Anti-Inflammatory and Immunomodulatory Potential of the Novel PDE4 Inhibitor Roflumilast in Vitro

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ABSTRACT

From a series of benzamide derivatives, roflumilast (3-cyclopropylmethoxy-4-difluoromethoxy-N-[3,5-di-chloropyrid-4-yl]benzamide) was identified as a potent and selective PDE4 inhibitor. It inhibits PDE4 activity from human neutrophils with an IC_{50} of 0.8 nM without affecting PDE1 (bovine brain), PDE2 (rat heart), and PDE3 and PDE5 (human platelets) even at 10,000-fold higher concentrations. Roflumilast is almost equipotent to its major metabolite formed in vivo (roflumilast N-oxide) and piclamilast (RP 73401), however, more than 100-fold more potent than rolipram and Ariflo (cilomilast; SB 207499). The anti-inflammatory and immunomodulatory potential of roflumilast and the reference compounds was investigated in various human leukocytes using cell-specific responses: neutrophils [N-formyl-methyl-leucyl-phenylalanine (fMLP)-induced formation of LTB_{3} and reactive oxygen species (ROS)], eosinophils (fMLP- and C5a-induced ROS formation), monocytes, monocyte-derived macrophages, and dendritic cells (lipopolysaccharide-induced tumor necrosis factor-α synthesis), and CD4^{+} T cells (anti-CD3/anti-CD28 monoclonal antibody-stimulated proliferation, IL-2, IL-4, IL-5, and interferon-γ release). Independent of the cell type and the response investigated, the corresponding IC values (for half-maximum inhibition) of roflumilast were within a narrow range (2–21 nM), very similar to roflumilast N-oxide (3–40 nM) and piclamilast (2–13 nM). In contrast, cilomilast (40–3000 nM) and rolipram (10–600 nM) showed greater differences with the highest potency for neutrophils. Compared with neutrophils and eosinophils, representing the terminal inflammatory effector cells, the relative potency of roflumilast and its N-oxide for monocytes, CD4^{+} T cells, and dendritic cells is substantially higher compared with cilomilast and rolipram, probably reflecting an improved immunomodulatory potential. The efficacy of roflumilast in vitro and in vivo (see accompanying article in this issue) suggests that roflumilast will be useful in the treatment of chronic inflammatory disorders such as asthma and chronic obstructive pulmonary disease.

Cyclic nucleotide hydrolyzing phosphodiesterases (PDEs) comprise a still-growing superfamily of isoenzymes with 11 members known at present (Beavo et al., 1994; Soderling and Beavo, 2000). These isoenzymes can be discriminated based on substrate specificity and/or affinity, and their regulation by specific activators and inhibitors. The complexity is further enhanced by the existence of two or more genes coding for different subtypes within one particular isoenzyme, and furthermore, two or more splicing variants derived from one gene (Loughney and Ferguson, 1996). In total, more than 50 different proteins can be expected for humans. The functional (patho)physiological importance of this diversity on the level of different genes and splicing variants is not well understood at the moment, mainly due to the lack of subtype- or even splicing variant-specific inhibitors. Among the cAMP-specific isoenzymes, PDE4 has received particular attention due to the fact that all of the inflammatory and immunomodulatory cells not only contain PDE4 (Tenor and Schudt, 1996) but also that specific functions of these cells are broadly inhibited by selective PDE4 inhibitors (Torphy, 1998; Barnette, 1999; Essayan, 1999). Although not generally demonstrated it can be assumed that many of the effects of PDE4 inhibitors are due to the inhibition of cAMP hydrolysis, leading to enhanced intracellular cAMP levels; cAMP itself is well known to be inhibitory for many inflammatory and immunomodulatory cells. Furthermore, in various animal models (e.g., for asthma and other allergic diseases, rheumatoid arthritis, multiple sclerosis, and others) PDE4 inhibitors show pronounced anti-inflammatory effects (Teixeira et al., 1997) and, therefore, have been proposed as a new therapeutic approach for a variety of inflammatory diseases such as asthma.

ABBREVIATIONS: PDE, phosphodiesterase; COPD, chronic obstructive pulmonary disease; BSA, bovine serum albumin; PBS, phosphate-buffered saline; fMLP, N-formyl-methyl-leucyl-phenylalanine; LPS, lipopolysaccharide; PGE_{2}, prostaglandin E_{2}; FBS, fetal bovine serum; IL, interleukin; GM-CSF, granulocyte macrophage-colony stimulating factor; DMSO, dimethyl sulfoxide; CL, chemiluminescence; AUC, area under the curve; MACS, magnetic cell separation; TNF_{α}, tumor necrosis factor-α; mAb, monoclonal antibody; IFN_{γ}, interferon-γ; LTB_{3}, leukotriene B_{3}; ROS, reactive oxygen species; HARBS, high-affinity rolipram binding site; LARBS, low-affinity rolipram binding site.
(Giembycz, 1992; Torphy, 1998; Schudt et al., 1999). However, despite the large effort of the pharmaceutical industries to identify selective PDE4 inhibitors in the last decade, for only a few of them effectiveness in patients has been reported. According to published data the most advanced PDE4 inhibitor in clinical development seems to be Ariflo (SB207499; cilomilast) from GlaxoSmithKline (King of Prussia, PA) (Barnette et al., 1998), which shows clinical efficacy both in asthma and in chronic obstructive pulmonary disease (COPD) patients (Barnette, 1999).

In our own screening program, we have identified roflumilast as a potent and selective PDE4 inhibitor from a series of benzamides (Amschsler, 1995). Similar to cilomilast, this compound is in advanced clinical development for asthma (phase III) and COPD (phase II). In the present article, the in vitro pharmacology of roflumilast is described in an accompanying article (Bundschuh et al., 2001). In both parts roflumilast is compared with cilomilast (see above), rolipram (as the archetypal PDE4 inhibitor), piclamilast/RP 73401 (be-}

Inhibition of PDE Isoenzymes

PDE activity was determined as described by Thompson et al. (1979) with some modifications (Bauer and Schwabe, 1980). The assay mixture contained 50 mM Tris (pH 7.4), 5 mM MgCl₂, 0.5 μM cAMP or cGMP, and [³H]cAMP or [³H]cGMP (about 30,000 cpm/assay), the indicated concentration of the inhibitor and an aliquot of the enzyme solution at a final assay volume of 200 μl.

Stock solutions of the compounds were diluted 1:100 (v/v) in the Tris buffer mentioned above; appropriate dilutions were prepared in 1% (v/v) DMSO/Tris buffer, which were diluted 1:2 (v/v) in the assays to obtain the desired final concentrations of the inhibitors at a DMSO concentration of 0.5% (v/v). DMSO itself affected none of the PDE activities.

After preincubation for 5 min at 37°C, the reaction was started by the addition of substrate (cAMP or cGMP) and the assays were incubated for further 15 min at 37°C. Then 50 μl of 0.2 N HCl was added to stop the reaction and the assays were left on ice for about 10 min. Following incubation with 25 μg of 5’-nucleotidase (Crotalus atrox snake venom) for 10 min at 37°C, the assays were loaded on QAE Sephadex A-25 (1 ml of bed volume in Poly-Prep chromatography columns; Bio-Rad, München, Germany). The columns were eluted with 2 ml of 30 mM ammonium formate (pH 6.0) and the eluate was counted for radioactivity. Results were corrected for blank values (measured in the presence of denatured protein) that were below 5% of total radioactivity. The amount of cyclic nucleotides hydrolyzed did not exceed 30% of the original substrate concentration.

PDE1 from bovine brain, kindly provided by Dr. Gietzen (Ulm, Germany), was prepared as described (Gietzen et al., 1982). This isoenzyme was assayed in the presence of Ca²⁺ (1 mM) and calmodulin (100 nM) using cGMP as substrate. A blank value measured in the presence of EGTA (1 mM) was subtracted from all values. PDE2 from rat heart was chromatographically purified as described by Schudt et al. (1991b) and was assayed in the presence of cGMP (5 μM) using cAMP as substrate. PDE3 and PDE5 were assayed in the cytosol of human platelets essentially as described by Schudt et al. (1991b) using cAMP and cGMP, respectively, as substrate. PDE4 was tested in the cytosol of human neutrophils as described by Schudt et al. (1991a) using cAMP as substrate. The PDE3-specific inhibitor motapizone (1 μM) was included to suppress PDE3 activity originating from contaminating platelets.

Functional Studies

All cells referred to were purified from human venous blood (200–250 ml) of healthy donors.

Neutrophils

The isolation of neutrophils (polymorphonuclear leukocytes) from blood (anticoagulated with sodium citrate) by dextran sedimentation, centrifugation on Ficoll Paque, and hypotonic lysis of remaining red blood cells has been performed essentially as described previously (Hatzelmann and Ulrich, 1987).

Chemiluminescence (CL) Assay. In buffer. CL measurements were performed in “CL-buffer” (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 10 mM HEPES, pH 7.4) containing 1 mM CaCl₂, 1 mg/ml glucose, 0.05% (v/v) BSA, 10 μM luminol, and 4 μM microperoxidase (all values correspond to final concentrations in the assay). Aliquots (0.4 ml) of the cell suspension (1.25 × 10⁶ cells/ml) were preincubated for 5 min at 37°C in the absence or presence of inhibitors (0.05 ml). Stock solutions of inhibitors were diluted 1:100 (v/v) in CL-buffer. Subsequent dilutions were made in 1% (v/v) DMSO/CL-buffer to

Materials and Methods

Reagents and Inhibitors

Bovine serum albumin (BSA fraction V powder), calmodulin, cAMP, cGMP, complement C5α, cytochalasin B, dextran (mol. wt. = 515,000), Dulbecco’s phosphate-buffered saline (PBS, pH 7.4), EGTA, N-formyl-methionyl-leucyl-phenylalanine (FMLP), glucose, HEPES, hydroxylamine, lipopolysaccharide (LPS, Salmonella abortus equi), microperoxidase, 5’-nucleotidase, prostaglandin E₂ (PGE₂), prostaglandin B₂, salbutamol, and thimerosal were purchased from Sigma Chemical (Deisenhofen, Germany). Human AB-serum was obtained from PAA Laboratories (Cölbe, Germany). Fetal bovine serum (FBS), gentamicin, Iscove’s medium, L-glutamine solutions, penicillin/streptomycin solution (5000 U/ml and 5000 μg/ml, respectively), RPMI 1640 medium, and sodium pyruvate were purchased from Life Technologies (Eggenstein, Germany). Recombinant human interleukin-4 (IL-4) and granulocyte macrophage-colony stimulating factor (GM-CSF) were purchased from Biozol (Eching, Germany). [5,8-³H]cAMP, [8-³H]cGMP, and [methyl-³H]thymidine aqueous solutions were purchased from Amersham Buchler (Braunschweig, Germany). Ficoll Paque, Percoll, and QAE Sephadex A-25 were obtained from Pharmacia Biotech (Uppsala, Sweden) and luminol from either Boehringer (Mannheim, Germany) or Sigma Chemical. Heparin (Liquemin N 25000) was obtained from Hoffmann-La Roche AG (Grenzach-Wyhlen, Germany). All other chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany).

From the inhibitors used, roflumilast and its N-oxide (WO8950138), piclamilast (WO9212961), and cilomilast (WO9319749) were synthesized at the chemical facilities of Byk Gulden essentially as described in the corresponding patents. Racemix R,S-rolipram was kindly provided by Schering AG (Berlin, Germany); of that material R(-)-rolipram as the more active enantiomer was purified by Grom Analytik + HPLC-GmbH (Herrenberg, Germany), which was used throughout the whole study. Motapizone was a generous gift from Rhone-Poulenc Rorer (Köln, Germany). Appropriate stock solutions of all compounds used were prepared in dimethyl sulfoxide (DMSO).
achieve the final drug concentrations in the assays at a DMSO concentration of 0.1% (v/v), which by itself only weakly affected the CL response. The assays were then transferred into a “Multi-Biolumat LB 9505C” from Berthold (Wildbad, Germany) and stimulated by the addition of 0.05 ml of FMLP (100 nM, final concentration). CL was continuously recorded for 3 min and the area under the curve (AUC) calculated.

In plasma. For this purpose blood was anticoagulated with heparin (8 U/ml), and centrifuged for 10 min at 900g using a GS-6KR centrifuge from Beckman Instruments (München, Germany). The supernatant (platelet-rich plasma) was removed and again centrifuged for 10 min at 3200g to obtain cell-free autologous plasma. The subsequent isolation of neutrophils from the sediment of the former centrifugation step was identical to the method described above. For CL measurements neutrophils were resuspended in heparinized plasma at 107 cells/ml. The final assay volume was 0.5 ml. To 0.4-ml aliquots of neutrophil suspensions, 0.025 ml of “testmix” (resulting in final concentrations of 100 μM luminol/0.1% v/v DMSO and 10 μM microperoxidase) was added and preincubated for 5 min at 37°C in the absence or presence of the compounds (0.05 ml) as described above. After preincubation, the assays were transferred into the Multi-Biolumat LB 9505C as described above and stimulated by the addition of 0.025 ml of FMLP (10 μM, final concentration). CL was continuously recorded for 5 min and the AUCs calculated. For the calculation of compound effects, the CL signal of unstimulated cells in the absence of inhibitor was subtracted as a blank value.

Leukotriene Synthesis. Experiments were performed in a buffer consisting of Dulbecco’s PBS (pH 7.4) containing 10 mM HEPES. The total assay volume was 0.85 ml. To aliquots (740 μl) of human neutrophils (about 107 cells/ml) suspended in buffer 8.5 μl of both thimerosal (5 mM) and Ca2+/Mg2+ (100 mM) were added to achieve final concentrations of 50 μM thimerosal and 1 mM Ca2+/Mg2+, respectively. After preincubation of the cells in the absence or presence of the inhibitors (85 μl) for 5 min at 37°C, the assays were stimulated for further 5 min with FMLP (8.5 μl, 1 μM final concentration). Stock solutions of the inhibitors were diluted 1:100 (v/v) in buffer; subsequent dilutions were made in 1% (v/v) DMSO/buffer to achieve final drug concentrations in the assays at a DMSO concentration of 0.1% (v/v), which by itself only weakly affected leukotriene synthesis. The assays were stopped by the addition of 0.85 ml of ice-cold methanol containing 2 mM EGTA, 0.01 N HCl, and about 200 to 250 ng/ml prostaglandin B2 (internal standard). The extraction as well as the analysis of 5-lipoxygenase metabolites by reverse phase high performance liquid chromatography using the system “LiChroGraph H” from Merck/Hitachi (Darmstadt, Germany) has been performed essentially as described previously (Hatzelmann et al., 1993). Percentage of inhibition is related to the synthesis of the sum of leukotriene B4 and its two 6-trans isomers.

Eosinophils
Eosinophils were purified essentially as described in detail elsewhere (Hatzelmann et al., 1995). Briefly, total granulocytes were first purified from blood (anticoagulated with 0.3% w/v sodium citrate) by dextran sedimentation, centrifugation on Ficoll Paque, and hypotonic lysis of remaining red blood cells. For the further purification of the eosinophil fraction, the magnetic cell separation (MACS) system from Miltenyi Biotec (Bergisch-Gladbach, Germany) was applied. Eosinophils were separated from neutrophils by negative selection using anti-CD16 microbeads in a two-step protocol using D- and BS- (formerly called B2-) separation columns. By this method, human eosinophils with a purity of >99% and a viability of >97% were obtained.

CL Assay. The CL measurements in eosinophils were performed identical to those described above for neutrophils (see protocol in buffer) with the following exceptions: 1) the assays contained a cell concentration of 107 cells/ml; and 2) during the preincubation of the cells further additions (0.01 ml) were included that were stimulus dependent: in the case of FMLP, the assays contained cytochalasin B at a final concentration of 5 μg/ml; in the case of C5a, salbutamol (100 nM final concentration) was included as additional cAMP trigger. After preincubulation, the assays were transferred into the Multi-Biolumat LB 9505C as described above and stimulated by the addition of 0.05 ml of FMLP or C5a (100 nM final concentration each). CL was continuously recorded for 1 min (C5a) or 3 min (FMLP), respectively, and the AUCs calculated.

Monocytes, Macrophages, and Dendritic Cells
Blood was anticoagulated with sodium citrate (0.3% v/v). The isolation of monocytes by combining Percoll gradient centrifugation, countercurrent centrifugal elution, and adherence on culture dishes has been performed essentially as described (Gantner et al., 1997a). Monocytes were then cultured for 6 days either in endotoxin-free RPMI 1640 medium containing 10% (v/v) heat-inactivated (at 56°C) human AB-serum, 1% (v/v) of a 100 mM sodium pyruvate solution, 2% (v/v) of a 200 mM l-glutamine solution, 1% (v/v) of a nonessential amino acid solution, and 1% (v/v) of an antibiotic solution (5000 IU/ml penicillin, 5000 μg/ml streptomycin) either toward macrophages (Gantner et al., 1997a) in Falcon Primaria 3872 tissue culture plates (Becton Dickinson, Lincoln Park, NJ) or, alternatively, using Costar cell culture dishes (medical grade polystyrene, Corning Costar Corporation GmbH, Bodenheim, Germany) in endotoxin-free Iscove’s modified Dulbecco’s medium containing 10% (v/v) FBS and 80 μg/ml (v/v) gentamicin toward dendritic cells (Gantner et al., 1999) in the presence of GM-CSF (10 ng/ml) and IL-4 (1000 U/ml) essentially according to the protocol described by others (Romani et al., 1994; Sallusto and Lanzavecchia, 1994). For this purpose cells (5 × 106) were cultured in a volume of 10 ml plate in an incubator (type BB 6220 CU; Heraeus Instruments GmbH, Hanau, Germany) at 37°C and 5% CO2. In the case of dendritic cells, the above-mentioned cytokines were added a second time after 3 days, and at day 6, cells were collected by vigorous pipetting, counted, and used for the experiments described below.

TNFα Assay. Cells were incubated in 96-well plates (Primaria 3872) at a density of 5 × 104 cells/well in a total assay volume of 200 μl (RPMI 1640 medium containing 10% AB-serum for monocytes and macrophages, and Iscove’s modified Dulbecco’s medium containing 10% FBS for dendritic cells). Compounds (10 μl) were added 30 min before stimulation of the cells with “LPS working solution” (10 μl): a stock solution of LPS (1 mg/ml, w/v) was prepared in 0.1% (v/v) hydroxyamine in PBS; after sonication for 5 min, 1-ml aliquots were stored at −20°C. Before starting the experiment, this solution was further diluted in the corresponding cell-specific culture medium (see below) to get the LPS working solution. The appropriate cell-specific submaximal final LPS concentrations have been determined in preliminary experiments (data not shown) and are 1 ng/ml for monocytes and 100 ng/ml for macrophages and dendritic cells. In the macrophage experiments, PGE2 (10 nM) was added as a CAMP trigger to provide responsiveness of the cells for PDE inhibitors.

Stock solutions of the compounds were diluted 1:50 (v/v) in medium; subsequent dilutions were made in 2% (v/v) DMSO medium to achieve the final drug concentrations in the assays at a DMSO concentration of 0.1% (v/v), which by itself did not affect TNFα synthesis. Starting from a 10 mM stock solution in DMSO, motapizone was further diluted in medium so that the resulting DMSO concentration at the final compound concentration (1 μM) could be neglected. After overnight culture (about 13 h) in the case of monocytes and macrophages or 24 h in the case of dendritic cells, supernatants (about 180 μl) were removed and stored at −20°C before TNFα measurement by a commercially available enzymimmunnoassay from Immunotech (Hamburg, Germany) performed essentially according to the manufacturer’s instructions.

Whole Blood
Blood was anticoagulated with heparin (8 U/ml).

TNFα Assay. The final assay volume was 0.5 ml. In 96-deepwell plates from Beckman, aliquots of blood (0.4 ml) were preincububed
for 15 min at 37°C in the absence or presence of the compounds (0.05 ml). Stock solutions of the compounds were diluted 1:100 (v/v) in PBS; subsequent dilutions were made in 1% (v/v) DMSO/PBS to achieve the final drug concentrations in the assays at a DMSO concentration of 0.1% (v/v), which by itself did not affect TNFα synthesis. After preincubation, the assays were stimulated by the addition of 0.05 ml of LPS working solution (1 μg/ml LPS, final concentration; see above) for 4 h at 37°C. Afterward, about 150 μl of supernatant (plasma) was removed and diluted 1:30 (v/v) in PBS containing 3% (w/v) BSA. The samples were stored at −20°C before TNFα measurement as described above.

**CD4+ T Lymphocytes**

For the purification of CD4+ T lymphocytes, blood was anticoagulated with citrate (0.3% w/v) and diluted 1:6-fold with PBS before centrifugation at room temperature for 20 min at 220g (centrifuge type CL-GS6 KR; Beckman Instruments). The lower phase was layered on a Percoll gradient (ρ = 1.077 g/ml) and the interface containing the peripheral blood mononuclear cells was obtained following centrifugation at 800g for 25 min at room temperature. Cells were washed in PBS and then resuspended in elutriation medium (PBS, 2% v/v heat-inactivated human AB-serum, 2 mM EDTA, 5 mM glucose, pH 7.4) before first countercurrent centrifugal elutriation of the cells using a J2-MC centrifuge equipped with a JE-6B rotor (Beckman Instruments). The lymphocyte-containing fraction (>95% purity) was obtained at a flow rate of 32.5 ml/min and a rotor speed of 3000 rpm. Cells were spun down for 5 min at 570g and resuspended in 10 ml of elutriation medium for a second countercurrent centrifugal elutriation step at 19 ml/min and 3000 rpm, which minimizes the contamination of the lymphocyte fraction by platelets. Cells were then resuspended in 700 μl of PBS containing 2% v/v FBS, and CD4+ T cells were obtained by negative selection of the whole lymphocyte fraction on MACS type C5 columns (Miltenyi Biotec, Bergisch Gladbach, Germany) using magnetic antibodies (MACS colloidal superparamagnetic micro beads; Miltenyi Biotec) directed against CD19, CD14, CD16, and CD8 (150 μl each) after incubation for 1 h at 4°C (shaking) to deplete B cells, monocytes, granulocytes and NK cells, and CD8+ T cells, respectively. CD4+ T cells obtained under these conditions were >99% pure as checked by flow cytometry as described elsewhere (Gantner et al., 1997b).

For the functional studies described below cells were suspended in RPMI 1640 medium containing 10% (v/v) heat-inactivated human AB-serum, 2 mM glutamine solution, and 1% (v/v) penicillin/streptomycin solution (referred to as “culture medium”).

**Functional Parameters.** CD4+ T cells were stimulated via the T-cell receptor CD3 and the costimulatory molecule CD28 by using corresponding mAbs. For this purpose, 96-well microtiter plates (microtiter tissue culture plates 3072; Becton Dickinson, Heidelberg, Germany) were prepared on the day before cell isolation: 50 μl of anti-CD3 mAb (Orthoclone OKT-3; Janssen-Cilag, Neuss, Germany) at a concentration of 0.3 μg/well in PBS was incubated for about 2.5 h at 37°C in an incubator (type BB6220 CU; Heraeus Instruments, Hanau, Germany) at 5% CO₂, then stored overnight at 4°C and washed three times with PBS (200 μl) before use. The total assay volume was 200 μl. Assays were started by adding first 100 μl of culture medium/well and 10 μl of inhibitors. Stock solutions of the inhibitors were diluted 1:50 (v/v) in culture medium; subsequent dilutions were made in culture medium/2% (v/v) DMSO to achieve the final drug concentrations in the assays at a DMSO concentration of 0.1% (v/v). Optimal, motapizone (in 5 μl of culture medium) was included at a final concentration of 1 μM. Afterward, CD4+ T-cell suspensions in culture medium (80 μl) were added resulting in a cell concentration of 2 × 10⁵ cells/well. Anti-CD28 (10 μl in PBS, clone CD28.2; Coulter-Immunotech Diagnostics, Hamburg, Germany) was added to the final concentration of 3 μg/ml and the plates were further incubated at 37°C and 5% CO₂ for 72 h.

For determination of cytokine levels, all assays were performed in quadruplicates, and at the end of the incubation supernatants were removed, pooled in deep-well plates (267001; Beckman Instruments) and stored at −20°C before measurement of IL-2, IL-4, IL-5, and/or IFNγ with commercially available enzynimmunoassay kits from Coulter-Immunotech Diagnostics. Due to the high variability of cytokine levels from different blood donors, for each experiment and cytokine appropriate dilution factors had to be determined to guarantee that the cytokine levels were in the linear range of the enzyme-linked immunosorbent assay standard curves. Dilutions were performed in PBS containing 3% (w/v) BSA, and all cytokines for one condition were determined from the pool fraction in duplicate.

For determination of proliferation, ¹H]thymidine (0.2 μCi/well, added in 10 μl of culture medium) was present during the last 18 h of culture (72 h). Incubations were done in triplicates and cells were harvested on a Tom tec Harvester 96 (Dunn Labortechnik, Asbach, Germany). Filter plates (Uni Filter-96, GPC; Canberra-Packard, Dreieich, Germany) were washed three times with water at room temperature, and then dried for about 1.5 h at 60°C; scintillator (Microscint O; Canberra-Packard) was added and then radioactivity (cpm) was measured using the TopCount microplate scintillation counter from Canberra-Packard.

**Statistics**

Results are given as mean ± S.D. from the number (n) of independent experiments indicated. Dependent on the efficacy of the PDE4 inhibitors in the various test systems, corresponding IC values for half-maximum inhibition were calculated from concentration-inhibition curves by nonlinear regression analysis using the program GraphPad Prism (version 3.00) from GraphPad Software Inc., San Diego, CA.

**Results**

**Roflumilast Is a Potent and Selective PDE4 Inhibitor.** The structure of roflumilast is shown in Fig. 1. It was identified from a series of benzamide derivatives as a potent inhibitor of PDE4 activity in human polymorphonuclear leukocyte cytosol (Amschler, 1995). With an IC₅₀ of 0.8 nM, roflumilast is equipotent to the structurally related reference compound RP73401 (piclamilast), however, more than 100-fold more potent than SB207499 (cilomilast) and rolipram (Table 1). Since roflumilast in vivo is efficiently metabolized to the corresponding pyridyl N-oxide in various species, including humans (M. David, E. Sturm, and K. Zech, manuscript in preparation), it is important to note that this compound is only 2- to 3-fold less potent than roflumilast itself. It is therefore possible that roflumilast N-oxide contributes to
the overall pharmacological effect(s) of roflumilast in vivo. Roflumilast is a monoselective PDE4 inhibitor since it does not affect other PDE isoenzymes, including PDE1, PDE2, PDE3, and PDE5 up to 10,000-fold higher concentrations (Table 1; for solubility reasons concentrations higher than 10 μM could not be tested in the case of the benzamides). This is also true for roflumilast N-oxide and the other inhibitors tested with the exception of cilomilast, which affects other PDE isoenzymes apart from PDE4 at concentrations higher than 10 μM. However, these high concentrations can assumed to be clinically not relevant.

The following cellular data are illustrated in the same graph format with identical x- and y-axis scales for clarity reasons.

**Roflumilast Inhibits Human Neutrophil Functions.**

The receptor agonist FMLP triggers the release of substantial quantities of LTB4 in the presence of thimerosal (Hatzelmann et al., 1990). Under these conditions, all of the PDE4 inhibitors tested inhibited LTB4 synthesis almost completely (Fig. 2A) in the rank order of potency (IC50 values given in parentheses): roflumilast and piclamilast (2 nM), N-oxide (5 nM), rolipram (11 nM), and cilomilast (40 nM). It should be mentioned that the PDE4 inhibitors affect a receptor-mediated event since the compounds were inactive when LTB4 synthesis was triggered by the calcium ionophore A23187 (data not shown). In addition to leukotriene synthesis, the formation of reactive oxygen species (ROS) along the so-called respiratory burst is an often-used read-out parameter for neutrophil function. We measured FMLP-stimulated ROS formation as luminol-enhanced CL. In contrast to LTB4 synthesis, the PDE4 inhibitors inhibited CL to a maximum of only about 70% under the conditions used (Fig. 2B). Therefore, for quantitative analysis IC50 values were calculated that were similar to the IC50 values determined for inhibition of LTB4 synthesis: roflumilast and piclamilast 4 nM, N-oxide 8 nM, rolipram 20 nM, and cilomilast 60 nM. These CL measurements were performed in a buffer system. In parallel, we adapted this test system to conditions where neutrophils were suspended in autologous heparinized plasma (80% v/v) to test the impact of plasma protein binding on the overall potency of the compounds under evaluation. It should be noted that other PDE4 inhibitors known to have a low plasma protein binding show the same potency under both test conditions (data not shown). The potency of all the PDE4 inhibitors tested in the present study shifted to the right (Fig. 2C), however, to a different extent. Based on the IC50 values determined in plasma (roflumilast 90 nM, N-oxide 110 nM, piclamilast 150 nM, rolipram 90 nM, and cilomilast 1000 nM), ratios can be calculated in comparison to the IC50 values obtained in buffer, which show an increase in the relative plasma protein binding in the rank order of rolipram (4.5), N-oxide (14), cilomilast (17), roflumilast (22.5), and piclamilast (37.5).

**Roflumilast Inhibits the CL Response of Human Eosinophils.**

Similar to neutrophils we assessed the effect of roflumilast on the eosinophil CL response as a representative functional parameter. We have shown previously, however, that other eosinophil functions such as the release of granule constituents (eosinophil cationic protein, eosinophil-derived neurotoxin), leukotriene C4 synthesis, or chemotaxis will be inhibited by PDE4 inhibitors similarly (Hatzelmann et al., 1995; Tenor et al., 1996). Eosinophil CL response was stimulated either by FMLP or complement C5a; in case of the latter stimulus, the β2-adrenoceptor agonist salbutamol (which by itself shows <10% inhibition at the concentration of 100 nM used) is necessary as a permissive CAMP trigger to render PDE4 inhibitors active (Hatzelmann et al., 1995). As shown in Fig. 3, A and B, the inhibition of CL by roflumilast and the other compounds investigated is almost identical for both stimuli with an efficacy of 60 to 80% and calculated IC35 values (given for FMLP/C5a) of 7/10 nM for roflumilast, 20/40 nM for the N-oxide, 10/6 nM for piclamilast, 200/70 nM for rolipram, and 120/230 nM for cilomilast.

**Roflumilast Inhibits TNFα Synthesis in Monocytes.**

The therapeutic potential of TNFα inhibition in various chronic inflammatory diseases, including airway inflammation (Renzetti et al., 1996) is widely accepted, and various strategies for inhibition of TNFα have been suggested (Newton and Decicco, 1999). Among these, PDE4 inhibition is known to be very effective. We therefore selected LPS-stimulated TNFα synthesis as a suitable functional parameter for monocytes as well as macrophages and dendritic cells. Under the conditions used, LPS stimulated the release of substantial amounts of TNFα (24.9 ± 8.4 ng/ml; n = 31) in monocytes. The PDE4 inhibitors tested inhibited TNFα synthesis to a maximum of about 80 to 85% (Fig. 4A) in agreement with the notion that PDE4 is the predominant PDE isoenzyme involved in the regulation of monocyte functions (Gantner et al., 1997a). Residual TNFα can be inhibited in an additive fashion by PDE3 inhibition (data not shown). The rank order of potency (IC40 values given in parentheses) is piclamilast (6 nM) > N-oxide (17 nM) = roflumilast (21 nM) ≫ rolipram (330 nM) > cilomilast (1300 nM). The absolute values for rolipram and cilomilast are substantially lower compared with the IC50 values (63 and 100 nM, respectively) reported by others under obviously similar conditions (Barnette et al., 1998), although relatively in both cases rolipram is more potent (2–4-fold) than cilomilast. In contrast, our values obtained for rolipram and piclamilast are identical to the IC50 values (397 and 6.9 nM, respectively) reported by Souness et al. (1996) for the same test parameter. These data suggest that rather the absolute potency than the rank order of

<table>
<thead>
<tr>
<th>Table 1: Selectivity of roflumilast and reference compounds to inhibit PDE4 activity.</th>
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<tbody>
<tr>
<td><strong>PDE1 (bovine brain)</strong></td>
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<td>-----------------------------</td>
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<tr>
<td>IC50 (nM)</td>
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<tr>
<td>Roflumilast</td>
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<tr>
<td>PDE2 (rat heart)</td>
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<td>PDE3 (human platelets)</td>
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<tr>
<td>PDE4 (human neutrophils)</td>
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<td>PDE5 (human platelets)</td>
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</table>
potency for the PDE4 inhibitors may be influenced by the experimental conditions applied when testing LPS-stimulated TNFα synthesis in human monocytes.

In whole blood, monocytes appear to be the main source for TNFα upon LPS stimulation. Surprisingly, the maximum inhibition of PDE4 inhibitors is only about 60 to 65% (Fig. 4B), and again, residual TNFα can be inhibited by PDE3 inhibition (data not shown). The reason(s) for this difference is not known. The IC₅₀ values calculated in whole blood for roflumilast (50 nM), N-oxide (50 nM), piclamilast (70 nM), rolipram (500 nM), and cilomilast (5000 nM) are generally higher than in monocytes. The differences can be explained by the plasma protein binding of the compounds (compare Fig. 2, B and C), and the loss of potency for the single compounds roughly follows the relative plasma protein bind-

Fig. 2. Inhibition of human neutrophil functions by roflumilast and reference compounds. Cells (10⁷/ml) were preincubated for 5 min at 37°C in the absence or presence of the inhibitors to be tested. A, for induction of leukotriene synthesis, thimerosal (50 μM) was included. After preincubation, cells were stimulated by fMLP (1 μM) for 5 min and leukotriene synthesis (LTB₄ plus its 6-trans isomers) was assessed by high performance liquid chromatography analysis. B, cells were stimulated by fMLP (100 nM) and the formation of reactive oxygen species (AUC) was measured by luminol-enhanced chemiluminescence for 3 min. C, chemiluminescence measurements (AUC for 5 min after stimulation with 10 μM fMLP) were performed essentially as shown in B except that the assays contained 80% (v/v) autologous plasma. Data are shown as mean ± S.D. from the number of independent experiments given in parentheses for each inhibitor.

Fig. 3. Inhibition of the human eosinophil chemiluminescence response by roflumilast and reference compounds. Cells (10⁶/ml) were preincubated for 5 min at 37°C before stimulation for the formation of reactive oxygen species (measured as luminol-enhanced chemiluminescence) by either 100 nM fMLP (A) in the presence of cytochalasin B (5 μg/ml) or 100 nM complement C5a (B) in the presence of 100 nM salbutamol (which by itself does not substantially affect chemiluminescence but is required to get the PDE4 inhibitors active using C5a as a stimulus). AUCs for chemiluminescence were recorded for 3 min (fMLP) or 1 min (C5a), respectively. Data are shown as mean ± S.D. from the number of independent experiments given in parentheses for each inhibitor.
ing factors estimated from the CL measurements in neutrophils.

**Roflumilast Inhibits TNFα Synthesis in Monocyte-Derived Macrophages in the Presence of the PDE3-Selective Inhibitor Motapizone.** We previously have characterized the in vitro differentiation of human monocytes to macrophages (Gantner et al., 1997a). It should be noted that the macrophage-like phenotype has been proven based on changes of surface markers (e.g., down-regulation of CD14) as well as the up-regulation of macrophage marker enzymes such as unspecific, NaF-insensitive esterase and acid phosphatase. In addition, the PDE isoenzyme pattern of these monocyte-derived macrophages closely resembles that of human lung macrophages from bronchoalveolar lavages (Tenor et al., 1995).

Under the experimental conditions used, LPS elicited a mean amount of 4.0 ± 2.0 ng/ml (n = 14) TNFα in macrophages that is about 7-fold lower compared with monocytes (see above). We previously demonstrated that LPS-stimulated TNFα synthesis in macrophages is rather insensitive to inhibition by PDE inhibitors unless an additional cAMP trigger such as PGE₂ is added (Gantner et al., 1997). Even in the presence of PGE₂ at 10 nM, which inhibited TNFα by about 20 to 25%, the overall effect of selective PDE4 inhibitors was rather weak (10–20% inhibition; Fig. 5A). PGE₂ (10 nM) in the presence of motapizone (1 μM) inhibited TNFα by 41.6 ± 10.2% (n = 11); under these conditions, the remaining TNFα was inhibited by the PDE4 inhibitors to a maximum of about 70% (Fig. 5B) allowing the comparison of the potency of roflumilast and the other PDE4 inhibitors tested. As shown in Fig. 5B, the potency (IC₃₅ values given in parentheses) of roflumilast (13 nM), N-oxide (12 nM), piclamilast (7 nM), and...
Roflumilast Inhibits TNFα Synthesis in Monocyte-Derived Dendritic Cells. Human dendritic cells were obtained by cultivating human monocytes in the presence of IL-4 and GM-CSF, and their qualities have been described by the expression of specific surface markers such as MHCII, CD1 or B7 by others (Sallusto and Lanzavecchia, 1994; Romani et al., 1994). In addition, our own previously reported functional studies have characterized these cells as being by far more potent in stimulating CD4+ T-cell proliferation in so-called “mixed lymphocyte reaction” as well as in presenting foreign antigens (tetanus toxoid, keyhole limpet hemocyanin) compared with monocytes or macrophages (Gantner et al., 1999). Together, these results demonstrate that the dendritic cells used in the present experiments show major features of “professional antigen-presenting cells”, which are important for the initiation of an immune response in vivo.

Under the experimental conditions used, LPS elicited substantial amounts of TNFα in dendritic cells (5.5 ± 3.5 ng/ml; n = 14) that were in the same order of magnitude as in macrophages (see above). Roflumilast as well as the other PDE4-selective compounds inhibited LPS-stimulated TNFα synthesis in dendritic cells only to a maximum of about 40% (Fig. 6A). The calculated IC50 values under these conditions demonstrate that roflumilast (5 nM), its N-oxide (4 nM), and piclamilast (3 nM) are almost equipotent, whereas rolipram (40 nM) and cilomilast (200 nM) are 10- to 50-fold less active. Compared with monocytes, dendritic cells up-regulate PDE3 activity in favor of PDE4 activity (which is down-regulated) during the differentiation process in vitro (Gantner et al., 1999). Therefore, it was not surprising that the PDE3-selective compound motapizone (1 μM) exerted a synergistic effect on the efficacy of the PDE4 inhibitors (Fig. 6B). Although motapizone alone at 1 μM inhibited TNFα synthesis only to 24.9 ± 7.6% (n = 10), it increased the maximum inhibition of remaining TNFα by PDE4 inhibitors to about 80%. Calculation of the corresponding IC40 values showed that unlike efficacy, the potency of the compounds were not influenced substantially by additional PDE3 inhibition: roflumilast (7 nM), its N-oxide (5 nM), piclamilast (2 nM), rolipram (30 nM), and cilomilast (400 nM).

Roflumilast Inhibits Proliferation and Cytokine Synthesis in CD4+ T Cells. Human CD4+ T cells were purified by a three-step protocol involving a Percoll gradient, countercurrent centrifugal elutriation, and negative selection by magnetic cell separation as described under Materials and Methods. For the stimulation of the cells in vitro, a protocol using anti-CD3 (0.3 μg/well) and anti-CD28 (3 μg/ml) mAbs was used to simulate the stimulation of T cells by professional antigen presenting cells via the T-cell receptor (CD3) and perhaps the most important (CD28) of several possible costimulatory mechanisms occurring in vivo under (patho)physiological conditions. From initial time course studies 72 h was selected as the optimal time to analyze all activation parameters (proliferation, IL-2, IL-4, IL-5, and IFNγ synthesis) simultaneously. These studies were performed in the presence of 0.1% (v/v) DMSO, which by itself did not affect proliferation (mean inhibition of 5.3 ± 17.4% in n = 14 experiments), IL-2 (mean inhibition of 3.7 ± 13.0% in n = 14 experiments) and IL-4 (mean inhibition of 7.4 ± 18.2% in n = 12 experiments), and slightly inhibited IFNγ (mean of 16.7 ± 14.5% in n = 15 experiments) and IL-5 (mean of 17.9 ± 13.0% in n = 15 experiments).

Proliferation was inhibited to a maximum of about 60% by roflumilast (Fig. 7A) with a potency (IC50) of 7 nM. Similar results (IC50 values in parentheses) were obtained for roflumilast N-oxide (10 nM) and piclamilast (10 nM), whereas rolipram (330 nM) and cilomilast (900 nM) were about 30- and 90-fold, respectively, less potent but more efficient at the highest concentrations (10 and 100 μM, respectively) used. The reason(s) for this higher efficacy is unclear. In the presence of the PDE3-selective inhibitor motapizone, which by itself did not influence proliferation, an almost complete inhibition of proliferation by additional roflumilast was obtained (data not shown); however, potency (IC40 of 9 nM) was not altered, which is also true for the other compounds (IC40 values in parentheses): N-oxide (6 nM), piclamilast (4 nM),
rolipram (230 nM), and cilomilast (800 nM). The results obtained for proliferation as a representative parameter indicate that under the stimulation conditions used, PDE4 inhibitors are effective but that complete inhibition of CD4+ T-cell activation can be achieved only by combined inhibition of PDE4 and PDE3. Similar to proliferation, all cytokines...
investigated were concentration dependently inhibited by roflumilast although with different efficacies (Fig. 7, B–E). For quantitative analysis, adequate IC values were calculated according to the parameter-specific efficacy of roflumilast as summarized in Table 2. From these results it is evident that roflumilast inhibits all parameters almost equipotently with IC values in the range of 7 to 13 nM except IL-2, which is inhibited more potently with an IC value of 1 nM. Roughly, identical results were obtained for roflumilast N-oxide and piclamilast, whereas rolipram and cilomilast were clearly less potent. As already mentioned for proliferation, motapizone also increased efficacy of PDE4 inhibitors to inhibit cytokine synthesis (about 30–35%) without affecting potency (data not shown).

Comparison of the Relative Potency of Roflumilast and the Other PDE4 Inhibitors to Inhibit the Function of Various Leukocytes. In the preceding text, the potency of roflumilast to inhibit cell-specific parameters of various leukocytes has been compared with its N-oxide as well as the reference compounds piclamilast, rolipram, and cilomilast. As a summary, all the IC values determined are listed in Table 2. Based on these data we addressed the question whether there are differences between the potency of each compound to inhibit a particular cell-type in comparison to the others. For this purpose, the neutrophil was arbitrarily selected as a reference cell. The corresponding potency of the respective compound to inhibit neutrophil function (mean inhibition of fMLP-stimulated chemiluminescence/buffer and the IC<sub>35</sub> for inhibition of LTB₄ synthesis) was standardized to the value 1. Analogously for the other cells, potency (at half-maximum inhibition) was calculated as (mean) value based on the inhibition of the following parameters: eosinophils (fMLP- and C5a-stimulated chemiluminescence), monocytes (TNFα synthesis), macrophages (TNFα synthesis in the presence of PGE₂ and motapizone), dendritic cells (TNFα synthesis in the absence and presence of motapizone), and CD4⁺ T cells (proliferation, IL-4, IL-5, and IFNγ synthesis). The potency of each PDE4 inhibitor for these cells relative to neutrophils is illustrated in Fig. 8. It is evident that eosinophils and macrophages are inhibited at the same relative potency by all five PDE4 inhibitors investigated, except rolipram, which seems to be a little more active in macrophages and a little less active in eosinophils. In contrast, both monocytes and CD4⁺ T-cells are inhibited with a substantial greater relative potency by roflumilast compared with piclamilast and rolipram. This feature is even more pronounced in the case of piclamilast and, importantly, also in the case of roflumilast N-oxide. In dendritic cells, roflumilast N-oxide and piclamilast show a greater relative potency than rolipram and cilomilast; compared with the latter two compounds, such difference is less evident for roflumilast.

Taken together, in contrast to rolipram and cilomilast the benzamide derivatives roflumilast, its N-oxide, and piclamilast show a relative higher potency for monocytes, CD4⁺ T-cells, and a little less active in eosinophils. In contrast, both monocytes and CD4⁺ T-cells are inhibited with a substantial greater relative potency by roflumilast compared with piclamilast and rolipram. This feature is even more pronounced in the case of piclamilast and, importantly, also in the case of roflumilast N-oxide. In dendritic cells, roflumilast N-oxide and piclamilast show a greater relative potency than rolipram and cilomilast; compared with the latter two compounds, such difference is less evident for roflumilast.

TABLE 2
Inhibition of leukocyte cell function(s) by roflumilast and reference compounds
This table summarizes the quantitative analysis of the data shown in Figs. 2 through 7. The two lower test systems are separated since the potency of the compounds is determined by plasma protein binding in addition to the cellular effect. IC values for half-maximum inhibition were calculated by nonlinear regression analysis.

<table>
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<tr>
<th>Cells</th>
<th>Stimulus</th>
<th>Parameter</th>
<th>Addition(s)</th>
<th>IC&lt;sub&gt;35&lt;/sub&gt;</th>
<th>Roflumilast</th>
<th>N-Oxide</th>
<th>Piclamilast</th>
<th>Rolipram</th>
<th>Cilomilast</th>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>5</td>
<td>2</td>
<td>11</td>
<td>40</td>
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<tr>
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<td>fMLP</td>
<td>CL</td>
<td>PGE₂ + motapizone</td>
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<td>10</td>
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<td>4</td>
<td>6</td>
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<td>12</td>
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<tr>
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<td>12</td>
<td>7</td>
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<td>4</td>
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<tr>
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<td>70</td>
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lymphocytes, and dendritic cells compared with neutrophils, eosinophils, and macrophages. In a previous section (Fig. 2C) we have shown that the compounds under investigation show substantial plasma protein binding although to a different degree. The functional studies were performed in buffers containing different protein additions (in the case of neutrophils and eosinophils, 0.05% w/v BSA; in the case of monocytes and macrophages, 10% v/v human AB-serum; and in the case of dendritic cells and CD4+ T cells, 10% v/v FBS, respectively). Therefore, the possibility might have existed that the differences in the inhibition of the various cell types between the PDE4 inhibitors tested could be a consequence of different compound protein binding in the buffers described above. We have carefully addressed this question by comparing the potency of the compounds in the neutrophil chemiluminescence assay (Fig. 2B) in an assay buffer containing either 0.05% w/v BSA, 10% v/v FBS, or 10% v/v AB-serum, respectively. We found that for each of the five PDE4 inhibitors, the corresponding IC values for half-maximum inhibition were almost identical in the three buffer systems (data not shown), indicating that the differences in potency shown in Fig. 8 are not the result of the different cell-specific buffers/media used.

Discussion

The present study demonstrates that the novel compound roflumilast is a potent and selective PDE4 inhibitor with a broad anti-inflammatory and immunomodulatory action in vitro. These features also translate to the main metabolite (N-oxide) of roflumilast formed in vivo; it is therefore likely that the N-oxide contributes to the overall action of roflumilast in animal species as well as humans.

For measuring PDE4 activity we have used human neutrophil cytosol as an easily accessible biological source. This material probably contains a mixture of several PDE4 species, based on polymerase chain reaction analysis mainly of the PDE4B and D subtypes (Ortiz et al., 2000). However, our own preliminary results indicate that roflumilast (as well as the other benzamides) does not discriminate between inhibition of various splicing variants among the PDE4A, B, and D genes except those of the PDE4C gene, which are inhibited with about 10-fold lower potency. Although we have investigated the PDE isoenzyme selectivity of roflumilast only among PDE4i, it is unlikely that roflumilast will inhibit any of the newer PDE isoenzymes (PDE7–11) in analogy to the archetypal PDE4 inhibitor rolipram, which doesn’t affect any of these PDE isoenzymes.

Among the human leukocytes investigated, neutrophils, eosinophils, and monocytes contain PDE4 as the prominent PDE isoenzyme (Tenor and Schudt, 1996), and therefore it was not surprising that roflumilast potently and effectively inhibits representative functions of these cells (Figs. 2–4). Neutrophils and eosinophils are assumed to be the terminal effector cells of inflammation although with different importance for various diseases. For example, in airway diseases, the eosinophil is accepted to play an important role in asthma, whereas the neutrophil is predominant in COPD. In both situations, however, the release of cell-specific mediators (such as the leukotrienes, reactive oxygen species (derived from superoxide anion radical), and various granule constituents (e.g., proteases in the case of neutrophils, cationic proteins in the case of eosinophils) seem to be involved in the amplification of the inflammatory response, smooth muscle contraction, and lung damage ultimately leading to declining lung function and/or bronchial hyperreactivity. It is therefore important to note that besides the representative functions of neutrophils (LTB4 synthesis and ROS formation) and eosinophils (ROS formation) investigated in the present study, roflumilast in analogy to other PDE4 inhibitors will potently affect other granulocyte functions.

For the other cells investigated (macrophages, dendritic cells, and CD4+ T cells) the addition of the PDE3-selective inhibitor motapizone enhanced the efficacy of all PDE4 inhibitors investigated to some extent. This functional synergism is mirrored by the presence of PDE3 in addition to PDE4 in these cell types. The need for additional PDE3 inhibition to completely abrogate activation of macrophages, dendritic cells, and CD4+ T cells by PDE4 inhibitors in vitro must not necessarily translate to the complex in vivo situation of an inflammatory reaction. For example, large amounts of nitric oxide synthesized by inducible nitric-oxide synthase stimulates guanylate cyclase and, consequently, substantially enhances intracellular cGMP levels. PDE3 is known as the cGMP-inhibited PDE isoenzyme and would be inhibited under such conditions, thereby reflecting the pharmacological PDE3 inhibition applied in vitro. Indeed, in support of this assumption, roflumilast is very effective in inflammatory animal models in vivo (see accompanying article by Bundschuh et al., 2001).

There is no doubt that, in contrast to macrophages, both antigen-presenting dendritic cells (Holt and Stumbles, 2000) and CD4+ T lymphocytes (Romagnani, 2000) play an important role in the initiation and propagation of the immune response in asthma. As discussed previously, it is rather unlikely that the process of antigen processing can be inhibited directly by PDE4 inhibitors in monocyte-derived dendritic cells (Ganttner et al., 1999). However, as shown in the present study, roflumilast potently inhibits TNFα synthesis of these cells (Fig. 6). Together with the even more efficient TNFα synthesis inhibition by roflumilast in monocytes (Fig. 4) and the large therapeutic potential of TNFα inhibition in various chronic inflammatory diseases (Newton and Decicco, 1999), this finding largely adds to the potential of roflumilast to be of therapeutic benefit not limited to airway diseases.

In addition to isolated monocytes we have also investigated inhibition of TNFα synthesis by PDE4 inhibitors in whole blood (Fig. 4B). Under these conditions, the potency of the compounds is determined by plasma protein binding as additional parameter besides cellular activity. Without requiring biophysical (analytical) methods, the relative plasma protein binding of the compounds was determined in a functional assay by comparing the potency of the compounds...
to inhibit chemiluminescence in neutrophils suspended either in buffer or in plasma (compare Fig. 2, B and C). From the compounds investigated in the present article, rolipram was found to have the lowest plasma protein binding (factor of 4.5) and plasma protein binding increases in the rank order roflumilast-N-oxide (14) < cilomilast (17) < roflumilast (22.5) < piclamilast (37.5). Since whole blood in small amounts can be easily obtained also during in vivo studies without further manipulation, the inhibition of TNFα synthesis in ex vivo LPS-stimulated whole blood samples can be taken to monitor the pharmacodynamic effect of a PDE4 inhibitor in whole blood after compound administration. We use this assay in clinical studies of roflumilast to monitor TNFα as a surrogate parameter.

The immunomodulatory potential of PDE4 inhibitors has been reviewed recently (Essayan, 1999). Our own results (Fig. 7) demonstrate that roflumilast as well as its N-oxide are potent inhibitors of human CD4+ T-cell functions, including proliferation and the release of various cytokines. The question whether PDE4 inhibitors might selectively affect either Th1- (IL-2, IFNγ) or Th2- (IL-4, IL-5) cytokine release in human T cells is discussed controversially in the literature. Under the experimental conditions applied, in the present study (costimulation with anti-CD3 and anti-CD28 mAbs) no preferential inhibition of either Th1- or Th2-cytokines was found. In our opinion the question whether PDE4 inhibitors may be of greater therapeutic potential either in typical Th1- (e.g., rheumatoid arthritis, inflammatory bowel disease) or Th2 (e.g., asthma, atopic dermatitis)-mediated diseases, is still open and will definitely be answered only in the clinics.

In an attempt to compare roflumilast (and its N-oxide) with well known reference compounds, we included piclamilast, rolipram, and cilomilast in the present in vitro studies. Although it is clear that all of these compounds are selective PDE4 inhibitors (Table 1), both quantitative and qualitative differences became evident. Compared with roflumilast itself, its N-oxide behaves almost identical in all test systems investigated; this is also true for piclamilast in vitro (Table 2), which may not be surprising due to the structural similarity of both compounds. However, in vivo (at least in animal models) roflumilast is clearly superior to piclamilast upon oral administration as demonstrated in the accompanying article by Bundschuh et al. (2001). In contrast, the overall in vitro potency of the benzamides (roflumilast, its N-oxide, and piclamilast) is substantially greater compared with rolipram and cilomilast at first glance. However, a more detailed reflection shows that the differences in potency are not uniform but rather cell-specific (Table 2). For example, although roflumilast inhibits TNFα synthesis in macrophages only 10-fold more potent than cilomilast, the difference in potency for inhibition of CD4+ T-cell functions for the same compounds is roughly 170-fold. In an attempt to illustrate such relative differences in more detail for all cells and inhibitors investigated, we first estimated the mean potency of each inhibitor to inhibit a certain cell type (under Results), and second put the mean potency of all other cell types (arbitrarily) in relation to neutrophils, which was standardized to the value 1 (Fig. 8). It should be noted that the mean potency was calculated for reasons of precision despite the fact that this value does not discriminate between different stimuli used (e.g., fMLP and C5a in the case of eosinophils) or different parameters measured (e.g., chemiluminescence and LTB4 synthesis in neutrophils, or proliferation; IL-4, IL-5, and IFNγ synthesis in T cells); however, this procedure seems to be justified since for all cells the single values are very close to the resulting mean value. As a result (Fig. 8), two things are obvious: first, both eosinophils and macrophages are inhibited by all compounds with the same relative potency compared with neutrophils; and second, the three benzamides inhibit monocytes, CD4+ T cells, and dendritic cells (although to a lesser extent) with a relatively higher potency compared with rolipram and cilomilast. In other words, at a given compound concentration (or plasma level under in vivo conditions), roflumilast and its N-oxide will likely have a greater potential to inhibit the function(s) of immunocompetent cells (CD4+ T cells, dendritic cells) and monocytes in addition to the postulated inhibition of inflammatory cells (neutrophils, eosinophils) compared with rolipram and cilomilast. This could be of particular importance for efficacy in vivo since the former cells are decisive both for the initiation and the propagation of an immune response.

What could be the explanation for these differences between the benzamides and rolipram/cilomilast? Since the first description of a high-affinity binding of the archetypal PDE4 inhibitor rolipram in rat brain (Schneider et al., 1986), the concept emerged (Christensen et al., 1996) that PDE4 can exist in at least two different conformations interacting with rolipram (and other PDE4 inhibitors) with either high (at HARBS, high-affinity rolipram binding site) or low affinity (at LARBS, low-affinity rolipram binding site). The expectation that HARBS is mediating side effects (e.g., emesis) and LARBS will mediate most of the anti-inflammatory and/or immunomodulatory effects of PDE4 inhibitors lead to the concept that PDE4 inhibitors with an improved therapeutic window (compared with rolipram) should favor the interaction with LARBS rather than HARBS. Since among the cells investigated in the present article the inhibition of monocytes and T lymphocytes seems to be correlated to LARBS, whereas the inhibition of neutrophils and macrophages seems to be correlated to HARBS (for review, see Tenor and Schudt, 1999), the conclusion can be drawn that the improved ability of roflumilast (and the other benzamides) to inhibit monocytes, T cells, and dendritic cells versus neutrophils, macrophages, and eosinophils provides indirect evidence for an improved ability of the benzamides to interact with LARBS versus HARBS. This is in contrast to rolipram but, surprisingly, in our hands also to cilomilast, which has been postulated as a second-generation PDE4 inhibitor having a favored interaction with LARBS opposed to HARBS. The reason for this discrepancy is unclear but may be resolved by a detailed analysis 1) of the interaction of these PDE4 inhibitors with all known PDE4 splicing variants and 2) the participation of (probably different) splicing variants in the regulation of the various inflammatory and immunomodulatory cells (corresponding experiments are in progress and will be reported separately).

In summary, we have shown that roflumilast as well as its primary metabolite roflumilast N-oxide are potent and selective PDE4 inhibitors with broad anti-inflammatory and immunomodulatory actions in vitro. These features, covered by a novel mode of action, translate to the in vivo situation of animal models (see the accompanying article by Bundschuh et al., 2001) and hopefully will also permit clinically relevant
benefits in patients. The testing of roflumilast in clinical studies covering airway diseases is ongoing.

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References


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