was the evaluation of a T cell subtype preference of AWD 12-281 since PDE4 inhibitors are said to preferentially inhibit Th2-type cytokines. Therefore, the effects of AWD 12-281 on a broad spectrum of Th1- and Th2-type cytokines were studied in tissue homogenates after allergen challenge in sensitized mice and in supernatants of anti CD3/anti-CD28-stimulated peripheral blood mononuclear cells (PBMCs). In both models, AWD 12-281 suppressed both T cell subtype cytokines indicating a broad spectrum activity of AWD 12-281. A further issue was to determine the duration of action and the concentration–response relationship of the topical activity of AWD 12-281 using a model of acute local inflammation — the arachidonic-acid-induced mouse ear oedema. The compound exhibited a dose-dependent effect with a minimally effective concentration of 0.3%; after repeated administration the minimally effective concentration was found to be 0.03%. A single administration of a 3% solution resulted in significant suppression of inflammation even 48 h after treatment. In conclusion, our results indicate that AWD 12-281 is a very promising drug candidate not only for the treatment of lung inflammation using inhalative administration but also for the treatment of atopic dermatitis.

Department of Pharmacology, elbion AG, Meissner Str. 191, D-01445 Radebeul, Germany

Joachim Hoppmann, Christin Galetzka, Norbert Höfgen, Chris Rundfeldt

Department of Pharmacology, Toxicology and Pharmacy, School of Veterinary Medicine, Buenteweg 17, D-30559 Hannover

Wolfgang Bäumer, Manfred Kietzmann

Correspondence: J. Hoppmann, Department of Pharmacology, elbion AG, Meissner Str. 191, D-01445 Radebeul, Germany. E-mail: joachim.hoppmann@elbion.de

Acknowledgements: We thank PharmaTec for supplying the water-in-oil cream. We also thank Angela Thomas and Katja Pfeifer for their valuable assistance in the laboratory.

Introduction

The potential use of phosphodiesterase 4 (PDE4) inhibitors as agents for the treatment of inflammatory disorders, like asthma, chronic obstructive pulmonary disease and multiple sclerosis, has received considerable attention (Dyke & Montana 2002). In addition to these diseases, atopic dermatitis is a possible target for PDE4 inhibitors. The PDE4 isoenzyme is the major cyclic-AMP metabolizing enzyme in immune and inflammatory cells, including leucocytes, from patients with atopic dermatitis (Grewe et al 1982; Hanifin et al 1996). The efficacy of PDE4 inhibitors in the treatment of atopic dermatitis has already been demonstrated in clinical studies. The PDE4 inhibitor arofylline (Almirall-Prodesfarma) was as effective as prednisone in controlling pruritus and skin lesions in dogs with atopic dermatitis but its efficacy was associated with characteristic PDE4 inhibitor side effects, such as nausea and vomiting, as a result of systemic exposure (Ferrer et al 1999). In two clinical trials, the efficacy of PDE4 inhibitors in the treatment of patients with atopic dermatitis was demonstrated with cipamfylline (Glaxo Smith

Kline, Leo Pharmaceuticals) and atizoram (Pfizer) (Hanifin et al 1996). However, again, systemic side effects limited further development even in the case of cipamphylline and atizoram, which were administered topically. A PDE4 inhibitor that is optimized for the topical route (i.e. which has a potent and long-lasting local effect after topical administration but at minimal systemic exposure) should be a very interesting drug candidate for treatment of atopic dermatitis. *N*-(3,5-Dichloropyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indole-3-yl]-glyoxylic acid amide (AWD 12-281) is a new selective PDE4 inhibitor being optimized for topical administration (Kuss et al 2003). The efficacy of AWD 12-281 was demonstrated in different models of airway inflammation (Kuss et al 2002) and bronchoconstriction (Kuss et al 2003). Currently AWD 12-281 is undergoing clinical development phase IIa for chronic obstructive pulmonary disease (COPD) (Kuss et al 2003). Recently, the anti-inflammatory capacity of AWD 12-281 was demonstrated in a model of immunological skin inflammation in mice — the toluene-2,4-diisocyanate mouse ear swelling test (TDI-MEST). In this model, prophylactic and therapeutic administration of AWD 12-281 was capable of suppressing ear swelling and infiltration of granulocytes after TDI-challenge in ears of sensitized mice. In these studies, only topical administration of AWD 12-281 was effective, whereas systemic administration failed to reduce the allergic response (Bäumer et al 2002, 2003). For the pharmaceutical development of topical drugs it is a prerequisite to document percutaneous penetration. This was previously demonstrated for AWD 12-281 in murine back skin in the Franz diffusion cell (Bäumer et al 2003). However, as mouse and human skin differ in anatomy, particularly the thickness of the stratum corneum, no clear prediction about the ability of AWD 12-281 to penetrate human skin can be made from these studies. For this reason, the main aim of this study was to develop a new pharmacological model able to predict human skin penetration. As the thickness of the stratum corneum, being the main barrier to percutaneous skin penetration, is similar in human and guinea-pig skin, this species was selected to develop such an in-vivo model. Guinea-pigs are frequently used for allergic reactions since they can be easily sensitized using ovalbumin (OVA) (Evilevitch et al 1999). If guinea-pigs are challenged by intradermal injection of the specific allergen (i.e. OVA), a so-called wheal and flare reaction is immediately induced (Derks et al 1997). If test substances are topically administered onto the skin, an effect on the size of the allergic skin wheal can be taken as a quantifiable pharmacological effect. Since the allergen is administered intradermally while the treatment occurs topically, this model can be taken as a proof of drug penetration through guinea-pig skin. The test compound, AWD 12-281, was administered as a solution in dimethyl sulfoxide (DMSO) and acetone for studying the overall anti-inflammatory potential of this PDE4 inhibitor in this model. However, depolar aprotic solvents, like DMSO, alter the barrier properties of the basal stratum corneum cells, as they cause a swelling of these cells and a disruption of the normal keratin pattern (Sharata & Burnette 1988). For this reason, in a separate set of experiments AWD 12-281 was also topically administered in a DMSO-free water-in-oil cream for studying skin penetration.

A further aim of this work was the determination of a potential T cell subtype preference of AWD 12-281. The discussion about a general T cell subtype preference of PDE4 inhibitors is still ongoing (Essayan et al 1997; Bielekova et al 2000). As both Th1 and Th2 cells are involved in the progression of atopic dermatitis, compounds suppressing both types of cytokines are needed to show good activity in atopic dermatitis. Recently, the inhibitory effect of AWD 12-281 on the release of interleukin (IL)-4, IL-6 and macrophage inflammatory protein-2 (MIP-2) was shown in a model of allergic skin reaction — the toluene-2,4-diisocyanate (TDI)-induced mouse ear swelling test (MEST) (Bäumer et al 2003). Because of the limited number of cytokines being analysed in these reported studies, no statement about a possible T cell subtype preference could have been made for AWD 12-281 up to now. By using the Bio-plex (Bio-Rad, Munich, Germany) cytokine assay it was possible to survey a broad panel of Th1- and Th2-type cytokines. In this study, samples for cytokine analysis were generated from TDI-challenged ears of sensitized mice and from anti-CD3/anti-CD28-activated peripheral blood mononuclear cells (PBMCs). An additional task was to characterize the duration of action and the concentration–response relationship of the anti-inflammatory property of AWD 12-281. As the TDI test in mice has a read out only 24–48 h after challenge, this is not well suited for time course experiments. Therefore the arachidonic-acid-induced mouse ear oedema was utilized, allowing also demonstration of the activity of AWD 12-281 in a non-immunological model of skin inflammation.

Material and Methods

All procedures were carried out in agreement with the current version of the German Law on the Protection of Animals.

Material

AWD 12-281 was synthesized by elbion AG (Radebeul, Germany). Arachidonic acid, toluene-2,4-diisocyanate (TDI), ovalbumin, indometacin and diflorasone diacetate were supplied by Sigma-Aldrich Chemie (Deisenhofen, Germany). Acetone and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). The protein content was measured using a Bio-Rad protein assay (Munich, Germany). Ketamine was obtained from Serumwerk Bernburg (Bernburg, Germany) and xylazine was supplied by WDT (Neustadt am Ruebenberge, Germany). Pyrilamine (mepyramine) maleate was purchased from ICN Biochemicals GmbH (Eschwege, Germany). RPMI 1640 medium was obtained from Invitrogen (Karlsruhe, Germany). The water-in-oil cream, in accordance with the European Pharmacopoeia 2005, was produced by Pharmatec (Kiel, Germany). The respective amounts of micronized AWD 12-281 to obtain a 1–5% concentration were worked into the cream formulation; the cream without AWD 12-281 served as vehicle.

OVA-sensitized guinea-pig model

The animal study was registered by Landesregierung Sachsen, Dresden, Germany (Az. 24-9168.21-2-2003-2). Male CRL (HA) BR guinea-pigs were obtained from Charles River (Sulzfeld, Germany). All guinea-pigs were healthy and housed in groups of 4 per cage at 22°C with a 12-h light–dark cycle. Water and a standard diet (sniff, Soest, Germany) were freely available. Before immunization, challenge or substance treatment was performed, guinea-pigs were anaesthetized by intraperitoneal administration of ketamine + xylazine ($40\,mg\,kg^{-1} + 5\,mg\,kg^{-1}$). The guinea-pigs were anaesthetized for approximately 70–80 min. Anaesthesia was found to be necessary to prevent the guinea-pigs from scratching, which would have biased the results of the assay for two reasons — on one hand, the scratching could have had effects on the allergic (inflammatory) reaction and on the other, scratching would have distributed active compound administered onto the skin into deeper layers of the skin possibly mimicking penetration, as indicated by Gummer & Maibach (1986). On two consecutive days, guinea-pigs were immunized by intraperitoneal injection of 20 μg OVA and 20 μg aluminium hydroxide. For assessing the sensitization status, guinea-pigs were challenged by intradermal injection of 50 μL OVA (0.05%) in 4 locations on the back skin 14 days after immunization. As successful intradermal administration of the solution results immediately in a visible papule (resulting from the injected volume), the areas of these non-allergic wheals were measured immediately after allergen challenge. The immunological reaction as a result of allergen challenge appeared as allergic wheals on the skin within 60 min after challenge. This time point was selected to evaluate drug effects. The area of the allergic skin wheals was measured and the area of the non-allergic wheal was subtracted to quantify the allergic response. The areas were obtained by drawing the circumferences of wheals on a transparent plastic film immediately and 60 min after antigen challenge. The circumferences were copied on white paper by using a photocopying machine (141% magnification) and cut out. Weights of paper cuts were converted into area of wheals by using a calibration curve (weight of paper with a defined area). Only those guinea-pigs showing a total allergic wheal area of at least $100\,mm^2$ (pooled from all 4 locations) were considered as sufficiently sensitized for drug testing. To prevent an anaphylactic reaction, the antihistamine mepyramine maleate ($10\,mg\,kg^{-1}$) was always intraperitoneally administered 30 minutes before antigen challenge. Five days after the first challenge, drug administration was initiated. Substances were topically applied to four locations of the back skin once daily on four consecutive days before challenging the guinea-pigs for the second time. As vehicle formulations, a solution in acetone–DMSO (9:1, v/v) and a water-in-oil cream were used. The last treatment was performed 5 h before allergen challenge. The application volume was 50 μL per location for the acetone–DMSO solution and $1\,mg\,cm^{-2}$ for the water-in-oil cream. As a reference compound, the potent steroid diflorasone diacetate was used.

Effect of AWD 12-281 in Th1- and Th2-type cytokines

Ex-vivo samples to evaluate cytokine levels were generated using the mouse ear swelling test in TDI-sensitized mice. In addition, the effect on cytokine release was evaluated in-vitro using anti-CD3/anti-CD28-stimulated human peripheral blood mononuclear cells (PBMCs). The TDI test and the generation of ear tissue homogenates were performed according to the method described previously (Gad et al 1986; Bäumer et al 2003). The animal study was registered by Bezirksregierung Hannover, Germany (Az. 509i-42502-98A839). Two hours before TDI challenge, the ears of sensitized mice were treated topically with 20 μL 3% AWD 12-281 or acetone–DMSO (9:1) as vehicle solution. Twenty-four hours later, mice were killed by cervical dislocation and their ears were resected, snap frozen and stored in liquid nitrogen immediately after sampling. For determination of biochemical parameters, ears were homogenized under liquid nitrogen. Homogenates were taken up in 200 μL RPMI 1640 medium containing 1 mmol protease inhibitor Pefabloc (Boehringer Mannheim, Germany). After centrifugation (10 000 g, 10 min, 4°C) supernatants were collected and the protein content was quantified using a Bio-Rad protein assay (Munich, Germany).

For the PBMC assay, human mononuclear cells were obtained from heparinized venous blood of healthy subjects through Ficoll Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation. The obtained mononuclear cells were washed with phosphate-buffered saline (PBS), resuspended in RPMI1640 with 25 mM Hepes supplemented with 10% fetal calf serum and seeded in 96-well microtitre plates (5×10^5 cells/50 μL/well). AWD 12-281 was dissolved in DMSO and diluted in RPMI 1640 medium to a final DMSO concentration of 0.1%. Then different test substance concentrations were added (25 μL/well). Thirty minutes later, the cells were stimulated with anti-CD3 ($0.5\,\mu g\,mL^{-1}$) and anti CD28 ($1\,\mu g\,mL^{-1}$) antibodies (25 μL/well) and mixed shortly. The plates were incubated for 18 h at 37°C in a humidified atmosphere of 5% CO_2. The microtitre plate was centrifuged at 200 g for 10 min. The supernatant was removed and frozen at −70°C until the measurement. The content of cytokines was measured with the Bioplex cytokine assay (Bio-Rad, Munich, Germany) and plotted against the concentration of AWD 12-281; IC50 values (half maximum inhibition base on maximum inhibition at $1\,\mu mol\,L^{-1}$ AWD 12-281) were calculated using 3 parameter Hill Fit (Sigma Plot for windows, version 7.0, SPSS Inc., Chicago, USA).

Arachidonic-acid-induced mouse ear oedema model

The animal study was registered by Landesregierung Sachsen, Dresden, Germany (Az. 24-9168.21-2-2002-27). The arachidonic-acid-induced mouse ear oedema model was performed according to the method described

previously (Rao et al 1993). In brief, in female NMRI mice, 6–8 weeks old, 20–25 g (Charles River, Sulzfeld, Germany), an inflammatory response was induced by application of 20 μL 5% arachidonic acid in acetone on left ears. The ear thickness was measured with a cutimeter (Model 7309; Mitutoyo, Neuss, Germany) before, as well as 60 min after, induction of inflammation. The arachidonic-acid-induced increase in ear thickness was calculated for each mouse as the difference between the left ear thickness before and 60 min after administration of arachidonic acid. Substances were administered topically on ears in an acetone–DMSO solution (9:1). The applied volume was 20 μL.

For testing the concentration–response relationship, mice were treated topically with AWD 12-281 in concentrations of 0.03–3% or its vehicle (acetone–DMSO, 9:1). One group was treated topically with 1% indometacin as reference. Treatment was performed 5 h before induction of inflammation. For testing the duration of action, mice were treated with 3% AWD 12-281 or its vehicle acetone–DMSO (9:1) up to 60 h before induction of inflammation. In a further study, the anti-inflammatory effect of single and multiple administration of AWD 12-281 was compared. Mice were treated with AWD 12-281 0.03% or 0.1%, or its vehicle, as a single dose or once daily on four consecutive days. The last treatment was performed 5 h before induction of inflammation.

Statistical analysis

A non-parametric analysis of variance, the Kruskal–Wallis test, followed by the Dunnett's test was used for multiple comparison. $P < 0.05$ was considered significant. For comparison of two different groups, the Mann–Whitney test U-test was used. Statistical analysis was performed by SigmaStat for windows 2.03 (SPSS Inc., Chicago, IL).

Results

Percutaneous penetration of AWD 12-281: OVA-sensitized guinea-pig model

Intracutaneous administration of OVA in mepyramine-pretreated guinea-pigs resulted in a reproducible allergic reaction, represented as allergic wheals. The combined area of the 4 wheals amounted to 127 ± 31 mm^2 in vehicle-treated guinea-pigs. Topical administration of AWD 12-281 on 4 consecutive days resulted in a dose-dependent reduction of the wheal area. With 1% AWD 12-281, a significant reduction of 37% could be observed while a 3% solution resulted in a 45% reduction, indicating that topical administration resulted in a pharmacological effect. When guinea-pigs were treated with the steroid diflorasone diacetate as reference, the increase in area of allergic wheals was reduced to one-third compared with vehicle (Figure 1).

In a separate experiment AWD 12-281 was administered in a water-in-oil cream. In vehicle-treated guinea-pigs the combined area of allergic wheals amounted to 206 ± 45 mm^2. Pre-treatment of guinea-pigs with 1% or 3% AWD 12-281 in a water-in-oil cream significantly reduced the area of allergic wheals by approximately 45%. On increasing the concentration of AWD 12-281 in the water-in-oil cream to 5%, a significant reduction by 65% could be observed (Figure 2).

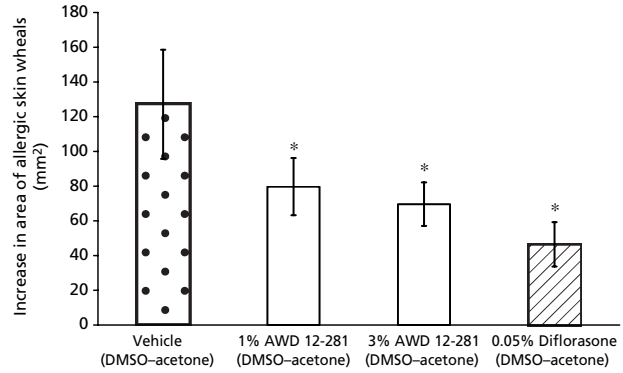

Figure 1 Development of allergic wheals following intradermal challenge with OVA in guinea-pigs. Bars represent mean increase in area of allergic wheals (± s.d.) at 60 min after allergen challenge. Compounds were topically administered as a DMSO–acetone solution. Guinea-pigs were treated with vehicle (dotted bars), 1% or 3% AWD 12-281 (white bars) or with 0.05% diflorasone diacetate (hatched bars) once daily on four consecutive days (last treatment 5 h before allergen challenge). *$P < 0.05$, compared with vehicle, n = 6.

Effect of AWD 12-281 on Th1- and Th2-type cytokines

One ex-vivo preparation in mice and one in-vitro model using human immune cells was used to evaluate the effect of AWD 12-281 on a panel of Th1- and Th2-type cytokines. Topical administration of TDI onto mice ears in the TDI-sensitized mice resulted in high contents of IL-1β, IL-6, IL-10 and granulocyte–macrophage-colony-stimulating factor GM-CSF in vehicle-treated ears, whereas the contents of tumour necrosis factor-α (TNF-α), IL-12 (p40), IL-2 and IL-3 were

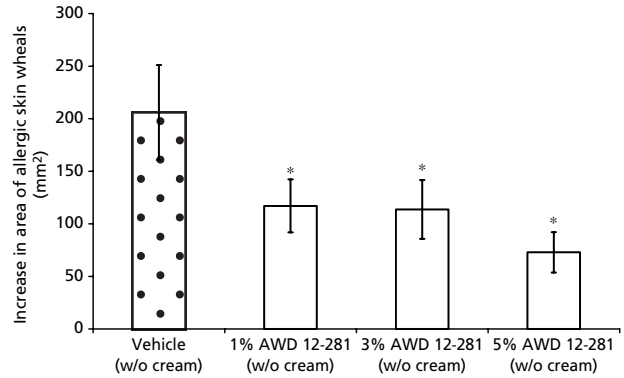

Figure 2 Development of allergic wheals following intradermal challenge with OVA in guinea-pigs. Bars represent mean increase in area of allergic wheals (± s.d.) at 60 min after allergen challenge. AWD 12-281 was topically administered as a water-in-oil cream. Guinea-pigs were treated with vehicle (dotted bars), 1%, 3% or 5% AWD 12-281 (white bars) once daily on four consecutive days (last treatment 5 h before allergen challenge). *$P < 0.05$, compared with vehicle, n = 6.

Table 1 Increase in ear swelling and content of cytokines and chemokines in ear-tissue homogenates 24 h after TDI challenge in mice

	Vehicle control	AWD 12-281 (3%)	x-fold decrease following treatment
Increase in ear swelling (mm)	0.108 ± 0.047	0.00 ± 0.06*	
Lymph node weight (mg)	4.9 ± 0.5	3.3 ± 0.3*	1.5
Cytokines (pg/500 μg protein)			
IL-1β	679 ± 99	90 ± 24*	7.5
IL-2	15 ± 0.66	10.00 ± 0.87*	1.5
IL-3	14 ± 2	8.0 ± 0.8*	1.8
IL-4	75 ± 8	35 ± 7*	2.1
IL-5	54 ± 10	23 ± 7*	2.3
IL-6	162 ± 29	42 ± 22*	3.9
IL-10	134 ± 25	33 ± 4*	4.0
IL-12 (p40)	12 ± 1	8 ± 2	1.5
IL-12 (p70)	86 ± 12	80 ± 16	1.1
IL-17	18 ± 0.9	1.0 ± 3.2*	1.5
TNF-α	6 ± 0.5	3.0 ± 0.7*	2.0
IFN-γ	48 ± 8	23 ± 8*	2.1
GM-CSF	136 ± 15	70 ± 18*	1.9
G-CSF	93 ± 50	19 ± 11*	4.9
Chemokines (pg/500 μg protein)			
KC	156 ± 67	71 ± 17*	2.2
MIP-1	1357 ± 67	574 ± 115*	2.4
RANTES	359 ± 57	205 ± 27*	1.8

Data are given as mean ± s.e.m. of five mice per group. *$P < 0.05$, compared with vehicle controls.

below 20 pg/500 μg protein. In addition, quantifiable contents of KC (keratinocyte-derived chemokine; functional homologue to human IL-8), MIP-1α and RANTES (regulated upon activation, normal T-cell expressed and secreted) were measured. Comparing these contents with the contents of ears pretreated with 3% AWD 12-281, an overall suppressive effect could be observed. The most pronounced suppression was observed for IL-1β (87% inhibition), granulocyte-colony-stimulating factor G-CSF (−80%), IL-10 (−75%) and IL-6 (−74%). Furthermore, pretreating ears with AWD 12-281 reduced the expression of KC (−54%), MIP-1α (−58%) and RANTES (−43%) (Table 1). In general, AWD 12-281 suppressed the concentration of all measured cytokines, which could be quantified following TDI challenge in sensitized mice.

To extend these results to human tissue, PBMCs from healthy subjects were used. Cytokine release was induced by anti-CD3/anti-CD28 stimulation. Again, pretreatment with AWD 12-281 resulted in an overall reduction of the content of measured cytokines and IC50 values could be determined for AWD 12-281. The compound was very effective in suppressing the release of IL-1β (IC50 29 ± 7 nmol L^{-1}), IL-2 (IC50 23 ± 4 nmol L^{-1}), IL-4 (IC50 29 ± 4 nmol L^{-1}), IL-5 (IC50 42 ± 13 nmol L^{-1}), IL-13 (IC50 77 ± 24 nmol L^{-1}), TNF-α (IC50 84 ± 19 nmol L^{-1}) and IFN-γ (IC50 49 ± 16 nmol L^{-1}). The estimated IC50 values for the cytokines IL-6, IL-8, IL-10 and IL-12 were between 110 and 189 nmol L^{-1} (Table 2).

Table 2 IC50 values of AWD 12-281 on cytokine release from human peripheral blood mononuclear cells (PBMCs) being stimulated with anti-CD3/anti-CD28 (0.5 μg mL^{-1}/1 μg mL^{-1})

Cytokine	IC50 (nmol L^{-1})
IL-1β	29 ± 7
IL-2	23 ± 4
IL-4	29 ± 4
IL-5	42 ± 13
IL-6	159 ± 91
IL-8	189 ± 111
IL-10	110 ± 12
IL-12 (p70)	135 ± 100
IL-13	77 ± 24
TNF-α	84 ± 19
IFN-γ	49 ± 16

Data are given as mean ± s.e.m. of 4 assays.

Dose–response and duration of action of AWD 12-281

To evaluate the dose–response effect of AWD 12-281 as well as the duration of action, the arachidonic-acid-induced mouse ear oedema model was used. Administration of arachidonic acid in vehicle-treated control mice induced a mean increase in ear thickness of 0.103 ± 0.028 mm 60 min after administration. Topical administration of AWD 12-281 5 h before arachidonic acid provocation dose dependently reduced the ear swelling. The minimum effective concentration of AWD 12-281 capable of significantly reducing the inflammatory reaction was 0.3%. AWD 12-281 5% was as effective as indometacin 1% (Figure 3).

To evaluate the duration of action after one single administration, AWD 12-281 (3%) was applied onto the mice ears at varying time points before arachidonic acid provocation.

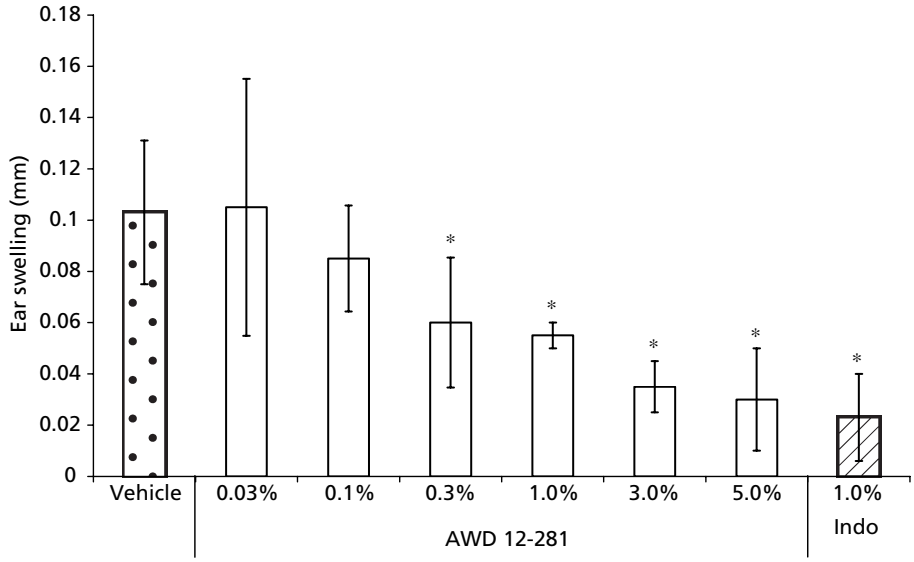

Figure 3 Effects of topical treatment with AWD 12-281 and indometacin on arachidonic-acid-induced ear swelling in mice. Bars represent mean ear swelling (± s.d.) at 60 min after induction of inflammation. Vehicle (dotted bars), AWD 12-281 (white bars) and indometacin (Indo, hatched bars) were applied topically to mouse ears 5 h before administration of arachidonic acid. $*P < 0.05$, compared with vehicle, n = 6.

While a similar level of anti-inflammatory effect was found if the compound was administered 2 h, 5 h or 24 h before arachidonic acid provocation, the effect was reduced but still significant for the time points 36 and 48 h pre-treatment indicating that a single administration resulted in a pharmacological effect lasting more than 24 h (Figure 4). To further support the long duration of action, low concentrations of AWD 12-281 (i.e. 0.03% or 0.1%) were repetitively applied once daily on 4 consecutive days. While repeated administration of vehicle had no effect on ear swelling, both concentrations of AWD 12-281, being inactive after a single administration, significantly reduced the ear swelling if tested 5 h after the fourth administration (Figure 5).

Discussion

Systemic bioavailability of PDE4 inhibitors includes the risk of inducing side effects, like nausea, vomiting and headache (Dyke & Montana 2002). One of the strategies

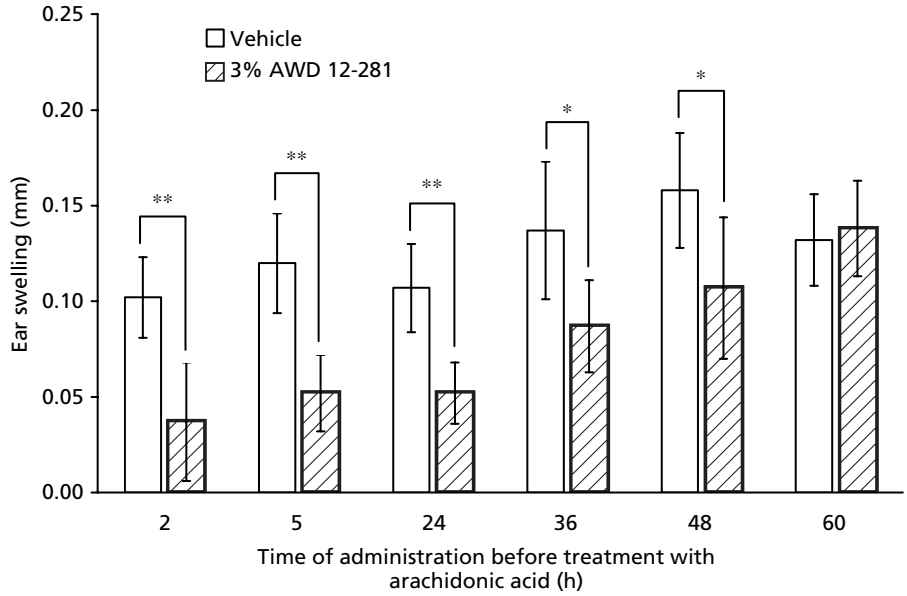

Figure 4 Effect of topical treatment with AWD 12-281 on arachidonic-acid-induced ear swelling in mice. Bars represent mean ear swelling (± s.d.) at 60 min after induction of inflammation. Mice were treated with 3% AWD 12-281 (hatched bars) or its vehicle (white bars) up to 60 h before induction of inflammation. $*P < 0.05$, $**P < 0.01$ compared with vehicle at corresponding pretreatment times, n = 6.

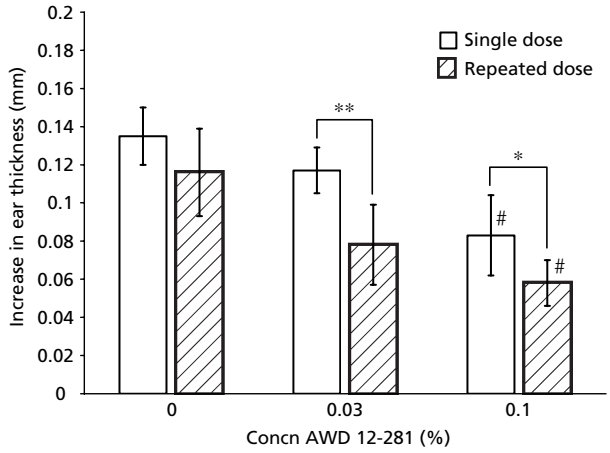

Figure 5 Effect of topical treatment with AWD 12 -281 on arachidonic-acid-induced ear swelling in mice. Bars represent mean ear swelling (± s.d.) at 60 min after induction of inflammation. Mice were treated with 0%, 0.03% or 0.1% AWD 12-281 as a single dose (white bars) 5 h before induction of inflammation or as repeated dose once daily (hatched bars) on four consecutive days (last treatment 5 h before administration of arachidonic acid). $*P < 0.05$, $**P < 0.01$, $\#P < 0.05$ compared with respective vehicle, n = 6.

to overcome this is to optimize a compound for topical administration. AWD 12-281 is currently under development as such a compound. Several factors contribute to this profile, the most important ones being very low oral bioavailability and extensive glucoronidation of the compound after absorption. A third important feature needed is long persistence in target tissue after topical administration. Indeed, AWD 12-281 was shown to have these features if administered as dry powder inhalation (Kuss et al 2002, 2003). While previous work has focused mainly on inhalative administration, similar features are not only needed for dermal administration to prevent systemic toxicity, but at the same time are also expected to be delivered by AWD 12-281. However, to be effective AWD 12-281 has to penetrate into the affected tissue. While for respiratory indications penetration into lung tissue after inhalation may be predictive for man, the situation is different for the skin. The stratum corneum, being the main barrier for skin penetration, varies grossly over the species. As the thickness of the stratum corneum is $8.8 \pm 1.0\,\mu$m in hairless mouse and $18.2 \pm 3.3\,\mu$m in human skin, mouse skin bears a poor resemblance to human skin, whereas the thickness of the stratum corneum in guinea-pigs ($18.6 \pm 1.2\,\mu$m) is similar to that in man (Sato et al 1991). Priborsky & Muhlbachova (1990) examined the permeability characteristics of human and laboratory animal skin. They demonstrated that skin permeability in rats is much higher than in man. In contrast, the permeability of human and guinea-pig skin was not significantly different in their experiments. For these reasons we developed a model for studying percutaneous penetration of AWD 12-281 in guinea-pigs. In this model, allergen was administered intracutaneously while drug treatment was performed topically. Therefore, inhibition of the development of allergic reaction following allergen challenge requires drug penetration through the stratum corneum. In a first experiment, the anti-inflammatory effect of AWD 12-281 was compared with that of the steroid diflorasone diacetate. As vehicle, a DMSO–acetone solution was used. As the nature of the model is rather severe, even the potent steroid diflorasone diacetate was unable to completely suppress the inflammatory response. Therefore, the concentration-dependent effect of AWD 12-281, although less potent than diflorasone, is very encouraging and indicates the potent activity of this compound. However, as DMSO alters the barrier properties of the stratum corneum, this may have altered the skin penetration of AWD 12-281. For this reason a second experiment was performed using a DMSO-free water-in-oil cream as a galenical formulation for topical administration of AWD 12-281. In this second experiment, AWD 12-281 was also able to significantly reduce the allergic reaction following topical administration in this formulation. The conclusion of this guinea-pig study is that AWD 12-281 is likely to penetrate through human skin after topical administration in a DMSO-free simple water-in-oil cream formulation. The secondary aim was the evaluation of a preference for Th1- or Th2-type cytokines by AWD 12-281. Some evidence can be taken as a hint that PDE4 inhibitors may lead to a preferential inhibition of Th2 responses, because Th1 cells showed reduced gene expression for the PDE4C and PDE4D isoforms (Essayan et al 1997). In contrast, published data document that PDE4 inhibitors predominantly suppress Th1-mediated immune responses in man (Bielekova et al 2000; Claveau et al 2004). Determination of cytokines in atopic dermatitis patients points out that both Th1 and Th2 mediated cytokines are involved in the pathology of the disease (Toda et al 2003). From these data it was concluded that atopic dermatitis comprises at least two different allergic responses. The Th2-type allergen-specific reaction is important in the onset of the disease, whereas the chronic phase characterized by maintenance and aggravation is a predominantly Th1-type immune response (Grewe et al 1998; Spergel et al 1999). Therefore, drugs for the treatment of atopic dermatitis should suppress both Th1- and Th2-mediated reactions (Inoue at al 2003).

In this study, the effect of AWD 12-281 on Th1- and Th2-type cytokines was investigated in tissue homogenates of TDI challenged mouse ears. The contact allergen TDI is capable of inducing IgE-dependent or IgE-independent allergic dermatitis. According to the sensitization regime the allergen response to TDI challenge in sensitized mice can be predominantly a Th1 or a Th2 cell driven process (Scheerens et al 1999). Bäumer et al (2003) demonstrated that the expression of cytokines like IL-1β, IL-4, IL-6 and MIP-2 is increased in TDI-challenged ears of sensitized mice. Topical administration of 3% AWD 12-281 reduced the amount of these cytokines in TDI-treated ears significantly. The inhibitory effect of AWD 12-281 on the expression of IL-4 and IL-6 was confirmed in our experiments. Furthermore, the expression of the growth, differentiation and activating factor for neutrophilic granulocytes and their progenitors, G-CSF, was decreased by

80% in TDI-challenged ears being pretreated with 3% AWD 12-281. Additionally, topical administration of AWD 12-281 reduced the expression of IL-1β in TDI-challenged ears by 87%. The chemokines RANTES, MIP-1α and KC were suppressed in TDI-challenged and AWD 12-281-treated ears. These chemokines have potential roles in allergic diseases (Kaburagi et al 2001). The conclusion of the cytokine analysis in ear-tissue homogenates of TDI-challenged mouse ears is that AWD 12-281 is capable of suppressing the release of both Th1- and Th2-type cytokines. These results are in line with cytokine measurements from anti-CD3/anti-CD28-activated PBMCs being pretreated with AWD 12-281. The functional responses following anti-CD8/anti-CD28 stimulation of T cells are comparable with antigen-induced responses in vivo. However, contrast to the in-vivo situation where antigens stimulate only specific T cells, anti-CD3 antibodies stimulate all T cells in a mixed population (Tiefenthaler & Hunig 1989). Human PBMCs following stimulation with anti-CD3 and co-stimulation with anti-CD28 released a broad spectrum of both Th1- and Th2-type cytokines. Pretreating cells with AWD 12-281 suppressed the release of both Th1- and Th2-type cytokines in anti-CD3/anti CD28-stimulated PBMCs. No preferred effect on either Th1- or Th2-type cytokines was observed. The IC50 for IL-2, IFNγ, IL-4 and IL-5 values were within a narrow range of 23–49 nmol L^{-1}. The effective inhibition of the release of Th1 and Th2 cytokines in the ex-vivo, as well as in the in-vitro, experiment underlines the likelihood of efficacy of AWD 12-281 in the treatment of the acute and chronic phase of atopic dermatitis.

A further aim of these studies was to characterize the dose–response relationship and duration of action of AWD 12-281 to see whether a long-lasting effect could be achieved, thus permitting topical treatment despite the rapid hepatic glucuronidation after absorption, which limits the possibility of systemic effects. The data generated indicate that the duration of action of a single administration clearly exceeded 24 h and that multiple administration resulted in a potentiation of the pharmacological effect. Despite the fact that we used the mouse for these experiments, these data are indicative that a long duration of action can be also expected in man, since the stratum corneum, being the main diffusion barrier and at the same time the reservoir for active drug, is thicker in man, allowing a build up of an even greater reservoir in the skin compared with mice.

Conclusion

The results show that AWD 12-281 is a very effective anti-inflammatory substance with outstanding properties concerning topical administration. As the substance suppressed the allergic response in the OVA guinea-pig model it can be predicted that AWD 12-281 penetrates human skin after topical administration.

Concerning the absence of a T cell subtype preference, it is expected that AWD 12-281 is effective in the acute and in the chronic phase of atopic dermatitis. Furthermore, the long-lasting effect following single administration and the enhanced anti-inflammatory efficacy after repeated administration indicate the storage of AWD 12-281 in the stratum corneum. Moreover, the topical administration provides a way of limiting systemic exposure and unwanted side effects. As clinical efficacy of PDE4 inhibitors has been demonstrated with cipamfylline and CP80633 (Hanifin et al 1996; Griffiths et al 2002), it is in all likelihood that topical treatment with AWD 12-281 is effective in patients with atopic dermatitis.

References

Bäumer, W., Gorr, G., Hoppman, J., Ehinger, A. M., Ehinger, B., Kietzmann, M. (2002) Effects of the phosphodiesterase 4 inhibitors SB 207499 and AWD 12-281 on the inflammatory reaction in a model of allergic dermatitis. *Eur. J. Pharmacol.* **446**: 195–200

Bäumer, W., Gorr, G., Hoppmann, J., Ehinger, A. M., Rundfeldt, C., Kietzmann, M. (2003) AWD 12-281, a highly selective phosphodiesterase 4 inhibitor, is effective in the prevention and treatment of inflammatory reactions in a model of allergic dermatitis. *J. Pharm. Pharmacol.* **55**: 1107–1114

Bielekova, B., Lincoln, A., Mc Farland, H., Martin, R. (2000) Therapeutic potential of phosphodiesterase-4 and -3 inhibitors in Th1-mediated autoimmune diseases. *J. Immunol.* **164**: 1117–1124

Claveau, D., Chen, S. L., O'Keefe, S., Zaller, D. M., Styhler, A., Liu, S., Huang, Z., Nicholson, D. W., Mancini, J. A. (2004) Preferential inhibition of T helper 1, but not T helper 2, cytokines in vitro by L-826,141, a potent and selective phosphodiesterase 4 inhibitor. *J. Pharmacol. Exp. Ther.* **310**: 752–760

Derks, M. G. M., van den Berg, B. T. J., van der Zee, J. S., Braat, M. C. P., Boxtel, C. J. (1997) Biphasic effect-time courses in man after formoterol inhalation: eosinopenic and hypokalemic effects and inhibition of allergic skin reactions. *J. Pharmacol. Exp. Ther.* **283**: 824–832

Dyke, H. J., Montana, J. G. (2002) Update on the therapeutic potential of PDE4 inhibitors. *Expert Opin. Invest. Drugs* **11**: 1–13

Essayan, D. M., Kagey-Sobotka, A., Lichtenstein, L. M., Huang, S. K. (1997) Differential regulation of human antigen-specific Th1 and Th2 lymphocyte responses by isoenzyme selective cyclic nucleotide phosphodiesterase inhibitors. *J. Pharmacol. Exp. Ther.* **282**: 505–512

Evilevitch, V., Wu, T. T., Lindgren, L., Greiff, L., Norrgren, K., Wollmer, P. (1999) Time course of the inflammatory response to histamine and allergen skin prick test in guinea pigs. *Acta Physiol. Scand.* **165**: 409–413

Ferrer, L., Alberola, J., Queralt, M., Brazis, P., Rabanal, R., Llenas, J., Puigdemont, A. (1999) Clinical anti-inflammatory efficacy of arofylline, a new selective phosphodieterase-4 inhibitor, in dogs with atopic dermatitis. *Vet. Rec.* **145**: 191–194

Gad, S. C., Dunn, B. J., Dobbs, D. W., Reilly, C., Walsh, R. D. (1986) Development and validation of an alternative dermal sensitization test: the mouse ear swelling test (MEST). *Toxicol. Appl. Pharmacol.* **84**: 93–114

Grewe, S., Chan, S. C., Hanifin, J. M. (1982) Elevated leukocyte cyclic AMP-phosphodiesterase in atopic disease: a possible mechanism for cyclic AMP-agonist hypo responsiveness. *J. Allergy Clin. Immunol.* **70**: 452–457

Grewe, M., Bruijnzeel-Koomen, C. A. F. M., Schöpf, E., Thepen, T., Langeveld-Wildschut, A. G., Ruzicka, T., Krutman, J. (1998) A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol. Today* **19**: 359–361

Griffiths, C. E. M., Van Lent, E. J. M., Gilbert, M., Traulsen, J. (2002) Randomized comparison of the type 4 phosphodiesterase 4 inhibitor cipamfylline cream, cream vehicle and hydrocortisone 17-butyrate cream for the treatment of atopic dermatitis. *Br. J. Dermatol.* **147**: 229–307

Gummer, C. L., Maibach, H. I. (1986) Animal models for percutaneous penetration: assessing contamination of the experimental system. *Br. J. Dermatol.* **115**: 335–339

Hanifin, J. M., Chan, S. C., Cheng, S. B., Tofte, S. J., Henderson, W. R., Kirby, D. S., Weiner, E. S. (1996) Type 4 phosphodiesterase inhibitors have clinical and *in vivo* anti-inflammatory effects in atopic dermatitis. *J. Invest. Dermatol.* **107**: 51–56

Inoue, Y., Isobe, M., Hayashi, H. (2003) The combined effect of topical CX-659S, a novel diaminouracil derivative, with topical corticosteroid on the three types of allergic responses in mice and guinea pigs. *J. Pharmacol. Sci* **91**: 71–78

Kaburagi, Y., Shimada, Y., Nagaoka, T., Hasegawa, M., Takehara, K., Sato, S. (2001) Enhanced production of CC-chemokines (RANTES, MCP-1, MIP-α, MIP-1β, and eotaxin) in patients with atopic dermatitis. *Arch. Dermatol. Res.* **293**: 350–355

Kuss, H., Hoefgen, N., Egerland, U., Heer, S., Marx, D., Szelenyi, I., Schupke, H., Gasparic, A., Olbrich, M., Hempel, R. (2002) AWD 12–281: antiasthmatic/antiinflammatory phosphodiesterase 4 inhibitor. *Drugs Future* **27**: 111–116

Kuss, H., Hoefgen, N., Johanssen, S., Kronbach, T., Rundfeldt, C. (2003) In vivo efficacy in airway disease models of N-(3,5-dichloro-pyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indole-3-yl]-glyoxylic acid amide (AWD 12–281), a selective phosphodiesterase 4 inhibitor for inhaled administration. *J. Pharmacol. Exp. Ther.* **307**: 373–385

Priborsky, J., Muhlbachova, E. (1990) Evaluation of in-vitro percutaneous absorption across human skin and in animal models. *J. Pharm. Pharmacol.* **42**: 468–472

Rao, T. S., Currie, J. L., Shaffer, A. F., Isakson, P. C. (1993) Comparative evaluation of arachidonic acid (AA)- and tetradecanoylphorbol acetate (TPA)-induced dermal inflammation. *Inflammation* **17**: 723–741

Sato, K., Sugibayashi, K., Morimoto, Y. (1991) Species differences in percutaneous absorption of nicorandil. *J. Pharm. Sci.* **80**: 104–107

Scheerens, H., Buckley, T. L., Muis, T. L., Garssen, J., Dormans, J., Nijkamp, F. P. and van Loveren, H. (1999) Long-term topical exposure to toluene di-isocyanate in mice leads to antibody production and in vivo airway hyperresponsiveness three hours after intranasal challenge. *Am. J. Respir. Crit. Care Med.* **159**: 1074–1080

Sharata H. H., Burnette R. R. (1988) Effect of dipolar aprotic permeability enhancers on the basal stratum corneum. *J. Pharm. Sci.* **77**: 27–32

Spergel, J. M., Mizoguchi, E., Oettgen, H., Bhan, A. K., Geha, R. F. (1999) Roles of Th1 and Th2 cytokines in a murine model of allergic dermatitis. *J. Clin. Invest.* **103**: 1103–1111

Tiefenthaler, G., Hunig, T. (1989) The role of CD2/LFA-3 interactions in antigen- and mitogen-induced activation of human T cells. *Int. J. Immunol.* **1**: 169–175

Toda, M., Leung, D. Y., Molet, S., Boguniewicz, M., Taha, R., Christodoulopoulos, P., Fukuda, T., Elias, J. A., Hamid, Q. A. (2003) Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. *J. Allergy Clin. Immunol.* **111**: 875–881