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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

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Abstract

AWD 12-281 (*N*-(3,5-dichloro-4-pyridinyl)-2-[1-(4-fluorobenzyl)-5-hydroxy-*1H*-indol-3-yl]-2-oxoacetamide), a phosphodiesterase 4 inhibitor, which is optimized for topical administration, was tested in a model of allergic dermatitis in mice. To obtain an allergic dermatitis, BALB/c mice were sensitized to toluene-2,4-diisocyanate (TDI). The allergic reaction was challenged by topical administration of TDI onto the mice ears. AWD 12-281 was tested for its anti-inflammatory potential by oral, intraperitoneal and topical administration. The phosphodiesterase 4 inhibitor, cilomilast (SB 207499), and/or the corticosteroid, diflorasone diacetate, were used as reference compounds. Given orally and intraperitoneally 2 h before as well as 5 and 24 h after TDI challenge, AWD 12-281 showed no, or only a transient inhibition of the allergen-induced ear swelling, whereas cilomilast significantly inhibited this ear swelling. Applied topically onto the ears before TDI challenge, AWD 12-281, cilomilast and diflorasone diacetate caused total inhibition of ear swelling 24 h after challenge, confirmed by a decrease of the pro-inflammatory cytokines interleukin-4, interleukin-6 and macrophage inflammatory protein-2. Administered topically after TDI challenge as therapeutic intervention, AWD 12-281 and diflorasone diacetate caused significant inhibition of ear swelling; cilomilast failed to do so. These results indicate that topically administered AWD 12-281 may be potent in the prevention and treatment of allergic/inflammatory skin diseases.

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Introduction

Phosphodiesterase (PDE) isoenzymes are involved in the regulation of cellular signal transduction cascades through the modulation of cyclic nucleotide levels. To date, 11 PDE isoenzyme gene families, differing in their cellular distribution and biochemical function, have been identified (Giembycz 2000). Leukocytes from patients with atopic dermatitis have abnormally high PDE4 activity (Butler et al 1983; Cooper et al 1985). PDE4 inhibitors exhibit very strong anti-inflammatory effects by an increase of the intracellular cAMP level. Through inhibition of cAMP degradation, PDE4 inhibitors modulate intracellular functions (e.g. attenuation of superoxide generation) and gene transcription (e.g. inhibition of synthesis and/or release of inflammatory cytokines) (Giembycz 2000; Hatzelmann & Schudt 2001; Kuss et al 2002). As PDE4 is also expressed in keratinocytes, these cells may be an additional potential pharmacological target by using PDE4 inhibitors for the control of inflammatory disorders in the skin (Chujor et al 1998).

Two PDE4 inhibitors were used in the present study. The PDE4 inhibitor cilomilast (SB 207499) was taken as a reference compound, as it is currently evaluated for the treatment of asthma (Griswold et al 1998; Giembycz 2000) and chronic obstructive pulmonary disease. Phase II/III clinical trials concerning chronic obstructive pulmonary disease have demonstrated a clinically significant improvement in lung function (Giembycz 2001; Dyke & Montana 2002). The PDE 4 inhibitor, AWD 12-281 (*N*-(3,5-dichloro-4-pyrid inyl)-2-[1-(4-fluorobenzyl)-5-hyd roxy-*1H*-indol-3-yl]-2-oxoa cetamide), was successfully examined in a model of allergic bronchoconstriction. It significantly

decrease the bronchospasmogenic effect of an allergen in passively sensitized human airways (Schmidt et al 2000). In-vivo, AWD 12-281 significantly reduced the accumulation of eosinophils in bronchoalveolar lavage in the late-phase airway reaction to antigen in sensitized Brown Norway rats. It also showed inhibitory effects in lipopolysaccharide-induced lung neutrophilia in domestic pigs (Kuss et al 2002).

We recently showed that the two PDE4 inhibitors, cilomilast (SB 207499) and AWD 12-281, were effective in the prevention of toluene-2,4-diisocyanate (TDI)-induced ear swelling, a model of allergic dermatitis (Bäumer et al 2002). This model was used as it has been demonstrated that TDI sensitization of the skin leads to Th2-type cytokine production in BALB/c mice (Dearman et al 1996). Activated lymph node cells from TDI-exposed animals produce substantial amounts of interleukin (IL)-4 and IL-10, but only low levels of interferon-γ. The contact allergen TDI can induce an IgE-independent or IgE-dependent allergic dermatitis. This depends on the duration of the sensitization (Scheerens et al 1999). As high IgE values are only obtained by long exposure sensitization, in one of the present experiments, sensitization was carried out over 120 days.

The present study was performed to test different routes of administration (parenteral vs topical application) and to compare the therapeutic effect of PDE4 inhibitors with preventive administration. The therapeutic effect was particularly important, as therapeutic intervention was considered to be closer to the clinical situation. Additionally, we intended to improve the model of TDI-induced ear swelling. For further characterization of the model, the cytokines IL-4, IL-6 and macrophage inflammatory protein 2 (MIP-2) were measured in challenged mouse ears. In contrast to our previous study (Bäumer et al 2002), where the cytokines IL-1β, IL-4 were measured at 4, 8, 16 h after challenge, in this study, we decided to measure the cytokines corresponding to the most distinct ear swelling (24 h after challenge).

Material and Methods

Materials

TDI was supplied by Sigma-Aldrich Chemie (Deisenhofen, Germany). AWD 12-281, ^{14}C AWD 12-281 and cilomilast were obtained from AWD (Dresden, Germany). Diflorasone diacetate was obtained from Basotherm (Biberach an der Riss, Germany). Acetone, PEG200 and dimethylsulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany), formaldehyde solution was from Fluka (Deisenhofen, Germany), miglyol and hydrocellulose were from Caesar & Loretz (Hilden, Germany), and RPMI 1640 medium was from Biochrom (Berlin, Germany). The enzyme-linked immunosorbent assays for the determination of cytokines were purchased from R&D Systems (Wiesbaden, Germany). Pefabloc was purchased from Boehringer Mannheim (Germany). The depilation cream (Veet) is a trademark of Reckitt & Colman (Hamburg, Germany). The adhesive tape (Tesafilm) was obtained from Beiersdorf (Hamburg, Germany). The protein content was measured using a Biorad assay (München, Germany).

Sensitization procedure

Female BALB/c-mice, 8 weeks old, 20 g, were obtained from Charles River (Sulzfeld, Germany). All animals were healthy and were housed in groups of six mice per cage at 22 °C with a 12-h light/dark cycle. Water and a standard diet (Altromin, Lage/Lippe, Germany) were freely available. The animal experiment was registered by Bezirksregierung Hannover, Germany (Az. 509i-42502-98A839). All procedures were carried out in agreement with the current version of the German Law on the Protection of Animals.

After settling in for 1 week, the abdominal skin of the mice was shaved and depilated with Veet. Subsequently, the horny layers of the abdominal skin were stripped off 10 times with adhesive tape. For active sensitization, 100 μL 5% TDI in acetone was administered to the stripped epidermis on 3 consecutive days.

The allergic reaction was boostered 21 days later by administration of 10 μL 0.5% TDI in acetone on both the inner and outer surfaces of the left ears (total volume of TDI: 20 μL) to examine the sensitization status. The ear thickness was measured with a cutimeter (Model 7309; Mitutoyo, Neuss, Germany) before, as well as 24 h after, TDI challenge. The swelling was calculated by comparison of the values before challenge with 24 h after challenge. Mice that had a mean difference in swelling of less than 20% at 24 h after challenge compared with the earlier assessed individual basal value (ca 230 μm) were excluded as being not sensitized ($< 5\%$ of all sensitized mice). The other mice were equally distributed to the treatment groups ($n = 6$) according to their swelling intensity, so that each group contained animals that had responded to varying degrees. The mice were allowed to rest until the ear thickness had reached almost a normal level after 7 days. To exclude residues of the allergen on the ears, the untreated right ears were used for the main experiment.

Oral application

At 2 h before, as well as 5 and 24 h after, TDI challenge, two groups of mice ($n = 6$ per group) were treated orally with AWD 12-281 or cilomilast (30 mg kg^{-1} suspended in 10 mL kg^{-1} miglyol). A third group was sham-treated orally with the vehicle (miglyol). The mice were challenged with 10 μL 0.5% TDI on both the inner and outer surfaces of the right ears. The ear thickness was measured before, as well as 6, 24 and 48 h after, TDI challenge.

Intraperitoneal application

At 2 h before, as well as 5 and 24 h after, TDI challenge, two groups of mice ($n = 6$) were treated intraperitoneally with AWD 12-281 or cilomilast (30 mg kg^{-1} suspended in 10% PEG200 and 0.5% hydrocellulose, 10 mL kg^{-1}). The animals treated orally with miglyol (see above) served as the control group for this experiment. The mice were challenged with

10 μL 0.5% TDI on both the inner and outer surfaces of the right ears. The ear thickness was measured before, as well as 6, 24 and 48 h after, TDI challenge.

Topical application

One group of mice (n = 6) was not sensitized and challenged. A second, sensitized group (n = 12) was treated with acetone/DMSO (9:1; 10 μL on both the inner and outer surfaces). After 2 h, the mice were challenged by topical administration of 20 μL (10 μL on both the inner and outer surfaces) 0.5% TDI in acetone onto the right ears. At 2 h before TDI challenge, a third and fourth group (each n = 6) were treated additionally with 20 μL (10 μL on both the inner and outer surfaces) AWD 12-281 (1% = 200 μg) and 20 μL cilomilast (3% = 600 μg) (each in acetone/DMSO 9:1) onto the right ears, respectively. Diflorasone diacetate (20 μL, 0.05% = 10 μg in acetone) 2 h before TDI challenge served as a corticoid positive control (n = 6). The dose of 0.05% is a common dosage used in therapy of allergic dermatitis (Packman 1992).

These mice were killed by cervical dislocation 24 h after TDI challenge and the ears were collected (see below).

To simulate therapeutic conditions, three additional groups (n = 6) received AWD 12-281 (1%), cilomilast (3%) or diflorasone diacetate (0.05%) topically 1 h after TDI challenge, that is directly after measurement of the ear thickness. The ear thickness was also quantified 5 and 24 h after TDI challenge.

To study dose dependency, the therapeutic intervention was repeated with a higher dose of cilomilast (10%) and AWD 12-281 (3%).

Five weeks after the first challenge, the animals treated therapeutically with AWD 12-281 (1%) and cilomilast (3%) were challenged again. Before challenge, one group was treated with AWD 12-281 (3%). These mice were killed before the determination of cytokines in the ears.

An experiment was carried out to obtain information on the therapeutic effects of AWD 12-281 in a model of long-term exposure to TDI. Ten mice were treated with 100 μL 0.5% TDI on the abdominal skin at intervals of 10 days for 120 days. Ten days after the last TDI treatment, the mice were challenged with 20 μL 0.5% TDI on the left ears, 10 μL on the outer surface and 10 μL on the inner surface. At 1 h after TDI challenge, five mice were treated with AWD 12-281 (3%) and five mice were sham-treated with the vehicle (acetone/DMSO, 9:1). The ear thickness was measured before, as well as 1, 5 and 24 h after, challenge.

Samples of the collected ears were fixed in 4% formaldehyde for histological section (paraffin-embedded, and stained with haematoxylin–eosin) to determine dermal thickness with respect to granulocyte accumulation. The analysis was performed semiquantitatively (− = no inflammatory cell influx; +++ = high inflammatory cell influx). These parameters were measured in 10 high-powered fields at 40× magnification in a blinded examination. The remaining tissue was snap-frozen and stored in liquid nitrogen immediately after sampling. For the determination of biochemical parameters, the mice ears were homogenized (pulverized) under liquid nitrogen. The homogenates were taken up in 200 μL RPMI 1640 medium, the protease inhibitor Pefabloc (1 mmol) was added and the samples were well mixed. After centrifugation (10 000 g, 10 min, 4 °C), the supernatant was collected and the protein content was quantified. The samples were stored at −80 °C until the cytokines were determined. IL-4, IL-6 and MIP-2 were measured in the samples by enzyme-linked immunosorbent assay using commercially available kits according to manufacturer's instructions.

Penetration of ^{14}C AWD 12-281 through murine back skin

The ability of ^{14}C AWD 12-281 to penetrate murine back skin was tested in a diffusion cell (Franz cell). Dry, shaved murine back skin was set onto the diffusion cell so that 1.5 cm (diameter) of the dermal side was in contact with warmed (34 °C) buffer (bovine serum). Then, 15 μL (110 322 970 d min^{-1}) of ^{14}C AWD 12-281 (dissolved in acetone/DMSO, 1:1) was applied to the shaved skin. Samples were taken after 15, 60, 120, 180, 240, 300, 360 min and the radioactivity was measured in a beta-counter (Beckman, München, Germany). The experiment was performed twice.

Statistical analysis

Results are presented as mean ± s.e.m. A one-way analysis of variance on ranks was performed followed by a multiple comparison method (Dunn's test). For comparison of two different groups, the Mann-Whitney test U-test was used.

Results

Oral application

At 6, 24 and 48 h after TDI challenge, the vehicle-treated TDI-challenged mice showed a mean increase of 27%, 40% and 46% in ear thickness. Cilomilast significantly inhibited this increase at all measured times, whereas AWD 12-281 had no significant effect on the ear thickness (Figure 1).

Intraperitoneal application

After intraperitoneal application, the increase in ear thickness was inhibited by approximately 50% by AWD 12-281 24 h after the challenge. This effect was attenuated after 48 h. Cilomilast showed a nearly 70% inhibition of swelling 24 h after the challenge. A significant reduction in ear swelling had already occurred after 6 h. However, there was no inhibitory effect 48 h after challenge. In contrast to cilomilast, AWD 12-281 was not able to inhibit ear swelling 6 h after the challenge (Figure 2).

Penetration of ^{14}C AWD 12-281 through murine back skin

In both experiments, an increase in radioactivity was measured in the buffer, indicating cutaneous penetration of AWD 12-281 through mouse skin. The amount of

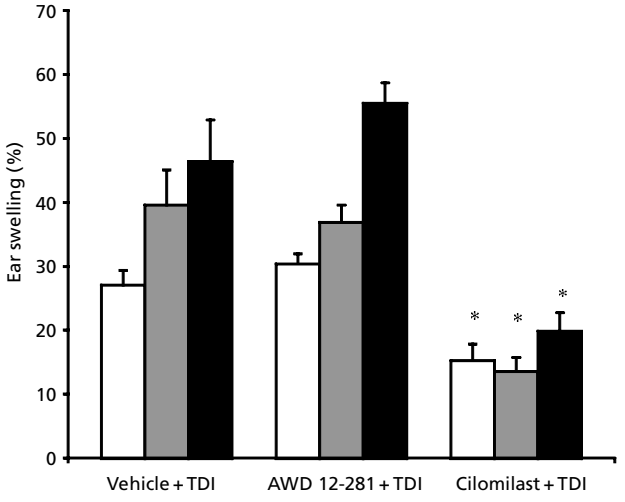

Figure 1 Effect of oral treatment with AWD 12-281 and cilomilast (SB 207499) in mice sensitized to TDI. Bars represent mean ear swelling (± s.e.m.) at 6 (white bars), 24 (grey bars) and 48 h (black bars) after TDI challenge. AWD 12-281 and cilomilast were administered orally 2 h before, as well as 5 and 24 h after, TDI challenge (30 mg kg^{-1}). Cilomilast significantly reduced the TDI-induced ear swelling. *$P < 0.05$, n = 6.

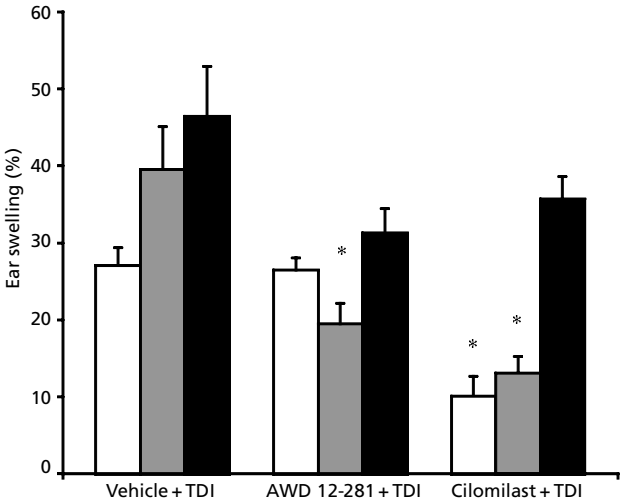

Figure 2 Effect of intraperitoneal treatment with AWD 12-281 and cilomilast (SB 207499) in mice sensitized to TDI. Bars represent mean ear swelling (± s.e.m.) at 6 (white bars), 24 (grey bars) and 48 h (black bars) after TDI challenge. AWD 12-281 and cilomilast were administered intraperitoneally 2 h before, as well as 5 and 24 h after, TDI challenge (30 mg kg^{-1}). Cilomilast significantly reduced the TDI-induced ear swelling 6 and 24 h after TDI challenge; AWD 12-281 significantly reduced the swelling 24 h after TDI challenge. *$P < 0.05$, n = 6.

radioactivity measured 360 min after application was 0.22% and 0.08% of the total activity (Figure 3).

Topical administration

The control mice showed a mean increase of approximately 30% (75 μm), 20% (50 μm) and 60% (146 μm) in

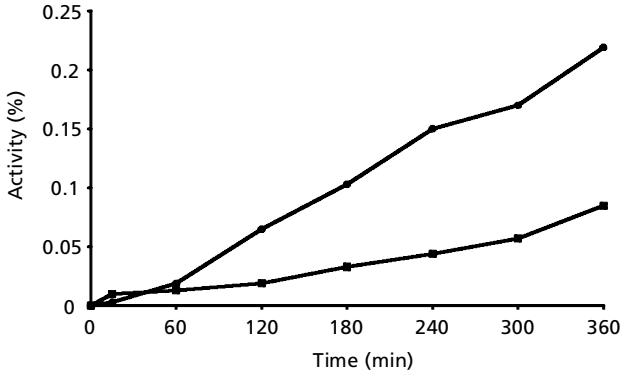

Figure 3 Cutaneous permeation of ^{14}C AWD 12-281 measured using a Franz diffusion cell and murine back skin. Activity of ^{14}C AWD 12-281 was measured in plasma during 360 min incubation. ^{14}C AWD 12-281 (15 μL, 110 322 970 d min^{-1}, in acetone/DMSO, 1:1) was applied to the shaved skin. Results of two independent experiments.

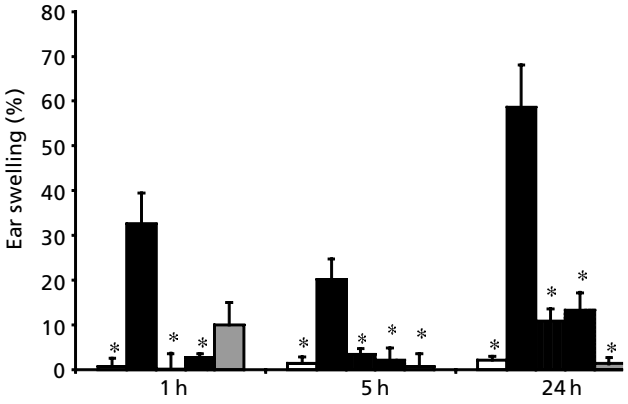

Figure 4 Effect of a single topical application of AWD 12-281, cilomilast and diflorasone onto the ears of mice sensitized to TDI 2 h before TDI challenge (preventive topical treatment). Bars represent mean ear swelling (± s.e.m.) 1, 5 and 24 h after TDI challenge. There was a significant increase of the ear swelling in TDI-treated mice (black bars) compared with untreated controls (white bars). AWD 12-281 (1%, vertical hatched bars) and cilomilast (3%, cross hatched bars) significantly inhibited the swelling at all measured times. Diflorasone (0.05%, grey bars) significantly inhibited the swelling 5 and 24 h after challenge. *$P < 0.05$ compared with TDI-treated control animals, n = 6 (TDI = 12) in each group.

ear thickness at 1, 5 and 24 h after TDI challenge. When administered 2 h before TDI challenge, topically administered AWD 12-281 (1%), cilomilast (3%) and diflorasone diacetate (0.05%) significantly inhibited the TDI-induced swelling at all measured times (Figure 4).

The groups used to test the therapeutic effect of AWD 12-281 showed swelling of between 25% and 30% 1 h after TDI challenge, that is directly before drug administration. No significant further swelling was observed 5 h after TDI challenge and the different treatment groups did not differ significantly. However, 24 h after challenge, AWD 12-281

as well as diflorasone diacetate significantly inhibited the TDI-induced ear swelling. Cilomilast (3%) induced a slight, but not significant, reduction in the swelling (Figure 5). Even a dose of 10% cilomilast did not result in statistically significant inhibition, whereas AWD 12-281 (3%) reduced the ear swelling to levels of untreated control ears (Table 1).

In the last experiment with long-term (120 days) sensitization, application of TDI onto the ears induced an increase in ear thickness of nearly 40% 1 h after challenge. Compared with the untreated control group 24 h after the challenge, the swelling was markedly inhibited by AWD 12-281 (Figure 6).

Table 1 Ear swelling (mean ± s.e.m.) in mice sensitized to toluene-2,4-diisocyanate (TDI) 1 and 24 h after TDI challenge. Cilomilast (10%) and AWD 12-281 (3%) were administered 1 h after the TDI challenge (therapeutic intervention).

	Vehicle	AWD 12-281 (3%)	Cilomilast (10%)
Ear swelling 1 h after TDI challenge	19 ± 5%	20 ± 8%	18 ± 6%
Ear swelling 24 h after TDI challenge	58 ± 12%	1 ± 1%*	26 ± 5%

*$P < 0.01$, n = 6.

Histological examination

The histological examination of the mouse ear skin 24 h after the TDI challenge showed distinct oedema and an influx of inflammatory cells (mainly granulocytes, score +++). In a previous study, a ratio of eosinophils to neutrophils of 1:5 was determined (Bäumer et al 2002). AWD 12-281, cilomilast and diflorasone diacetate markedly inhibited these inflammatory processes (granulocyte score −/+).

Inflammatory mediators in the mouse ear skin

The concentration of the cytokines was generally higher in ears of mice rechallenged 5 weeks after the first treatment. This was due to residues of inflammatory cells in the ear skin. The results (TDI compared with 3% AWD 12-281) are presented separately in Table 2.

There was a significant increase in IL-4 in TDI-treated mouse skin 24 h after the challenge (Figure 7A). AWD 12-281 (1%) showed a slight inhibitory effect, whereas 3% AWD 12-281 (Table 2), cilomilast (3%) and diflorasone diacetate (0.05%) significantly inhibited the increase.

The concentration of IL-6 was also significantly increased by TDI 24 h after challenge. AWD 12-281 (3%), cilomilast (3%) and diflorasone diacetate (0.05%) significantly inhibited this response. The inhibitory effect of AWD 12-281 (3%), cilomilast and diflorasone diacetate was comparable, whereas 1% AWD 12-281 showed only a slight effect (Figure 7B; Table 2).

MIP-2, a functional homologue of human IL-8, was also increased after TDI challenge. Enk & Katz (1992) reported an increase in MIP-2 mRNA in mice skin induced by the hapten trinitrochlorobenzene. Whereas 1% AWD 12-281 reduced this increase slightly, 3% AWD 12-281, cilomilast (3%) and diflorasone diacetate (0.05%) significantly diminished the increase of the MIP-2 concentration (Figure 7C; Table 2).

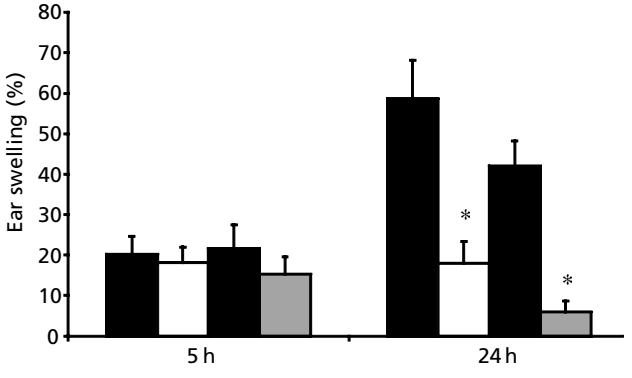

Figure 5 Effect of a single topical application of AWD 12-281, cilomilast and diflorasone onto the ears of mice sensitized to TDI 1 h after TDI challenge (therapeutic intervention). Bars represent mean ear swelling (± s.e.m.) 5 and 24 h after TDI challenge. There was no significant difference between the treated groups 5 h after the challenge. Compared with only TDI challenged control mice (black bars), AWD 12-281 (1%, white bars) and diflorasone (0.05%, grey bars) significantly inhibited the swelling 24 h after TDI challenge. Cilomilast (3%, cross hatched bars) caused no significant inhibition. *$P < 0.05$, n = 6 (TDI = 12) in each group.

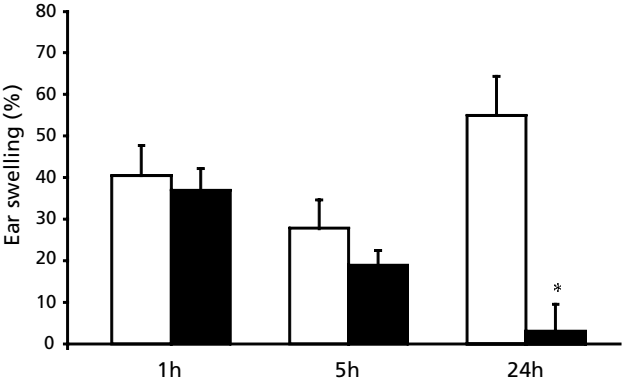

Figure 6 Effect of AWD 12-281 on TDI-induced ear swelling in mice sensitized to TDI using a long-term (120 days) repeated exposure sensitization paradigm. Bars represent ear swelling (± s.e.m.) 1, 5 and 24 h after TDI challenge. There was no significant difference between the groups at 1 and 5 h after the challenge. Compared with TDI-treated control mice (white bars), AWD 12-281 (3%, black bars) administered 1 h after TDI challenge significantly inhibited the swelling 24 h after the challenge. *$P < 0.05$, n = 5 in each group.

Table 2 Ear swelling 24 h after TDI challenge and mean values (±s.e.m.) of IL-4, IL-6 and MIP-2 (pg/400 μg protein) in homogenized mouse ears 24 h after TDI challenge.

	Challenge with TDI	
	Vehicle (Control)	AWD 12-281 (3%)
Ear swelling (%)	51 ± 7	1 ± 2*
IL-4	67 ± 2	12 ± 3*
IL-6	89 ± 5	42 ± 6*
MIP-2	286 ± 39	72 ± 8*

AWD 12-281 (3%) was administered 2 h before TDI challenge (preventive topical treatment). *$P < 0.01$ (n = 6 each group).

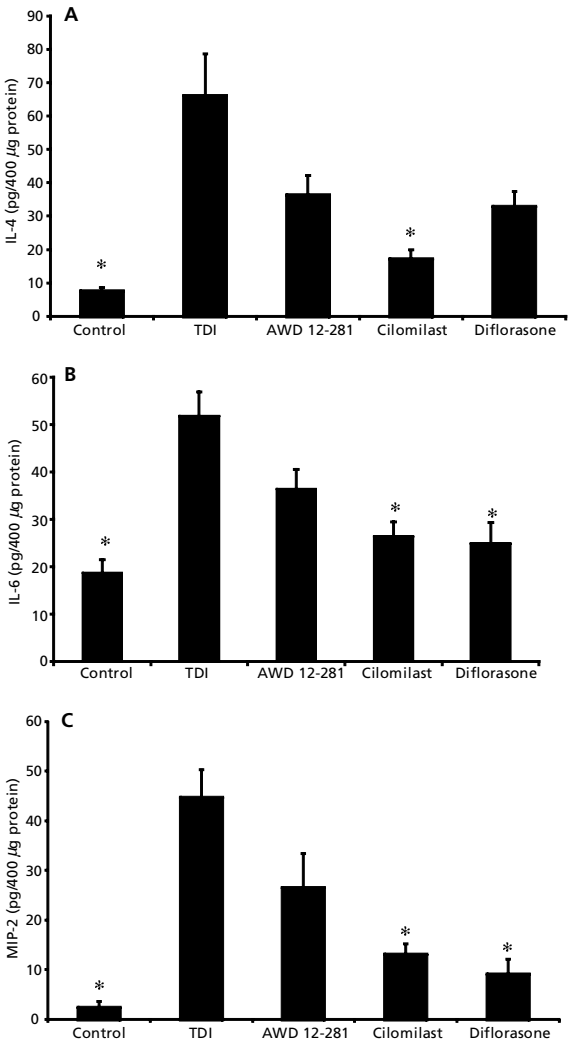

Figure 7 Mean concentration (± s.e.m.) of interleukin-4 (IL-4; A), interleukin-6 (IL-6; B) and macrophage inflammatory protein-2 (MIP-2; C) in homogenized mouse ears 24 h after TDI challenge. Samples were taken from mice in the experiment with preventive topical treatment (see Figure 4). *$P < 0.05$ compared with TDI-treated control animals, n = 6 (TDI = 12) in each group.

Discussion

This study has demonstrated the preventive and therapeutic effect of PDE4 inhibitors on TDI-induced mouse ear swelling via different routes of application, complementing our previous findings (Bäumer et al 2002). TDI, administered to the skin of sensitized mice, induces a predominantly Th2-type cytokine-mediated reaction with high amounts of IL-4 and IL-10 in activated lymph node cells from TDI-exposed animals, but only low levels of interferon-γ (Dearman et al 1996; Hayashi et al 2001). Our own results indicate that, besides IL-4, additional cytokines such as IL-1β (Bäumer et al 2002), IL-6 and MIP-2 are also involved in the allergic/inflammatory skin reaction to TDI. The increase in pro-inflammatory cytokines is accompanied by an influx of inflammatory cells such as neutrophils and eosinophils, and a distinct oedema. Therefore, ear swelling is useful as a functional parameter.

Orally administered AWD 12-281 had no inhibitory effect on the TDI-induced ear swelling. This is explained by the low oral bioavailability of less than 3% AWD 12-281 (Kuss et al 2002). In contrast, it was demonstrated that the oral bioavailability of cilomilast is sufficient to significantly reduce the inflammatory response (Figure 1). For cilomilast, the high oral bioavailability has already been shown in BALB/c mice (Griswold et al 1998) and confirmed in humans (Giembycz 2001).

The intraperitoneal application of AWD 12-281 and cilomilast results in a significant decrease in ear swelling 24 h after TDI challenge. Cilomilast also reduces this swelling 6 h after the challenge. Both substances failed to inhibit the swelling 48 h after TDI challenge, which may be due to metabolism. This is supported by the fact that topically administered AWD 12-281 and cilomilast inhibit the swelling until 48 h after only one administration 2 h before the challenge (Bäumer et al 2002).

As the oral bioavailability of AWD 12-281 is less than 3%, a skin permeability test was performed. In our experiment, AWD 12-281 was well absorbed and could penetrate through the skin. The observed cumulative absorption of 0.08% and 0.22% after 6 h amounts to a 10-fold higher absorption compared with hydrocortisone, measured for human skin in Franz diffusion cells (Hueber et al 1994). However, further experiments are needed to evaluate the skin permeability of AWD 12-281 using formulations without DMSO, which could have served as permeation enhancer. As the permeation test was a pilot study, a relatively high amount of DMSO was used (DMSO/acetone, 1:1). For the main experiments, the DMSO content was reduced to minimize the permeation enhancing effect (DMSO/acetone, 1:9), but a small amount of DMSO was necessary to maintain the solubility of the substances.

The results relating to topical treatment of the PDE4 inhibitors before TDI challenge confirm previous findings (Ehinger et al 2000; Bäumer et al 2002; Kietzmann et al 2002). In the present study, AWD 12-281 was administered in a lower dose to take into account the different IC50 values for PDE4. The IC50 of AWD 12-281 is about 10

times lower than that of cilomilast (Griswold et al 1998; Kuss et al 2002). Furthermore, a glucocorticoid control was added to compare the results with a reference compound.

In contrast to previous results, it was possible to obtain a more distinct positive control by modifying the sensitization protocol. By repeated use of the sensitization protocol described in this study, it was possible to reproduce and standardize the high response to TDI 24 h after challenge (mean swelling of 60% vs 25%). The magnitude of the response (60% or 146 μm 24 h after challenge) is comparable with, or even higher than, the swelling achieved with the frequently used contact sensitizer oxazolone in BALB/c mice (Särnstrand et al 1999; Wille et al 2000). Obviously, due to the high response at that time point, we were able to detect higher amounts of cytokines than in our previous study (Bäumer et al 2002). To get closer to clinical conditions, it was decided to examine effects of the PDE4 inhibitors in treating an allergic/inflammatory reaction that had already set in. Therefore, the PDE4 inhibitors were given 1 h after TDI challenge, where the beginning of inflammatory processes is reflected by a mean ear swelling of 30%. Although administered at a lower dose (1%), AWD 12-281 significantly reduced the inflammatory response to TDI 24 h after the challenge. This was confirmed in the study in which the effects of a long-term exposure to TDI were examined. One effect of this long-term exposure was an elevated ear swelling 1 h after challenge (Figure 6), which might be due to a more IgE-mediated response (Scheerens et al 1999). Administered at a higher concentration (3%), AWD 12-281 nearly abolished the TDI-induced swelling in this long-term exposure study (Table 1; Figure 6). The histological examination of the AWD 12-281-treated mice ears showed (in marked contrast to the positive control) nearly total absence of inflammatory cells and vascular leakage (result not shown).

To examine inflammatory mediators responsible for the TDI-induced ear swelling, the cytokines IL-4, IL-6 and MIP-2 were measured in treated mice ears. In acutely affected skin lesions of patients suffering from atopic dermatitis, IL-4 overproduction is apparent (Hanifin et al 1996; Spergel et al 1999). The inhibitory effect of cilomilast on IL-4 secretion in-vivo has also been demonstrated in a model of chronic oxazolone-induced contact sensitivity (Griswold et al 1998). The insufficient modulatory effect of AWD 12-281 (1%) is due to the low dose, as 3% AWD 12-281 results in 80% inhibition of IL-4 concentration (Table 3).

IL-6 is described as being elevated through TDI in-vitro (Mattoli et al 1991). IL-6 is secreted by keratinocytes after an inflammatory stimulus (McKenzie & Sauder 1990) and allergic reaction (Kondo et al 1994). Inhibition of IL-6 release by PDE4 inhibitors is also described for lipopolysaccharide-stimulated macrophages (Kambayashi et al 1995).

MIP-2 is a crucial cytokine for the chemotaxis of neutrophils produced by keratinocytes. Demonstrated here, and in a previous study (Bäumer et al 2002), TDI-induced ear swelling is accompanied by a vast influx of neutrophils. The inhibitory effect of cilomilast, diflorasone diacetate and AWD 12-281 (3%) may explain the reduced influx of neutrophils after treatment with a PDE4 inhibitor or a glucocorticoid.

Table 3 Comparison of the inhibitory effect (mean inhibition (%) of the TDI induced synthesis) of AWD 12-281 (3%), cilomilast (3%) and diflorasone (0.05%).

Cytokine	AWD 12-281 (3%)	Cilomilast (3%)	Diflorasone (0.05%)
IL-4	81	74	50
IL-6	52	49	52
MIP-2	75	66	76

In conclusion, our results suggest that AWD 12-281, as well as cilomilast, can inhibit inflammatory reactions in a model of allergic dermatitis. The anti-inflammatory response of AWD 12-281 is reliable when given via the topical route and there is greater inhibition by administration of 3% compared with 1% (Table 1; Figures 5 and 6). Although cilomilast (3%) also has inhibitory effects via oral and intraperitoneal routes, it lacks significant inhibitory effects when administered (3%, 10%) after the TDI challenge (Table 1; Figure 5). This lack of inhibition cannot be explained by a disadvantage in permeability due to the molecular weight of the compounds (cilomilast: MW 343.4; AWD 12-281: MW 458.2). Taking into account that the treatment of allergic reactions is in some situations more important than preventive administration, these data may indicate an advantage of AWD 12-281 in the therapeutic treatment of an allergic/inflammatory reaction in the skin. However, the permeability-enhancing effect of DMSO has to be taken into account.

There are two studies that demonstrate a clinical activity for two other PDE4 inhibitors (CP80633 and cipamfylline) in atopic dermatitis (Hanifin et al 1996; Griffiths et al 2002).

Therefore, it seems meaningful to continue studies relating to the treatment of allergic skin diseases with PDE4 inhibitors such as cilomilast and AWD 12-281.

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

Butler, J. M., Chan, S. C., Stevens, S. R., Hanifin, J. M. (1983) Increased leukocyte histamine release with elevated cyclic AMP-phosphodiesterase activity in atopic dermatitis. *J. Allergy Clin. Immunol.* **71**: 490–497

Chujor, S. N. C., Hammerschmid, F., Lam, C. (1998) Cyclic nucleotide phosphodiesterase 4 subtypes are differentially expressed by primary keratinocytes and human epidermoid cell lines. *J. Invest. Dermatol.* **110**: 287–291

Cooper, K. D., Kang, K. F., Chan, S. C., Hanifin, J. M. (1985) Phosphodiesterase inhibition by Ro 20-1724 reduces hyper-IgE synthesis by atopic dermatitis cells in vitro. *J. Invest. Dermatol.* **84**: 477–482

Dearman, R. J., Basketter, D. A., Kimber, I. (1996) Characterisation of chemical allergens as a function of divergent cytokine secretion profiles induced in mice. *Toxicol. Appl. Pharmacol.* **138**: 308–316

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

Ehinger, A. M., Gorr, G., Hoppmann, J., Telser, E., Ehinger, B., Kietzmann, M. (2000) Effects of the phosphodiesterase 4 inhibitor RPR 73401 in a model of immunological inflammation. *Eur. J. Pharmacol.* **392**: 93–99

Enk, A. H., Katz, S. I. (1992) Early molecular events in the induction phase of contact sensitivity. *Proc. Natl Acad. Sci. USA* **89**: 1398–1402

Giembycz, M. A. (2000) Phosphodiesterase 4 inhibitors and the treatment of asthma: where are we now and where do we go from here? *Drugs* **59**: 193–212

Giembycz, M. A. (2001) Cilomilast: a second generation phosphodiesterase 4 inhibitor for asthma and chronic obstructive pulmonary disease. *Expert Opin. Investig. Drugs* **10**: 1361–1379

Griffiths, C. E. M., Van Leent, E. J. M., Gilbert, M., Traulsen, J. (2002) Randomized comparison of the type 4 phosphodiesterase inhibitor cipamfylline cream, cream vehicle and hydrocortisone 17-butyrate cream for the treatment of atopic dermatitis. *Br. J. Dermatol.* **147**: 299–307

Griswold, D. E., Webb, E. F., Badger, A. M., Gorycki, P. D., Levandoski, P. A., Barnette, M. A., Grous, M., Christensen, S., Torphy, T. J. (1998) SB 207499 (Ariflo), a second generation phosphodiesterase 4 inhibitor, reduces tumor necrosis factor α and interleukin-4 production *in vivo*. *J. Pharmacol. Exp. Ther.* **287**: 705–711

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

Hatzelmann, A., Schudt, C. (2001) Anti-inflammatory and immunomodulatory potential of the novel PDE4 inhibitor roflumilast in vitro. *J. Pharmacol. Exp. Ther.* **297**: 267–279

Hayashi, M., Higashi, K., Kato, H., Kaneko, H. (2001) Assessment of preferential Th1 or Th2 induction by low-molecular-weight compounds using a reverse transcription-polymerase chain reaction method: comparison of two mouse strains, C57BL/6 and BALB/c. *Toxicol. Appl. Pharmacol.* **177**: 38–45

Hueber, F., Schaefer, H., Wepierre, J. (1994) Role of transepidermal and transfollicular routes in percutaneous absorption of steroids: in vitro studies on human skin. *Skin Pharmacol.* **7**: 237–244

Kambayashi, T., Jakob, C. O., Zhou, D., Mazurek, N., Fong, M., Strassmann, G. (1995) Cyclic nucleotide phosphodiesterase type IV participates in the regulation of IL-10 and in the subsequent inhibition of TNF-α and IL-6 release by endotoxin-stimulated macrophages. *J. Immunol.* **155**: 4909–4916

Kietzmann, M., Ehinger, A. M., Gorr, G., Hoppmann, J., Telser, E. (2002) Effects of phosphodiesterase-4 inhibitors in a model of allergic dermatitis. In: Thoday, K. L., Foil, C. S., Bond, R. (eds) *Advances in Veterinary Dermatology 4*. Blackwell Science, Oxford, pp. 196–201

Kondo, S., Pastore, S., Shivji, G. M., McKenzie, R. C., Sauder, D. N. (1994) Characterization of epidermal cytokine profiles in sensitization and elicitation phases of allergic contact dermatitis as well as irritant contact dermatitis in mouse skin. *Lymphokine Cytokine Res.* **13**: 367–375

Kuss, H., Höfgen, N., Egerland, U., Heer, S., Marx, D., Szelenyi, I., Schupke, A., Gasparic, M., Olbrich, M., Hempel, R., Hartenhauer, H., Krone, D., Berthold, K., Kronbach, T., Rundfeldt, C. (2002) AWD 12-281. *Drug Future* **27**: 111–116

Mattoli, S., Colotta, F., Fincato, G., Mezzetti, M., Mantovani, A., Patalano, F., Fascoli, A. (1991) Time course of IL1 and IL6 synthesis and release in human bronchial epithelial cell cultures exposed to toluene diisocyanate. *J. Cell Physiol.* **149**: 260–268

McKenzie, R. C., Sauder, D. N. (1990) The role of keratinocyte cytokines in inflammation and immunity. *J. Invest. Dermatol.* **95** (Suppl.) 105S–107S

Packman, A. M. (1992) Diflorasone diacetate. In: Maibach, H. I., Surber C. (eds) *Topical Corticosteroids*. Karger, Basel, pp. 403–414

Särnstrand, B., Jansson, A. -H., Matuseviciene, G., Scheynius, A., Pierrou, S., Bergstrand, H. (1999) *N*,*N*′-Diacetyl-L-cystine – the disulfide dimer of *N*-acetylcysteine – is a potent modulator of contact sensitivity/delayed type hypersensitivity reactions in rodents. *J. Pharmacol. Exp. Ther.* **288**: 1174–1184

Scheerens, H., Buckley, T. L., Muis, T. L., Garssen, J., Dormans, J., Nijkamp, F. P., Van Loweren, H. (1999) Long-term topical exposure to toluene diisocyanate 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

Schmidt, D. T., Watson, N., Dent, G., Rühlmann, E., Branscheid, D., Magnussen, H., Rabe, K. F. (2000) The effect of selective and non-selective phosphodiesterase inhibitors on allergen- and leukotriene C_4-induced contractions in passively sensitized human airways. *Br. J. Pharmacol.* **131**: 1607–1618

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

Wille, J. J., Kydonieus, A., Kalish, R. S. (2000) Inhibition of irritation and contact hypersensitivity by phenoxyacetic acid methyl ester in mice. *Skin Pharmacol. Appl. Skin Physiol.* **13**: 65–74