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Anti-inflammatory Potential of the Selective PDE4 Inhibitor N-(3,5-Dichloro-pyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indole-3-yl]-glyoxylic Acid Amide (AWD 12-281), in Human Cell Preparations

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Anti-inflammatory Potential of AWD 12-281 in Human Cells

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In the Material and Methods:	1229 words
In the Results:	604 words
In the Discussion:	1487 words

List of non-standard abbreviations:

COPD, chronic obstructive pulmonary disease; PDE, phosphodiesterase; cilomilast, SB 207499; RPR 73401, piclamilast; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide; BAL, bronchoalveolar lavage; BHR, bronchial hyperresponsiveness; PBS, phosphate buffered saline; FCS, fetal calf serum; TNFα, tumor necrosis factor α; IL, interleukin; PBMC, peripheral blood mononuclear cell; PMNL, polymorphonuclear leukocytes; ELISA, enzyme-linked immunosorbent assay;

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Abstract

AWD 12-281 is a potent (IC_{50} 9.7 nM) and highly selective inhibitor of the phosphodiesterase 4 (PDE4) isoenzyme with low affinity to the high-affinity rolipram-binding site. The compound was optimized for topical treatment of asthma, chronic obstructive pulmonary disease (COPD) and allergic rhinitis. The aim of the present study was to assess the effect of AWD 12-281 in human inflammatory cells. Peripheral blood mononuclear cells (PBMC), diluted whole blood and human nasal polyp cells derived from surgically resected nasal polyps from patients with polyposis comprise sources of target tissue cells that can be used to predict anti-inflammatory effects in patients. AWD 12-281 was capable of suppressing the production of cytokines in stimulated PBMC: Interleukin-2 (IL-2, phytohemagglutinin (PHA)-stimulation), IL-5 (concanavalin A (Con A) stimulation), IL-5 and IL-4 (anti-CD3/anti-CD28 co-stimulation) and LPS-stimulated release of tumor necrosis factor α (TNFα). The corresponding values for half-maximum inhibition, $EC_{50,}$ for AWD 12-281 were within a narrow range (46–121 nM). Comparing the effect of AWD 12-281 with roflumilast, cilomilast (SB 207499), rolipram, RPR-73401 and RS-253444-000, it could be shown that the PDE4 inhibitory activity was closely correlated with inhibitory potential as measured by the above assays. AWD 12-281 was also shown to suppress TNFα release in dispersed nasal polyps (EC_{50} 111 nM) and in diluted whole blood (EC_{50} 934 nM). The reduced activity in human blood may be related to high plasma protein binding. Currently, Phase-II clinical studies are under way to evaluate the therapeutic potential of AWD 12-281 in asthma, COPD and allergic rhinitis.

To date, 11 families of phosphodiesterases (PDEs) have been identified. These enzymes are critically involved in the modulation of signal transduction, and act by degradation of cyclic nucleotides (cAMP and/or cGMP). Among the PDEs, PDE4, PDE7 and PDE8 show specificity for cAMP (Soderling and Beavo 2000, Conti and Yin 1999). In the search for an anti-inflammatory treatment alternative to corticosteroids, selective PDE4 inhibitors have received considerable attention, because the PDE4 isoenzyme is the major cAMP-metabolizing enzyme in immune and inflammatory cells (Schmidt *et al*. 1999; Torphy *et al*. 1992). Selective inhibition of PDE4 has anti-inflammatory effects in patients with inflammatory diseases such as asthma or chronic obstructive pulmonary disease (COPD) (Barnes 2003; Compton *et al*. 2001; Leichtl *et al*. 2002).

Currently, two compounds, i.e. roflumilast and cilomilast, are in clinical development (Phase III) for the treatment of COPD and, in the case of roflumilast, for asthma as well. PDE4 inhibitors are potentially superior to corticosteroids in clinical use, since they not only induce anti-inflammatory effects but also influence diverse other cell types involved in these diseases; these cell types include respiratory epithelial cells, smooth muscle cells and sub-mucosal glands (Wright *et al*.1998, Le Jeune *et al*. 2002, Wenzel *et al*. 2003, Braunstahl *et al*. 2003). Furthermore, there are data indicating that PDE4 inhibitors of the new generation lack the side effects frequently associated with corticosteroid therapy, including *inter alia* adverse effects on the pituitary–hypophyseal axis and on bone density (Burnouf and Pruniaux 2002, Tattersfield *et al*. 2002, Leonard and Sur 2002, Cave *et al*. 1999). However, further side effects – such as nausea, vomiting, increased gastric acid secretion and headache – have so far limited the therapeutic use of PDE4 inhibitors (Montana and Hazel 2002).

AWD 12-281 is a structurally new and highly selective PDE4 inhibitor (Kuss *et al*, 2002, Marx *et al*, 2002, Kuss *et al*., 2003). This compound was shown to have a better safety profile than other PDE4 inhibitors in clinical development. Furthermore, it was designed for topical treatment. It has a low oral bio-availability and a low solubility, and it exerts strong and long-

lasting pharmacological effects after intratracheal administration in various animal models, indicating persistence in lung tissue. High plasma-protein binding and efficient hepatic metabolism by glucuronidation are additional factors contributing to low systemic exposure after intratracheal dosing. These factors contribute to a unique difference between emetic and anti-inflammatory dose levels – e.g., by a factor of >100 in ferrets (Kuss *et al*., 2003).

The aim of the present study was to assess the effect of AWD 12-281 in human inflammatory cells. The human cell preparations – PBMC, diluted whole blood and human nasal polyp cells derived from surgically resected nasal polyps in patients with polyposis – comprise sources of target tissue cells that can be used to predict anti-inflammatory effects in patients.

We selected the release of tumor necrosis factor alpha (TNFα) as marker for inflammatory processes in all cell preparations tested. In addition the release of the cytokines IL-2, IL-4 and IL-5 was determined in PBMC. TNFα is released by activated monocytes, macrophages and lymphocytes and the activity of TNFα is mediated through the binding to membrane-bound TNF receptors.

IL-4 is critically important for the development of allergic inflammation. It induces the IgE production of B lymphocytes (Coffman *et al*., 1986) and causes activation of the mast cells. IL-4 is essential to allergic inflammation because it is able to drive the differentiation of Th0 lymphocytes into Th2 lymphocytes (Hsieh *et al*., 1992). IL-5 plays a key role in the development and activation of eosinophils and therefore also in eosinophilic inflammation (Leckie and Walker, 2001). Th2 lymphocytes are the principal source of IL-5 (Ying *et al*., 1995). IL-2 is a cytokine derived from Th1 lymphocytes and is involved in the growth and differentiation of T cells and also in eosinophilic inflammation *in vivo*. Some of these data have already been published in abstract form (Heer *et al*. 1999, Heer *et al*. 2000, Kuesters *et al*. 1999).

Materials and Methods

Reagents and inhibitors

The following reagents were used: fetal calf serum (FCS), Dulbecco's phosphate-buffered saline (PBS), Hank's balanced salts solution, trypan blue solution, penicillin/streptomycin, Biocoll (Biochrom AG seromed, Germany); human immunoglobulin E (IgE) and anti-human IgE (Calbiochem, California, USA); RPMI 1640 medium, PBS (Gibco BRL, life technologies Ltd., Germany), Ficoll-Paque (Pharmacia,), [^{3}H] cAMP and SPA-beads (Amersham Biosciences, Freiburg, Germany), LPS (Sigma-Aldrich Chemie GmbH, Germany), anti-CD28 and IL-2, -4, -5, TNFα antibody pairs (PharMingen, Germany), IL-4, -5, TNFα: also kits with precoated plates (Coulter Immunotech Diagnostics, Germany). Anti-CD3 (Okt 3) was a gift from Thomas Noll, Forschungszentrum Jülich. Dexamethasone was obtained from Sigma-Aldrich Chemie GmbH, Germany. Dexamethasone (cristalline) was dissolved in DMSO and aliquots of the stock solution (10^{-2} M) were stored at –20°C.

All other reagents were purchased from standard laboratory suppliers including Sigma-Aldrich Chemie GmbH, Germany, and Merck, Germany. Rolipram, Cilomilast, RPR-73401, RS-25344-000, and Roflumilast were synthesized by the Chemical Department of Arzneimittelwerk Dresden GmbH, Radebeul, Germany (now elbion AG).

Blood from healthy volunteers was obtained in full compliance with ethical standards.

Determination of PDE4 activity in human PMNL (polymorphonuclear leukocytes)

Phosphodiesterase isoenzyme (PDE) activity was determined in human PMNL isolated from buffy coats. Peripheral blood from 12 healthy donors was collected and treated with 0.3% sodium citrate in PBS as anticoagulant. To remove the platelets, the cells were centrifuged at 700 g for 20 minutes at room temperature. After aspiration of the platelet-rich supernatant, the residue was topped up to its intial volume with 0.13% sodium citrate in PBS. A volume of 45 ml was mixed with 5 ml of 5% dextran and allowed to stand for 1 hour. After this dextran sedimentation, 20 ml of the supernatant was layered on the top of 15 ml of Ficoll-Paque and

centrifuged for 30 minutes at 500 g at room temperature. The sediments were re-suspended and washed twice with 50 ml of PBS by centrifugation at 500 g for 5 minutes at 4 °C. The remaining red blood cells were lysed in 10 ml of a hypotonic buffer (155 mM NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH=7.4) for 6 minutes at 4 °C. Afterwards, the cells were washed twice with 50 and 20 ml of PBS; this was followed by sonication of the cells. After centrifugation at 48000 g for 1 h the cytosolic PDE4 was present in the supernatant; this was stored at –70 °C.

PDE activity was determined in a one-step procedure in microtiter plates. The reaction mixture of 100 µl contained 50 mM Tris.HCl/5 mM $MgCl_2$ buffer (pH 7.4), 100 µM cGMP, 0.5 µM [^{3}H]-cAMP and the enzyme. Non-specific enzyme activity was tested in the presence of 100 µM rolipram. The enzyme reaction was started by the addition of the substrate solution and was continued at 37 °C for 30 minutes. It was stopped by the addition of 25 µl YSi-SPA beads. One hour later the radioactivity of the mixture was measured in a liquid scintillation counter (Microbeta Trilux). A Biomek 2000 robot (Beckman Coulter) was routinely used for pipetting the incubation mixture. PDE4 activity was measured by quantification of [^{3}H] 5'AMP, and concentrations that caused 50% inhibition (IC_{50}) were determined using hill equation.

Interleukin (IL)-2, -4, -5 and TNFα release from PBMC

PBMC (>90% lymphocytes) were extracted from heparinized blood of healthy donors by means of density centrifugation on Biocoll (density 1.077 g/l). Cells were harvested and washed three times and then re-suspended in RPMI 1640 medium supplemented with 10% FCS, HEPES 25 mM, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 2-mercaptoethanol (50 µM). PBMC were cultured in 96-well tissue-culture plates (1×10^6 cells/ml for IL-2, 2×10^6 cells/ml for IL-4/5 and 0.5×10^6 cells/ml for TNFα, 100 µl/well) at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air for 18 hours (IL-2, TNFα) or 48 hours (IL-4/5). Inhibitors were dissolved in DMSO, diluted in RPMI 1640 medium

(supplemented), and were added (in triplicate) to wells at a range of concentrations (final DMSO concentration 0.1%, final concentrations of inhibitors: 0.01 nM to 10,000 nM). Viability as determined by trypan blue exclusion was uniformly >95 %. Cells were stimulated as follows: (i) for IL-2 release, with 50 µl per well of stimulation agent, i.e. with phytohemagglutinin P (PHA-P, 20 µg/ml; Konno *et al*. 1994) (ii) for IL-4/5 release, with concanavalin A (Con A, 20 µg/ml; Endo *et al*. 1993) or anti-CD3 (1 µg/ml) plus anti-CD28 (0.5 µg/ml, applied in a solution) (iii) for release of TNFα, with LPS (1 µg/ml *Salmonella abortus equi*, ; Schindler *et al*. 1990). Each drug was pre-incubated for 30 minutes before stimulation. After incubation cells were pelleted, and IL-2, IL-4,IL-5 or TNFα in the supernatants (fresh or frozen at –80 °C) were measured by ELISA according to the manufacturer's instructions.

TNFα release from dispersed human nasal polyp cells and LPS-induced TNFα Release in Human Blood

Nasal polyps were obtained from patients with nasal polyposis after polypectomy. Patients were free of any medication for at least two weeks. This study was approved by the local ethics committee (No. EK 109072000, Ethics Committee of the University of Dresden), and all patients had given informed consent for the use of the resected tissue. The polyps were washed in RPMI 1640 medium and dissected, and cell dispersal was achieved by enzymatic digestion (Campbell and Bousquet, 1993). Briefly, the minced polyps were incubated for 2 hours at 37 °C in RPMI 1640 (1 g tissue per 4 ml) containing 2.0 mg/ml protease, 1.5 mg/ml collagenase, 0.75 mg/ml hyaluronidase and 0.05 mg/ml DNase. The cell suspension was then filtered to remove any undigested tissue and washed three times. The cell pellet was resuspended in erythrocyte-lysis buffer and incubated for 5 minutes at 37 °C under 5% CO_2 and then washed with RPMI 1640 complete medium (see below). The cells (lymphocytes, mast cells, macrophages, epithelial cells, neutrophils and eosinophils) were re-suspended at a concentration of 2×10^6 cells/ml in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 25 mM HEPES, penicillin (100 U/ml) and streptomycin (100 µg/ml). Viability as

determined by trypan blue exclusion was uniformly >95 %. For passive sensitization, the cells were incubated in the presence of human IgE (1 µg/ml) for 1 hour at 37 °C and washed three times with complete medium. The cell suspension (1 ml per well, 6-well plates) was pre-incubated with the inhibitors for 30 min before stimulation with 7.2 µg anti IgE per well. After an incubation period of 18 hours (37 °C and 5% CO_2), the cell suspension was centrifuged and the supernatant was stored at –80 °C until determination of TNFα. The TNFα ELISA (antibody pairs) was performed according to PharMingen's instructions. LPS-induced TNFα Release in Human Blood was carried out as described previously (Marx *et al.*, 2002).

Statistics

For *in vitro* experiments, data are shown as mean ± standard error of the mean (SEM). The percentage inhibition was calculated for each experiment using the following formula:

$$\%\,\text{inhibition} = 100 - \frac{100 \text{ x cytokine release compound}}{\text{cytokine release control}}$$

The IC_{50} values and SEM for the inhibition of phosphodiesterase 4 were determined by using the in-house program "Inhibit" (non-linear regression, Hill-Plot). The EC_{50} values (half-maximum inhibition, relative to the maximal effect obtained) and SEM for the inhibition of IL-2, IL-4, IL-5 and TNFα release in human PBMC, TNFα release in whole blood or polyps were determined by using the program SigmaPlot (nonlinear regression, sigmoidal curve, Hill Plot, four parameters).

Results

Effects of AWD 12-281 on IL-2, IL-4, IL-5 and TNFα release of stimulated human PBMC

AWD 12-281 was found to inhibit the PDE4 activity in human PMNLs with an IC_{50} of 9.71 ± 0.51 nM (mean ± SEM). The compound inhibited the following processes in a concentration-dependent manner (Table 1): phytohemagglutinin-induced IL-2 release (EC_{50} 50 nM), concanavalin A-induced IL-5 production (EC_{50} 46 nM), anti-CD3 and anti-CD28 co-stimulated IL-4 release (EC_{50} 121 nM) and IL-5 release (EC_{50} 77 nM) and LPS-stimulated TNFα release in human PBMC (EC_{50} 88 nM). AWD 12-281 was compared with other PDE4 inhibitors (rolipram, RPR-73401, RS-25344-000, cilomilast and roflumilast). It could be shown that all these PDE4 inhibitors suppressed the release of IL-2, IL-4, IL-5 and TNFα in a concentration-dependent manner (Figures 1–5). This activity could be correlated with the inhibitory activity of these compounds at the catalytic site of the PDE4 isoenzyme (Table 1 and Figure 6). The greatest inhibition, achieved at the highest concentration tested, amounted to about 90% for IL-2, IL-4 and IL-5 and about 70–75% for TNFα, with the exception of RS-253444-000 (82%), indicating that additional factors beside PDE4-dependent cAMP levels may contribute at least to the release of TNFα.

Despite the difference between the mechanisms of action of PDE4 inhibitors on the one hand and corticosteroid receptor activation on the other hand, dexamethasone also suppressed powerfully the release of IL-2, IL-4, IL-5 and TNFα. The inhibition by dexamethasone was likewise concentration-dependent and reached a similar maximum response (about 74% for TNFα and 90% for the interleukins). However, one difference is to be noted with regard to the release of IL-4. While all PDE4 inhibitors tested up to a final concentration of 1 µmol/l potently suppressed the IL-4 and IL-5 release when a co-stimulation protocol with anti-CD3 and anti-CD28 was followed (Figures 3 and 4), these compounds showed no – or only a poor – inhibition of concanavalin A-stimulated IL-4 release in human PBMC (data not shown). As mentioned above, the concanavalin A-stimulated IL-5 release was powerfully suppressed

(Figure 2). Dexamethasone, on the other hand, was capable of suppressing not only the concanavalin A-induced release of IL-5 (Figure 2), but also the release of IL-4 (regardless of the stimulation protocol used). Dexamethasone suppressed concanavalin A-stimulated IL-4 release with an IC_{50} value of 9.9 ± 1.4 nM (mean ± SEM, four experiments, data not shown), The effect on the release of IL-4 induced by anti-CD3 and anti-CD28 was tested only at the concentration of 10 nM, which resulted in 59±4.2% inhibition.

Inhibition of TNFα release in dispersed human polyp cells

The *in vitro* effect of AWD 12-281 on TNFα release was investigated in allergically stimulated human dispersed nasal polyp cells; it was compared with the effects of the reference compounds rolipram, cilomilast and roflumilast (Table 2). AWD 12-281 inhibited the allergically induced TNFα release with an EC_{50} value of 111 nM. In comparison, rolipram, cilomilast and roflumilast suppressed the release of TNFα with EC_{50}values of 156 nM, 190 nM and 13 nM, respectively.

Inhibition of TNFα release in human whole blood

The effect of AWD 12-281 on TNFα release was investigated and compared with that of rolipram, cilomilast and roflumilast in human blood diluted 1:5 (Table 2). The selective PDE4 inhibitor AWD 12-281 suppressed the LPS-induced TNFα release in a concentration-dependent manner with an EC_{50} of 900 nM. In comparison with effects of the reference compounds cilomilast (EC_{50} 1400 nM) and rolipram (EC_{50} 500 nM) AWD 12-281 was not a stronger inhibitor of LPS-induced TNFα release, despite its higher PDE4 inhibitory activity. The strongest inhibition in this assay was that by roflumilast, with an EC_{50} of 17 nM.

Discussion

AWD 12-281 is a highly potent and selective PDE4 inhibitor that has been optimized for topical administration. The compound was shown to suppress the allergen-induced cell infiltration in bronchoalveolar lavage fluid of sensetized Brown Norway rats and the LPS induced lung neutrophila in Lewis rats ferrets and domestic pigs. In sensitized BP-2 mice, AWD 12-281 abolished the allergen-induced bronchial hyperresponsiveness (Kuss et al., 2003). In passively sensitized human airways, AWD 12-281 was shown to have a protective effect against allergen-induced contractions and to also have a weak relaxant effect on the spontaneous tone (Schmidt *et al*., 1999). These data indicate that AWD 12-281 is suitable for the treatment of asthma and COPD.

The aim of the current experiments was to evaluate the anti-inflammatory potential of AWD 12-281 in comparison with other PDE4 inhibitors and dexamethasone in various human inflammatory-cell with the aim of predicting pharmacological activity in patients. The inhibitory power of AWD 12-281 on the PDE4 isoenzyme was found to be 9.71 nM (IC_{50}), and was thus higher than that of cilomilast (17.8 nM) and lower than that of roflumilast (0.27 nM). Our data for the inhibition of PDE4 activity of the reference compounds are in agreement with literature data (Hatzelmann and Schudt 2001, Barnette *et al*.1998, Giembycz 2001). On the basis of these data AWD 12-281 – like cilomilast and roflumilast – has the potential to be active in patients.

All PDE4 inhibitors investigated and dexamethasone were able to inhibit the release of IL-2, IL-4, IL-5 and TNFα from human PBMC. Our data for roflumilast, cilomilast and rolipram are in agreement with published data from different sources using similar methods (Hatzelmann and Schudt 2001, Barnette 1998, Jimenez *et al*. 2001).

We could show that AWD12-281 and other PDE4 inhibitors suppressed anti-CD3/anti-CD28-stimulated release of IL-4 while they had little effect on concanavalin A-induced IL-4 release. Dexamethasone was capable of suppressing the IL-4 release induced by either of the two

stimulation methods. In this context, T-cell receptors (i.e., stimulation with anti-CD3/anti-CD28) can be seen as a model for immunological diseases such as allergy and asthma. Stimulation with concanavalin A utilizes lectin receptors instead; this stimulation can be seen as unspecific, and not related to any particular disease. While this difference does not help to identify the mechanistic basis for the difference in sensitivity to PDE4 inhibition, we can conclude that PDE4 inhibitors may be more selective than steroids and therefore especially suitable for the treatment of immunological diseases. However, the inhibition of IL-4 release by dexamethasone, but not by PDE4 inhibitors, may be also misleading, since a corresponding difference was not evident for the inhibition of IL-5 release.

If PDE4 inhibitory activity was plotted against inhibitory potential, a good correlation ($p < 0.05$) between PDE4 inhibitory activity of the inhibitors investigated and the inhibition of cytokine release in PHA-P-stimulated PBMC (IL-2), concanavalin A-stimulated PBMC (IL-5) and LPS-stimulated PBMC (TNFα) could be shown (Figure 6). This close correlation is suggestive. It indicates that the parameters measured are indeed closely related to PDE4-dependent cAMP levels, further supporting the belief that PDE4 inhibition is relevant for anti-inflammatory activity. The assays employed here may therefore be used to predict the anti-inflammatory potential of new PDE4 inhibitors. While the correlation between PDE4-inhibitory potential and inhibition of mediator release is very close for IL-2 and IL-5 release ($r^2 > 0.9$), it is somewhat lower for TNFα release ($r^2 = 0.82$). In addition, if we inspect the individual concentration–response curves (Figure 5), we can see that the maximum TNFα inhibition achieved amounted to approximately 70–75% at 1 µM of each test compound. This was also shown by Hatzelmann and Schudt (2001), who found that the maximum inhibition of TNFα release in LPS-stimulated whole blood was only about 60–65%. This indicates that other factors beside PDE4 activity may contribute to TNFα release. Candidates are other PDE subtypes – i.e., PDE3 (Hatzelmann and Schudt 2001) or PDE7 – that are also cAMP-PDE isoenzymes and are found in activated T cells (Glavas 2001, Smith 2003). However, since

the maximum suppression achieved with dexamethasone was also of the order of 70%, other factors besides PDE enzymes may also contribute to TNFα release.

We were interested in comparing the effects of AWD 12-281 on TNFα release in different cell preparations containing human inflammatory cells (i.e. PBMC, whole blood and nasal polyps) in relation to roflumilast and dexamethasone, in order to identify the preparation best suited to test PDE4 inhibitors. Cells dispersed from human nasal polyps differ from PBMC and human whole blood in that inflammatory cells are derived directly from inflamed tissue. It can be expected that these cells are pre-activated, owing to the ongoing inflammatory process. Furthermore, the stimulation mechanism used in the two preparations differs considerably. While PBMC and whole blood were stimulated according to standard methods, using bacterial endotoxin (LPS), which is a physiological stimulator only under conditions of bacterial infection and sepsis, we had established a stimulation protocol for nasal polyps which is closely related to an allergic reaction. The cells were first incubated with IgE, this was followed by the addition of anti-IgE according to the modified method described by the group of Bousquet (Campbell et al. 1993). While the concentration for half maximal inhibition for suppression of TNFα release was similar between PBMC and dispersed nasal polyp cells for both PDE4 inhibitors tested, dexamethasone inhibited approximately 5 times more powerfully in human polyp cells (Tables 1 and 2). (It should be noted that data reported previously in abstract form on TNFα release in whole blood and polyps (Heer *et al*.1999 and 2000, Kuesters *et al*.1999) are based on the same row data but had been calculated using a (less suitable) in-house program.)

The data indicate that PDE4 inhibitors can exert potent anti-inflammatory effects even in activated immune cells derived from inflamed cells by an allergy-related stimulation mechanism. The abilities of the most potent PDE4 inhibitors to suppress TNFα were comparable to that of dexamethasone, which, on the basis of these data, was also active in the nanomolar range (1.3–20 nM) in suppressing TNFα release in PBMC and nasal polyp

cells. In contrast to these two cell preparations, the ability of AWD 12-281 to suppress LPS-induced TNFα release was approximately 1/10 as great in human whole blood. This difference may be related to the high plasma protein binding of AWD 12-281, which was found to be >99% (Kuss *et al*., 2003; Krone 2000, unpublished). Isolated PBMC as well as nasal polyp cells are cultivated *in vitro* in artificial media containing (after addition of both stimulation medium and compound solution) 10% or 9% of FCS as the only source of plasma proteins. They therefore do not have the problem of binding the compounds to plasma proteins to the same extent as in whole blood. This may result in a reduced systemic availability of the compound. However, since AWD 12-281 is optimized for topical administration, a high level of protein binding can contribute to the advantageous profile of AWD 12-281, i.e. long persistence in target tissue after administration by inhalation and low systemic availability (Kuss *et al*., 2003). This, in addition to the low oral bioavailability and rapid metabolism outside the target organ may result in a low emetic potential, especially after inhalative administration. Indeed, it could be shown that AWD 12-281 was exceptionally well tolerated and did not induce emesis while being highly potent in animal models of inflammation when given via the inhalative route (Kuss *et al*., 2003). In addition, AWD 12-281 was found to have a low affinity to the rolipram binding site with an IC_{50} of 104 nM (Hoefgen *et al*. 1998). Since this site is discussed to be associated with emesis and gastro-intestinal disturbances (Torphy, 1998), this may also contribute to the good tolerability found in vivo.

Comparing the three cell preparations to assess antiinflammatory potential, it can be concluded that PBMC are well suited for the evaluation of the anti-inflammatory potential of PDE4 inhibitors. While nasal polyp cells are more closely related to an ongoing inflammatory process, data obtained in this preparation did not differ from results obtained in PBMC, at least with regard to PDE4 inhibitors. Whole blood in small amounts can be easily obtained from donors, without further preparation. This method is therefore also well suited for *in vitro* determination of TNFα release.

In summary, AWD 12-281 was shown to suppress powerfully the release of inflammatory mediators in three different human cell preparations. The ability of AWD 12-281 to inhibit PDE4 was found to be as great as that of other PDE4 inhibitors. These results, in combination with the potent activity of AWD 12-281 in animal models and the good separation between its emetic and anti-inflammatory activities, especially after topical administration, make AWD 12-281 an interesting candidate for the topical treatment of airway diseases such as asthma, COPD and allergic rhinitis. Currently, phase II clinical studies are under way to evaluate the therapeutic potential of AWD 12-281 in these diseases.

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Footnotes

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Legends for Figures

Figure 1:

Concentration–response curves for the inhibition of PHA-P-induced IL-2 release by AWD 12-281, other PDE4 inhibitors and dexamethasone in human PBMC. Data are shown as mean ± SEM from 3 to 4 experiments.

Figure 2:

Concentration–response curves for the inhibition of concanavalin A-induced IL-5 release by AWD 12-281, other PDE4 inhibitors and dexamethasone in human PBMC. Data are shown as mean ± SEM from 2 to 4 experiments.

Figure 3:

Concentration–response curves for the inhibition of anti-CD3- and anti-CD28-induced IL-5 release by AWD 12-281 and roflumilast in human PBMC. Data are shown as mean ± SEM from 4 experiments.

Figure 4:

Concentration–response curves for the inhibition of anti-CD3/anti-CD28-co-stimulated IL-4 release by AWD 12-281 and roflumilast in human PBMC. Data are shown as mean ± SEM from 3 to 4 experiments.

Figure 5:

Concentration–response curves for the inhibition of LPS-stimulated TNFα release by six selective PDE4 inhibitors and dexamethasone in human PBMC. Data are shown as mean ± SEM from 3 to 4 experiments.

Figure 6:

Plots of linear regression (IL-2: dashed line, IL-5: solid line, TNFα: dotted line, $p< 0.05$) for inhibition of PDE4 versus inhibition of IL-2, IL-5 and TNFα release (EC_{50} values are given in Table 1).

Tables

TABLE 1

Inhibition of PDE4 activity in human PMNLs (polymorphonuclear leukocytes) by AWD 12-281 and other PDE4 inhibitors and inhibition of cytokine release in PBMC by AWD 12-281, other PDE4 inhibitors and dexamethasone. PDE4 activity was measured by quantification of [^{3}H] 5'AMP, and concentrations that caused 50% inhibition (IC_{50}) were determined. Values are displayed as standard error of the mean (SEM) in parentheses and numbers of experiments in brackets. Cytokines released by differently stimulated human PBMC were measured by ELISA, and concentrations causing half maximum inhibition (EC_{50}) were determined. Values are displayed as SEM in round brackets and numbers of experiments in square brackets.

TABLE 1

Compound	PDE4 inhibition IC_{50} (nM)	IL-2 [a] EC_{50} [f] (nM)	IL-5 [b] EC_{50} [f] (nM)	IL-5 [c] EC_{50} [f] (nM)	IL-4 [c] EC_{50} [f] (nM)	TNFα [d] EC_{50} [f] (nM)
AWD 12-281	9.71 (0.51) [4]	50.0 (10.84) [4]	46.3 (7.25) [3]	77.0 (10.68) [4]	121.5 (55.04) [3]	87.9 (26.60) [3]
Rolipram	75.50 (4.56) [4]	207.0 (43.62) [4]	338.6 (32.49) [2]	N.D.	N.D.	322.4 (87.50) [4]
Cilomilast	17.80 (2.32) [4]	160.0 (33.03) [4]	165.3 (54.53) [2]	N.D.	N.D.	699.7 (179.64) [3]
RPR-73401	0.41 (0.0075) [4]	N.D.	2.0 (0.19) [3]	N.D.	N.D.	4.97(0.78) [4]
RS-25344-000	0.28 [e]	N.D.	0.3 (0.37) [4]	N.D.	N.D.	5.4 (0.75) [4]
Roflumilast	0.27 (0.02) [8]	4.7 (0.88) [4]	3.4 (1.22) [4]	8.7 (0.36) [4]	11.2 (0.78) [4]	21.2 (1.94) [3]
Dexamethasone		7.5 (0.51) [3]	2.9 (0.79) [4]	88±1.6% inhibition at 10 nM [4]	59±4.2% inhibition at 10 nM [4]	6.4 (1.42) [4]

N.D.= not determined [a] PHA-P-stimulated [b] Con A-stimulated

[c] Co-stimulated [d] LPS-stimulated [e] Data from Alvarez *et al*., 1994 [f] half-maximum inhibition

TABLE 2

Inhibition of TNFα release in LPS-stimulated human blood and inhibition of TNFα release in anti-IgE-stimulated human nasal polyp cells by AWD 12-281, rolipram, cilomilast, roflumilast and dexamethasone. Levels of cytokines released by anti-IgE-stimulated dispersed human nasal polyp cells were measured by ELISA, and concentrations causing half maximum inhibition (EC_{50}) were determined. Values are displayed as geometric means with SEM in parentheses and numbers of experiments in brackets.

TABLE 2

Compound	TNFα, whole blood EC_{50}[a] (nM)	TNFα, polyp EC_{50}[a] (nM)
AWD 12-281	934 (213) [6]	111 (59) [6]
Rolipram	511 (*) [4]	156 (*) [4]
Cilomilast	1425 (171) [4]	190 (*) [5]
Roflumilast	17 (6) [5]	13 (*) [2]
Dexamethasone	20 (4) [10]	1.3 (0.3) [4]

* EC_{50} estimated, limited number of data points [a] half-maximum inhibition

Figure 1

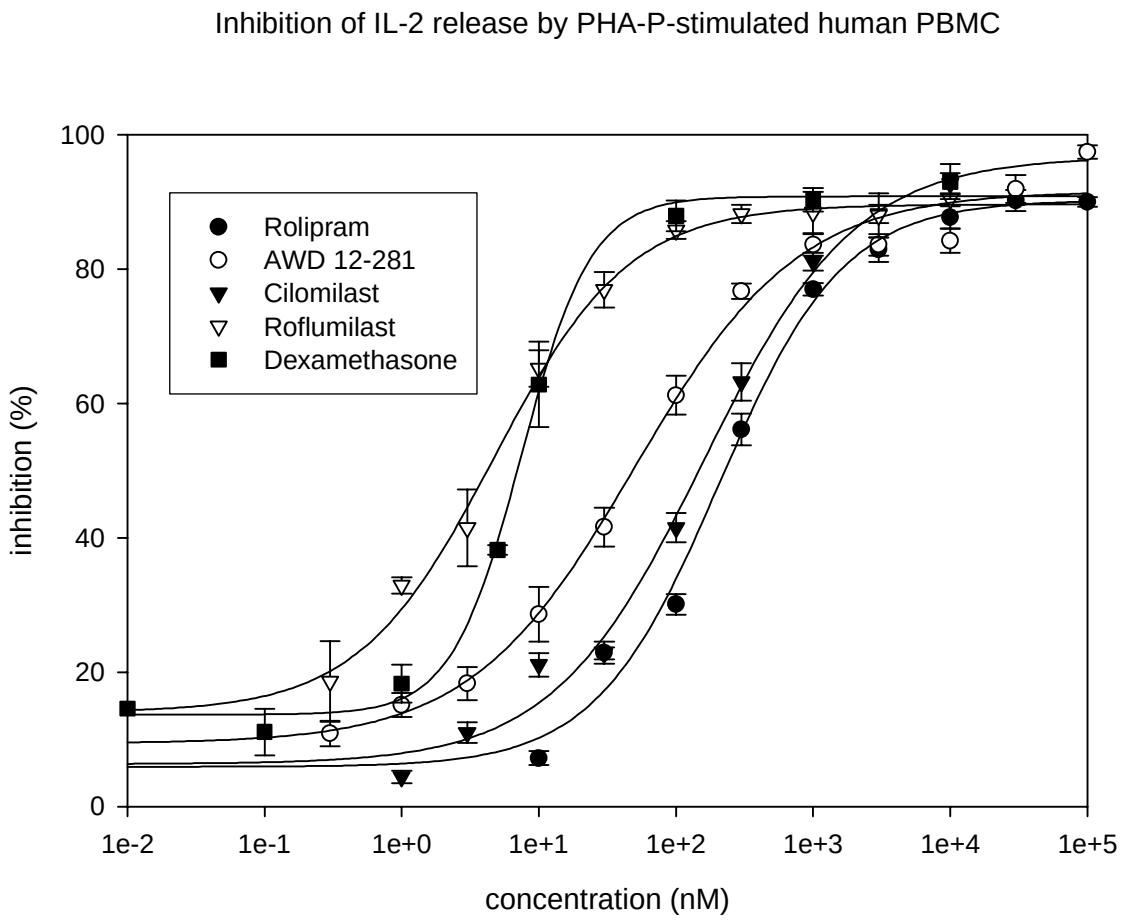
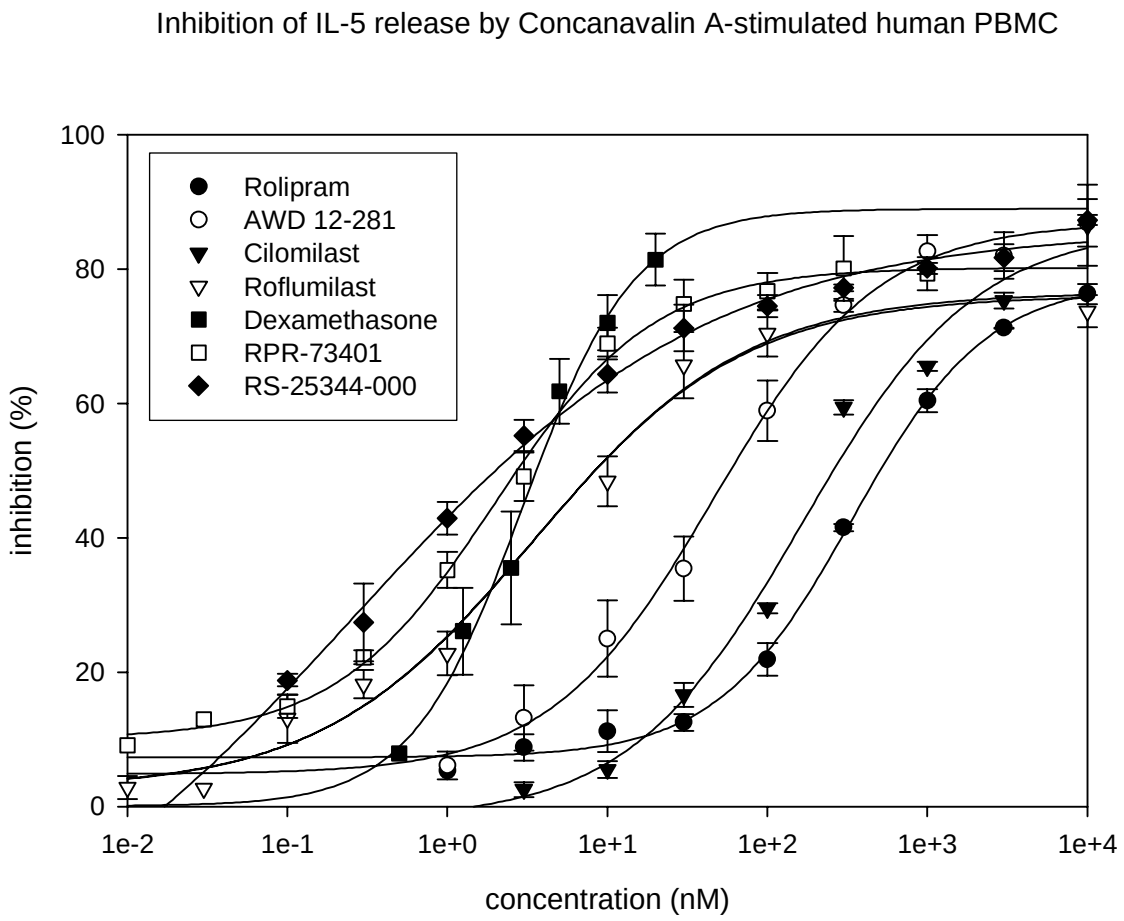


Figure 2



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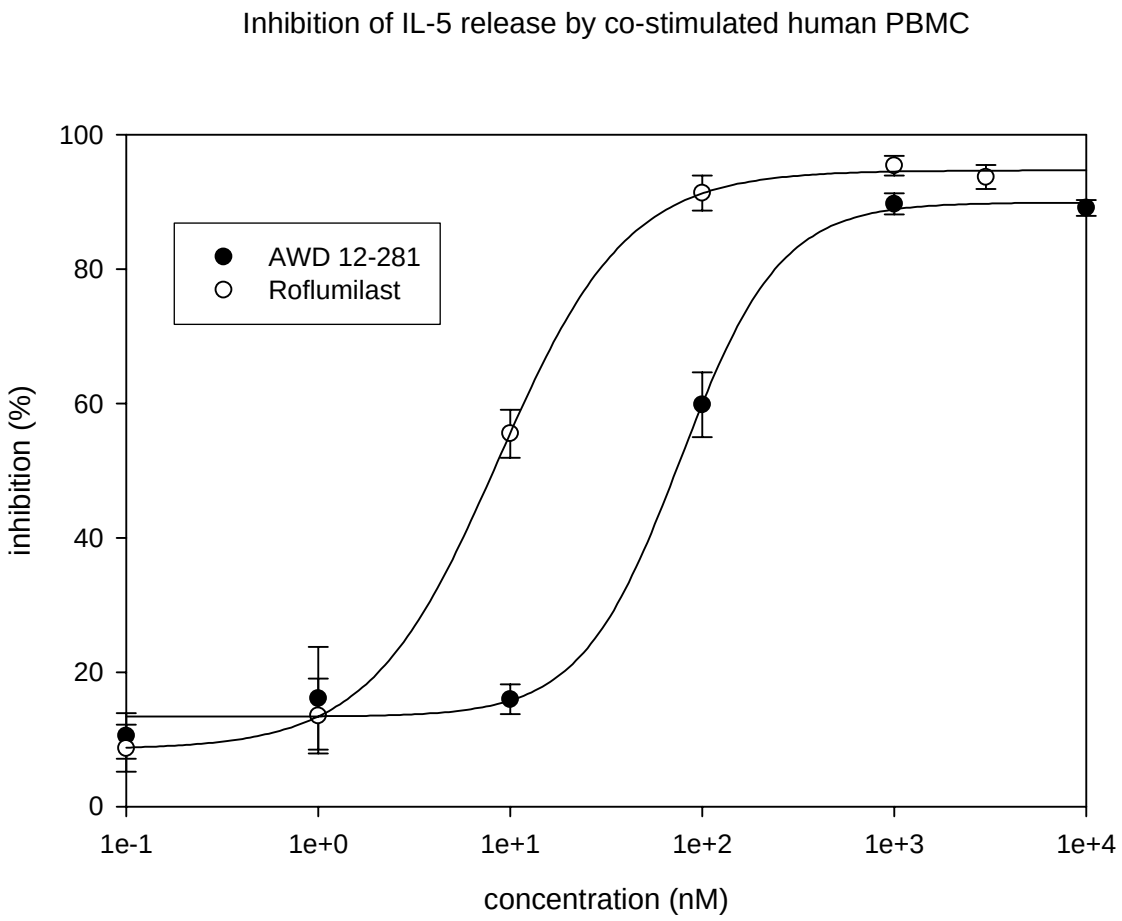

Figure 4

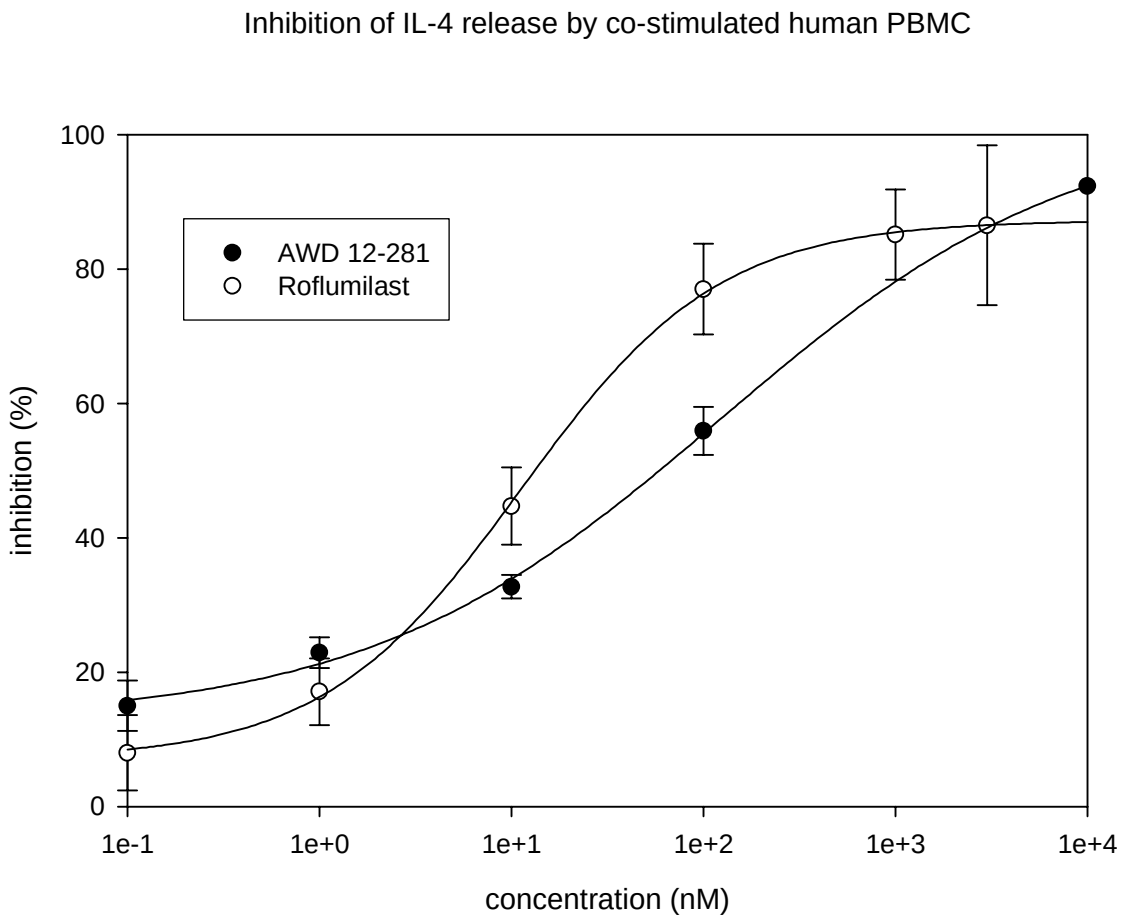

Figure 5

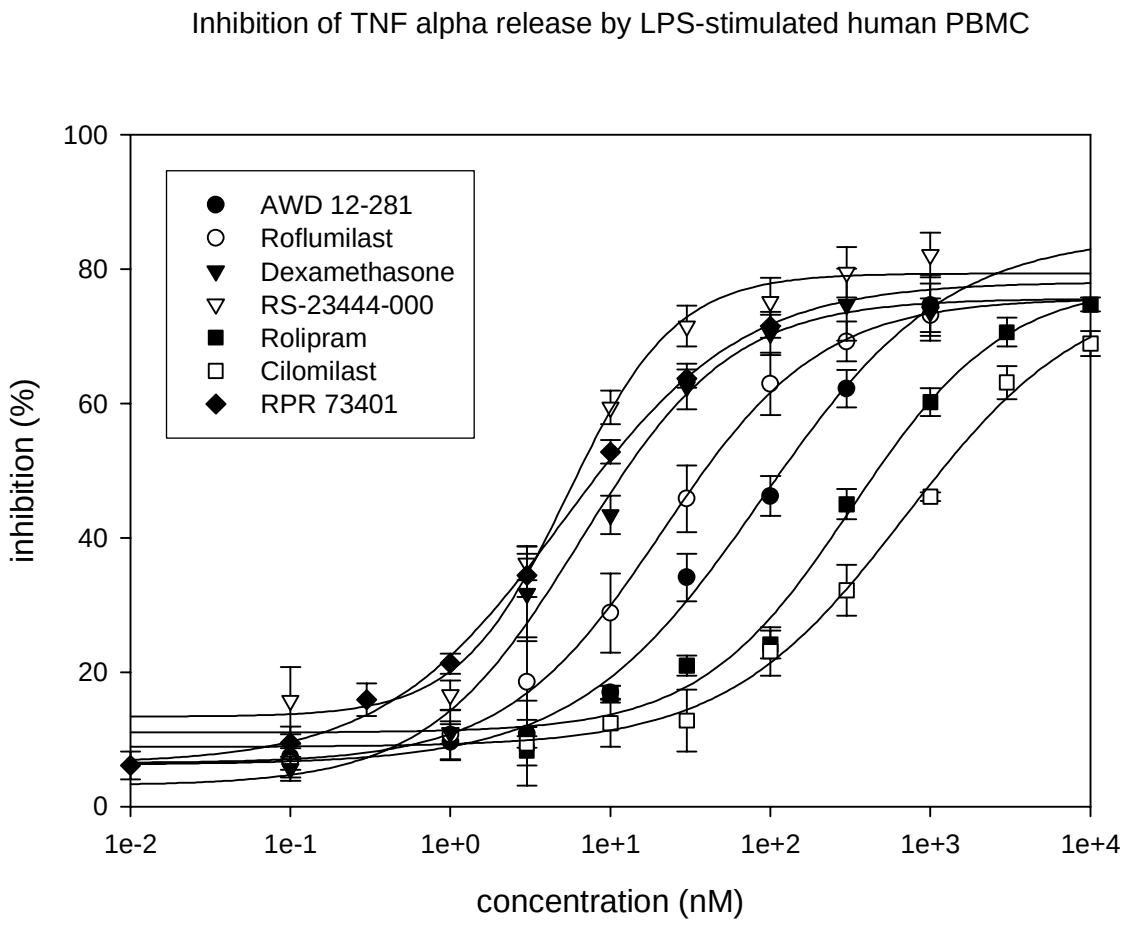

Figure 6