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RESEARCH PAPER

Inhibition of cyclooxygenase-2 prevents adverse effects induced by phosphodiesterase type 4 inhibitors in rats

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BACKGROUND AND PURPOSE

Phosphodiesterase type 4 (PDE4) inhibitors such as roflumilast are currently being developed as anti-inflammatory treatments for chronic airway disorders. However, high doses of PDE4 inhibitors have also been linked to several side effects in different animal species, including pro-inflammatory effects in the rat. Here, we analysed PDE4-related toxicological findings in a rat model and how these side effects might be therapeutically prevented.

EXPERIMENTAL APPROACH

Wistar rats were treated orally once daily with 10 mg·kg^{-1} roflumilast for 4 days. Macroscopic changes were monitored throughout the study and further parameters were analysed at the end of the experiment on day 5. In addition, the effects of concomitant treatment with cyclooxygenase (COX) inhibitors were assessed.

KEY RESULTS

Supratherapeutical treatment with roflumilast induced marked body and spleen weight loss, diarrhea, increased secretory activity of the harderian glands, leukocytosis, increased serum cytokine-induced neutrophil chemoattractant-1 (CINC-1) levels, and histopathological changes in thymus, spleen, mesentery and mesenteric lymph nodes. All these toxicological findings could be prevented by the non-steroidal anti-inflammatory drug (NSAID) and non-selective COX inhibitor, diclofenac, given orally. Similar protective effects could be achieved by the COX-2 selective inhibitor lumiracoxib, whereas the COX-1 selective inhibitor SC-560 was generally not effective.

CONCLUSIONS AND IMPLICATIONS

Treatment with an NSAID inhibiting COX-2 prevents the major effects found after subchronic overdosing with the PDE4-specific inhibitor roflumilast. If this effect translates into humans, such combined treatment may increase the therapeutic window of PDE4 inhibitors, currently under clinical development.

Abbreviations

BAL, bronchoalveolar lavage; CINC-1, cytokine-induced neutrophil chemoattractant-1; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; KC, keratinocyte-derived chemokine; LPS, lipopolysaccharide; NSAID, non-steroidal anti-inflammatory drug; PDE4, phosphodiesterase type 4; WBC, white blood cells

Introduction

It has been well-recognized that the anti-inflammatory effects of the second messenger cAMP on a broad range of pro-inflammatory and immunocompetent cells can be reinforced by blocking the main cAMP degrading capacity in these cells, the phosphodiesterase type 4 (PDE4) family (Torphy,

1998; Souness *et al.*, 2000). Having demonstrated efficacy in various *in vitro* settings and *in vivo* models, PDE4 inhibitors are currently developed as a therapeutic treatment option for chronic inflammatory diseases such as chronic obstructive pulmonary disease. PDE4 inhibitors were shown to actively suppress inflammation in the airways, with roflumilast [3-cyclo-propylmethoxy-4-difluoromethoxy-N-(3,5-di-chloropyrid-4-yl)-benzamide] being the most advanced PDE4 inhibitor (Lipworth, 2005; Spina, 2008; Cazzola *et al.*, 2010). However, the clinical development of PDE4 inhibitors has been accompanied and hampered by the occurrence of adverse gastrointestinal and emetic side effects, limiting the therapeutic doses and clinical efficacy of PDE4 inhibitors. Although second-generation PDE4 inhibitors display an improved tolerability window, common adverse effects that were occasionally reported encompass nausea, vomiting, diarrhea, headache, abdominal pain and dyspepsia (Rabe *et al.*, 2005; Giembycz, 2006; Calverley *et al.*, 2007; Rennard *et al.*, 2008).

In preclinical studies using supratherapeutical doses of PDE4 inhibitors, similar and additional disadvantages of targeting PDE4 have been demonstrated. Repeated administration of doses up to 100 $mg \cdot kg^{-1}$ of the archetypical PDE4 inhibitor rolipram for up to 2 weeks in Sprague-Dawley rats induced a loss of body weight and emaciation as well as histopathological changes of the heart, vasculature, mesentery, stomach and salivary glands (Larson *et al.*, 1996). Also more recent second-generation PDE4 inhibitors of different compound classes caused toxicological anomalies in rodent species, interestingly of a generally pro-inflammatory nature. Thus, the PDE4 inhibitor CI-1018 given orally at 750 $mg \cdot kg^{-1}$ for 4 days to Wistar rats has been shown to induce vascular lesions in the mesentery characterized by medial necrosis, haemorrhage, and/or oedema accompanied by perivascular mixed inflammatory cell infiltrates (Slim *et al.*, 2002). Follow-up experiments using 80 $mg \cdot kg^{-1} \cdot day^{-1}$ for 3 days of CI-1044, a very similar but more potent PDE4 inhibitor than CI-1018, resulted again in vascular injury in mesenteric tissue (Dagues *et al.*, 2007b). In another study, the authors concluded that the PDE4 inhibitor BYK169171, given at a dose of 10 $mg \cdot kg^{-1} \cdot day^{-1}$ for up to 28 days to Wistar rats, did not cause a primary vasculitis/arteritis, but induced a non-purulent inflammation in the mesentery that preceded the segmental necrosis of the vessels (Mecklenburg *et al.*, 2006). In a toxicological study from Dietsch *et al.* (2006) with Sprague-Dawley rats that received 100 $mg \cdot kg^{-1} \cdot day^{-1}$ of the PDE4 inhibitor IC542 for up to 2 weeks, clinical signs such as body weight loss, evidence of diarrhea, piloerection or decreased activity were observed. Histopathologically, an inflammatory response was noted that resulted in tissue damage primarily in the mesentery and the gastrointestinal tract (Dietsch *et al.*, 2006). IC542 treatment was also reported to lead to increased peripheral neutrophil numbers and serum IL-6, haptoglobin and fibrinogen levels and to reduced serum albumin levels that may have prognostic value as biomarkers to identify rats that have no or only minimal histological changes. In a murine study using BALB/c mice and subcutaneous administration of up to 100 $mg \cdot kg^{-1}$ of the PDE4 inhibitors piclamilast or roflumilast, both anti- and pro-inflammatory properties were reported (McCluskie *et al.*, 2006). Both PDE4 inhibitors were effective in suppressing neutrophil influx into the bronchoalveolar lavage (BAL) and tumour necrosis factor (TNF)α production in the BAL after lipopolysaccharide (LPS) challenge, but the inhibitors were shown to increase plasma/lung tissue KC (keratinocyte-derived chemokine, the mouse homologue of human IL-8) and lung tissue neutrophils if administered alone at 100 $mg \cdot kg^{-1}$. Although the authors proposed endothelial cells as potential source for KC/IL-8, the mechanism of the reported pro-inflammatory activities of the two PDE4 inhibitors used in this study as well as the underlying pro-inflammatory mechanism of the other PDE4 inhibitors mentioned earlier remains elusive.

In the present study, the objective was to clarify whether the PDE4 inhibitor roflumilast may also have direct anti- and pro-inflammatory properties in rats, and if so, how these unwanted adverse effects may be reduced. Thus, we initially explored the efficacy of roflumilast to block LPS-driven neutrophil influx into the BAL of rats and demonstrated that roflumilast suppressed the acute lung inflammation with an ID_{50} of 1 $mg \cdot kg^{-1}$. Next, we investigated whether a 5 day short-term tolerability model in rats would be useful as an early predictive model for the rapid toxicological assessment of PDE4 inhibitors. For this purpose, rats were treated orally for 4 consecutive days with 10 $mg \cdot kg^{-1} \cdot day^{-1}$ of roflumilast followed by the evaluation of toxicity at day 5. Remarkably, the observed adverse effects could be substantially reduced or even abolished by coadministration of therapeutic doses of the cyclooxygenase (COX)-1/-2 non-selective inhibitor diclofenac and the COX-2 selective inhibitor lumiracoxib, whereas the COX-1 selective inhibitor SC-560 had no protective effects on roflumilast-mediated effects. Because the inhibition of COX-2 had no effect on the efficacy of roflumilast in an acute LPS-driven lung inflammation model, we con-

clude that coadministration of non-steroidal anti-inflammatory drugs (NSAIDs) inhibiting COX-2 with PDE4 inhibitors could be beneficial by increasing the therapeutic window of PDE4 inhibitors.

Methods

Animals

All animal care and experiments were conducted in accordance with German national guidelines and legal regulations with approval from the Veterinary Authorities of the Regierungspräsidum Tübingen, Germany. Male Wistar rats (200–250 g for acute lung inflammation experiments; or 300–350 g for tolerability experiments) were obtained from Charles River Laboratories (Kißlegg, Germany). Before the experiments, animals were group-housed for at least 1 week in a climate-controlled environment with a 12-hour light/dark cycle in the animal facility of Boehringer Ingelheim Pharma GmbH & Co. KG. For the acute lung inflammation experiments, the animals were fasted overnight. For the tolerability experiments, food and water were provided *ad libitum*.

Design: LPS-driven acute lung inflammation model

The amount of compounds used for the different experiments is indicated in the figures or mentioned in the text as data not shown. Roflumilast and lumiracoxib were given p.o. in 0.5% natrosol (Merck, Darmstadt, Germany). Roflumilast or vehicle were administered 2 h, lumiracoxib or vehicle were administered 1 h before the aerosol challenge with LPS (L2880, Charge 066K4039; 3 mg·mL^{-1}; 30 min). Animals used for the negative control group or the LPS control group were also treated p.o. with the respective solvent used for the test compound. Four hours after the end of LPS nebulization, BAL was prepared as previously described (Dong *et al.*, 2003). Aliquots of lavage fluids were measured in a haemacytometer (Bayer Advia 120 Hematology Analyzer, Siemens Healthcare Diagnostics, Deerfield, USA) to calculate the neutrophil content.

Design: 5-day short-term tolerability rat model

In order to design a predictive short-term tolerability rat model that allows a comprehensive monitoring of roflumilast-mediated effects with predefined read-outs within 5 days, six male Wistar rats per group received daily oral dosages of 0, 2.5 and 10 mg·kg^{-1} roflumilast respectively, for 4 consecutive days. One animal per dose group was sacrificed on day 4 for peak pharmacokinetic analysis of plasma drug concentrations. During the study, body weight and clinical signs (e.g. diarrhea and harderian gland secretion) were monitored. At the end of the study at day 5, animals were sacrificed and their spleen weights were determined. Additionally, haematology, plasma cytokine-induced neutrophil chemoattractant-1 (CINC-1) analysis and histopathological examination of thymus, spleen, mesentery and mesenteric lymph nodes were conducted. Because the daily dose of 10 mg·kg^{-1} roflumilast induced clear adverse effects but was just tolerated, it was used as standard dose in further 5-day short-term tolerability experiments. In these follow-up experiments, 1 mg·kg^{-1} of the NSAID diclofenac (non-selective inhibitor of COX-1 and COX-2) was given orally 5 h before and 4 h after each administration of roflumilast on day 1 to 4 respectively. The latter experiment was repeated using 2 mg·kg^{-1} lumiracoxib (a COX-2 selective inhibitor) or 2 mg·kg^{-1} SC-560 (a COX-1 selective inhibitor), given orally 5 h before and 4 h after each administration of roflumilast respectively.

Histopathology

For standardized histopathological examination, tissue samples from mesentery, thymus, spleen and mesenteric lymph nodes from the 5-day short-term tolerability experiment were fixed in 10% neutral-buffered formalin, trimmed, dehydrated in a graded series of ethanol, cleared in xylol, embedded in paraffin, sectioned at a thickness of 3 µm and stained with haematoxylin/eosin. Pertinent histopathological changes were cellular depletion of thymus, spleen and mesenteric lymph nodes, and vascular/perivascular inflammation of arteries in the mesentery accompanied by hemorrhages, plasma exudates and fibroblast proliferation. The mesenteric changes were graded according to a combined set of criteria previously described by Joseph *et al.* (1996). The MSB (Martius, Scarlet, Blue) van Gieson and Giemsa techniques were used to stain fibrin, collagen fibres and inflammatory cells respectively.

Clinical pathology: haematology and enzyme-linked immunosorbent assays

At the end of the 5-day short-term tolerability experiment, blood was collected for haematology and clinical chemistry analysis. Blood samples (~1 mL volume) for haematological analyses were collected in Microvette capillary blood tubes (Sarstedt, Nümbrecht, Germany) containing lithium heparin, mixed by inversion and analysed using a haemacytometer (Advia 120) for white blood cell count and percentages of neutrophils. In parallel, for clinical chemistry determinations, blood

samples (~1 mL volume) were collected and centrifuged for 10 min at 120× *g*. Supernatant was transferred to polystyrene multiwell plates for use in an enzyme-linked immunosorbent assay for the detection of CINC-1 (the rat orthologue of human CXCL1; R&D Systems, Minneapolis, MN, USA), according to the protocol provided by the supplier.

Detection of plasma levels of the administered inhibitors

In order to control peak levels of all administered compounds, one animal per group was anaesthetized with isoflurane and retro-orbital blood withdrawn (~0.2 mL) on day 4 at time points when peak levels of the respective compounds were expected, that is, 30 min after roflumilast administration, 30 min after diclofenac administration and 1 h after lumiracoxib or SC-560 administration respectively. Plasma was transferred to polystyrene multiwell plates for standard HPLC analysis. The selection of the time points for plasma level determination was based on previous pharmacokinetic experiments or was extrapolated from published data. Additionally, plasma roflumilast levels were determined at the end of the 5-day experiment in all animals.

Statistical analysis

Averages are presented as mean ± SEM. For statistical analysis, unpaired Student's *t*-test, one-way ANOVA with Dunnett's post test, or two-way ANOVA with Bonferroni's post test was performed using GraphPad Prism 5.02, GraphPad Software, San Diego, USA. Using the same software, the ID_{50} concentration of roflumilast for half-maximal inhibition in the LPS model was calculated from inhibition curves by nonlinear regression analysis [log(inhibitor) vs. normalized response, variable slope].

Materials

Roflumilast [3-cyclo-propylmethoxy-4-difluoromethoxy-N-(3,5-di-chloropyrid-4-yl)-benzamide] and lumiracoxib ({2-[(2-chloro-6-fluorophenyl)amino]-5-methylphenyl} acetic acid) were synthesized at the chemical facilities of Boehringer Ingelheim Pharma GmbH & Co. KG (Biberach, Germany) essentially as described in the corresponding patents and were authenticated using mass and NMR spectroscopy. Additionally, lumiracoxib was purchased from Sequoia Research (Pangbourne, UK). Diclofenac {2-[(2,6-Dichlorophenyl)amino]benzene acetic acid sodium salt} was acquired from Sigma-Aldrich (Munich, Germany). SC-560 [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole] was purchased from AXXORA (Loerrach, Germany). All other chemicals not specifically mentioned were of analytical grade and were obtained from Sigma-Aldrich. For all *in vivo* studies, inhibitors were resuspended in 0.5% aqueous hydroxymethylcellulose (natrosol) and administered at 10 mL·kg^{-1} by oral gavage. The control groups received vehicle only. Drug and receptor nomenclature follows Alexander *et al.* (2009).

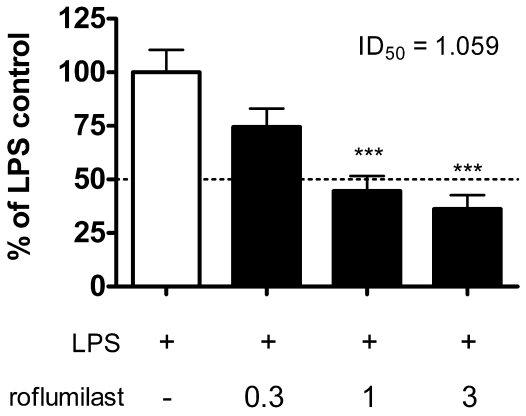

Figure 1

Roflumilast significantly decreases lipopolysaccharide (LPS)-induced acute lung inflammation. Two hours prior challenge, rats were treated with vehicle or with roflumilast (0.3, 1 or 3 mg·kg^{-1} respectively). Neutrophil influx into the bronchoalveolar lavage (BAL) was measured 4 h after challenging with aerosolized LPS (3 mg·mL^{-1} for 30 min). Values are in % of LPS control (set to 100%) and are expressed as mean + SEM using eight animals per treatment. Absolute neutrophil numbers in the BAL of LPS-treated animals were 1.4 ± 0.1 × 10^3 cells·µL^{-1}. The ID_{50} value was calculated from inhibition curves by nonlinear regression analysis [log(inhibitor) vs. normalized response, variable slope]. Statistical analysis was performed using one-way ANOVA followed by a Dunnett's post test; ***$P < 0.001$; compared to LPS group.

Results

Roflumilast inhibits LPS-driven lung inflammation in the rat

As roflumilast has proven efficacy in various preclinical models of inflammation, we investigated whether roflumilast suppressed neutrophil influx into the BAL in an acute LPS-driven lung inflammation model in the rat. Inhalation of aerosolized LPS resulted in a profound invasion of neutrophils into the lungs of Wistar rats, peaking at 1.4 ± 0.1 × 10^3 neutrophils·µL^{-1} in the BAL of LPS-treated animals. Roflumilast, administered p.o. 2 h before exposure to LPS, dose-dependently inhibited BAL neutrophilia with an ID_{50} of 1 mg·kg^{-1} (Figure 1).

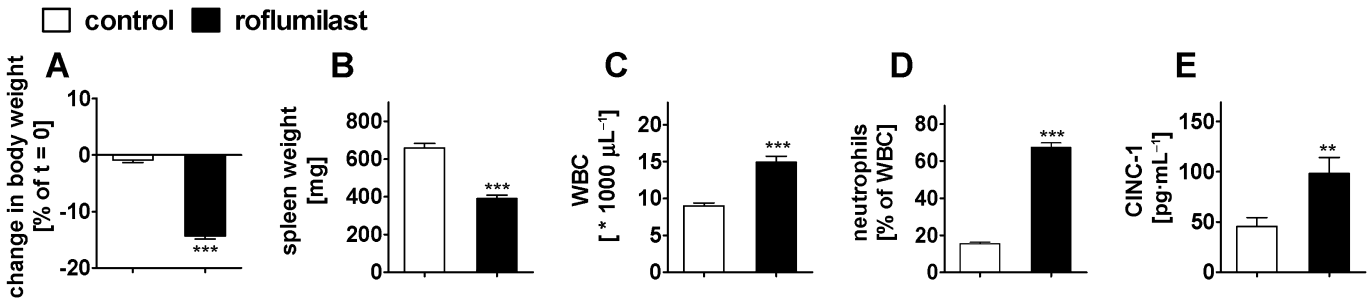

Figure 2

Roflumilast significantly decreases body weight and spleen weight, and significantly increases leukocytosis, blood neutrophilia and plasma cytokine-induced neutrophil chemoattractant-1 (CINC-1) levels after 5 days. Rats were treated with vehicle (group 'control') or with roflumilast (10 mg·kg^{-1}·day^{-1}; group 'roflumilast') for 4 consecutive days. (A) Body weight at the end of the experiment (day 4 for one animal per group; day 5 for five animals per group respectively) is shown as % change from the corresponding pretreatment value at the start of the experiment on day 1 at 8 a.m. (t = 0) and is given as mean + SEM. The initial mean absolute body weight was 326 ± 7 g. Reported data are from three independent experiments with total animal numbers of n = 18. At day 5, animals were killed for (B) macroscopic analysis (spleen weight), for (C, D) haematological analysis [absolute white blood cells (WBC) and % blood neutrophils] and for (E) ELISA measurement of plasma CINC-1. Values are expressed as mean + SEM of four independent experiments with total animal numbers of n = 19–20. One animal in the roflumilast group died early, during the night of day 4 and was excluded. Statistical analysis was performed using unpaired Student's t-tests; **P < 0.01, ***P < 0.001; compared to control group.

A rat 5-day short-term tolerability model reflects major characteristic roflumilast-mediated effects

In order to get a comparatively short, but predictive assessment of the effects of a PDE4 inhibitor, we designed a short-term tolerability model in rats that allowed a comprehensive monitoring of roflumilast-mediated effects with predefined read-outs within 5 days. Consequently, six male Wistar rats per group initially received a daily oral dose of 0, 2.5 and 10 mg·kg^{-1} roflumilast respectively, for 4 consecutive days. The daily oral dose of 10 mg·kg^{-1} roflumilast generated higher and more robust changes compared to the 2.5 mg·kg^{-1} group, but was generally still tolerated (data not shown). Thus, 10 mg·kg^{-1} (which was 10 times the ID_{50} of roflumilast in our LPS-driven acute lung inflammation model) was chosen as standard dose in the 5-day short-term tolerability model for further experiments. This dose induced pertinent changes of clinical, haematological and clinical chemistry parameters, such as significant body weight loss (up to 14% at day 5), spleen weight loss (1.7-fold decrease), leukocytosis (1.7-fold increase in white blood cells), blood neutrophilia (4.3-fold increase in per cent blood neutrophils) and elevated plasma CINC-1 levels (2.2-fold increase; see Figure 2). Additionally, animals treated with roflumilast showed a substantial incidence of diarrhea and increased secretion of harderian glands on day 4 (Table 1). Plasma concentrations at the estimated peak time of roflumilast (30 min after administration) were 119 ± 24 nM for the parental compound and 992 ± 383 nM for the major, similarly active N-oxide metabolite. At the termination of the experiment on day 5 (18 h after the last roflumilast administration), roflumilast levels were 48.6 ± 16.3 nM and roflumilast N-oxide levels were 439 ± 109 nM.

Most pertinent histopathological changes observed in the roflumilast-treated groups were cellular depletion of the thymus, the spleen and the mesenteric lymph nodes, and multifocal perivascular mononuclear/polymorphonuclear infiltration with plasma exudates and fibroblast proliferation in the mesentery (Table 1). Minimal to moderate focal/multifocal hemorrhages in the mesentery observed in the roflumilast group also occurred in the control group, indicating that these hemorrhages are most likely due to the preparation technique and are not mechanism-related.

The NSAID diclofenac prevents roflumilast-mediated adverse effects

Because COX-2 may be induced by cAMP-elevating agents (Klein *et al.*, 2007), we next assessed whether the roflumilast-mediated effects may be triggered via the COX pathway. For therapeutic modulation of COX, we used the NSAID diclofenac, an almost equipotent inhibitor of the two isoenyzmes of COX, COX-1 and COX-2, as measured biochemically and in human whole blood assays (Esser *et al.*, 2005; Sud'ina *et al.*, 2008). Considering the rapid and short half-life of COX-inhibitors, the non-ulcerogenic dose of 1 mg·kg^{-1} of diclofenac was given orally 5 h before and 4 h after each administration of roflumilast on day 1 to 4 respectively. The plasma concentration at the estimated peak time of diclofenac at 30 min after administration (Esser

Table 1

Summary of 5-day short-term tolerability observations proposed as standard parameters for the rapid assessment of the toxicity of roflumilast

Short-term tolerability observations	Control [incidence (%)]	Roflumilast [incidence (%)]
Ante-mortem observations:		
Diarrhea at day 4	0/24 (0 out of 24; 0%)	11/24 (46%)
Secretion of harderian glands at day 4	0/24 (0%)	16/24 (67%)
Histopathological findings at day 5:		
Thymus:		
Minimal to moderate cellular depletion	3/20 (15%)	0/19 (0%)
Marked cellular depletion	0/20 (0%)	18/19 (95%)
Spleen:		
Minimal to moderate lymphoid depletion	2/20 (10%)	5/19 (26%)
Marked lymphoid depletion	0/20 (0%)	14/19 (74%)
Mesenteric lymph node:		
Minimal to moderate cellular depletion	0/20 (0%)	12/19 (63%)
Marked cellular depletion	0/20 (0%)	4/19 (21%)
Mesentery:		
Minimal to moderate focal/multifocal hemorrhages	17/20 (85%)	19/19 (100%)
Minimal to moderate multifocal perivascular mononuclear/polymorphonuclear infiltration with plasma exudates and fibroblast proliferation	0/20 (0%)	8/19 (42%)
Marked multifocal perivascular mononuclear/polymorphonuclear infiltration with plasma exudates and fibroblast proliferation	0/20 (0%)	11/19 (58%)

In each experiment, six male Wistar rats were treated for 4 consecutive days with 10 $mg \cdot kg^{-1} \cdot day^{-1}$ roflumilast. Data shown are accumulated from four independent experiments; total animal numbers $n = 20–24$ (control group) and $n = 19–24$ (roflumilast group). One animal in the roflumilast group died early, during the night of day 4 and no samples were taken from this animal.

et al., 2005) was 616 nM. The plasma concentrations of roflumilast analysed at the end of the experiment at day 5 in the roflumilast + diclofenac group were lower than in the roflumilast group, but these levels still provoked toxicological effects in other short-term tolerability experiments (data not shown).

Coadministration of diclofenac significantly prevented roflumilast-mediated body weight loss, spleen weight loss, leukocytosis, blood neutrophilia and induction of serum CINC-1 (Figure 3). Notably, diclofenac could totally prevent the spleen weight loss, increase in absolute white blood cells and elevation of CINC-1 levels. Furthermore, as summarized in Table 2, coadministration of diclofenac fully prevented roflumilast-mediated diarrhea and increased secretion of harderian glands as well as all the monitored histopathological changes described above. Diclofenac alone had no effects on all monitored parameters.

The COX-2 selective inhibitor lumiracoxib, but not the COX-1 selective inhibitor SC-560 prevents roflumilast-mediated effects

In order to distinguish the role of COX-1 versus COX-2 in mediating the effects induced by roflumilast, a 5-day short-term tolerability experiment was performed using roflumilast with and without coadministration of 2 $mg \cdot kg^{-1}$ SC-560 or lumiracoxib, given orally 5 h before and 4 h after administration of roflumilast respectively. SC-560 is a COX inhibitor of the diaryl heterocycle class that is highly selective for COX-1 (Smith *et al.*, 1998; Sud'ina *et al.*, 2008), whereas lumiracoxib is chemically related to diclofenac, but is highly selective for COX-2 (Esser *et al.*, 2005; Sud'ina *et al.*, 2008). Coadministration of lumiracoxib but not of SC-560 substantially prevented roflumilast-mediated body weight loss, spleen weight loss, leukocytosis, blood neutrophilia and induction of serum CINC-1 (Figure 4). The protective effect of lumiracoxib on spleen weight was not statistically significant, but lumiracoxib did fully block the increase in white blood cells and CINC-1, and almost fully blocked the increase in blood neutrophil levels, whilst SC-560 had no protective effects. The selective COX inhibitors alone had no effects on these investigated parameters (Figure 4). Although the coadministration of SC-560 decreased diarrhea and secretion of harderian glands in the roflumilast + SC-560 group, SC-560 had overall no or little protective effects on

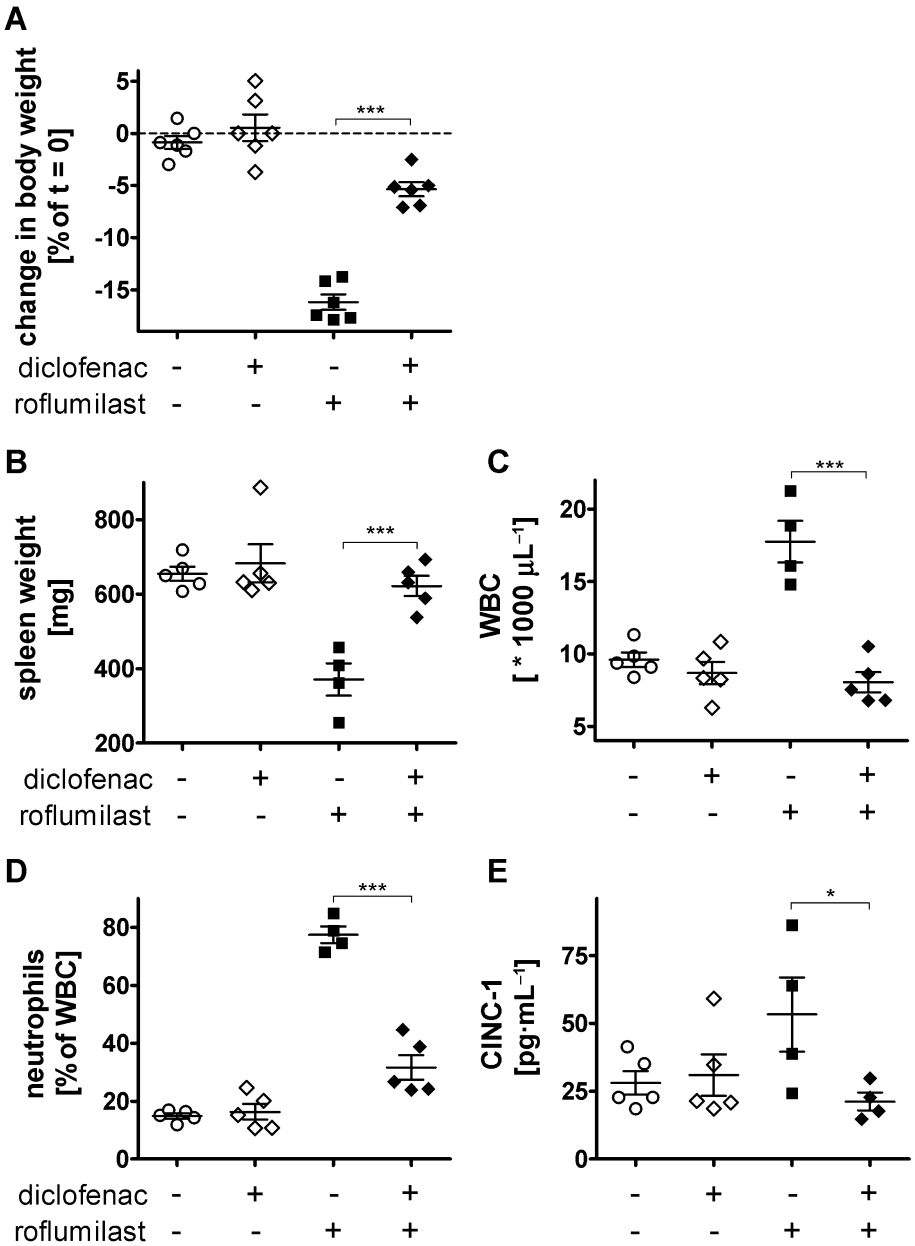

Figure 3

Diclofenac prevents roflumilast-mediated adverse effects. Rats were treated with vehicle (group 'control'), with diclofenac alone (2 mg·kg^{-1}·day^{-1}; group 'diclofenac'), with roflumilast alone (10 mg·kg^{-1}·day^{-1}; group 'roflumilast'), or with roflumilast and diclofenac (10 mg·kg^{-1}·day^{-1} roflumilast and 2 mg·kg^{-1}·day^{-1} diclofenac; group 'roflumilast + diclofenac') for 4 consecutive days. (A) Body weight at the end of the experiment (day 4 for one animal per group; day 5 for five animals per group respectively) is shown as % change from the corresponding pretreatment value at the start of the experiment on day 1 at 8 a.m. (t = 0) and is given as mean ± SEM (scatter dot plot). The initial mean absolute body weight was 353 ± 9 g. At day 5, animals were killed for (B) macroscopic analysis (spleen weight), for (C, D) haematological analysis [absolute white blood cells (WBC) and % blood neutrophils] and for (E) ELISA measurement of plasma cytokine-induced neutrophil chemoattractant-1 (CINC-1). Values are expressed as mean ± SEM (scatter dot plot) of one experiment with five animals per group. Statistical analysis was performed using two-way ANOVA followed by a Bonferroni's post test; *P < 0.05, ***P < 0.001.

the histopathological changes (Table 2). In contrast, coadministration of lumiracoxib had profound protective effects on all monitored ante- and post-mortem parameters. More precisely, lumiracoxib fully blocked roflumilast-mediated diarrhea and secretion of harderian glands, cellular depletion of the thymus and the mesenteric lymph nodes, and minimal to moderate and marked multifocal perivascular mononuclear/polymorphonuclear infiltration with plasma exudates and fibroblast

Table 2

Summary of protective effects of the COX-inhibitors diclofenac, lumiracoxib and SC-560 on pertinent roflumilast-mediated effects

Short-term tolerability observations	Control	Roflumilast	Roflumilast + diclofenac [incidence (%)]	Roflumilast + SC-560	Roflumilast + lumiracoxib
Ante-mortem observations:					
Diarrhea at day 4	0/12 (0%)	6/12 (50%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
Secretion of harderian glands at day 4	0/12 (0%)	11/12 (92%)	0/6 (0%)	2/6 (40%)	0/6 (0%)
Histopathological findings at day 5:					
Thymus:					
Minimal to moderate cellular depletion	0/10 (0%)	0/9 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Marked cellular depletion	0/10 (0%)	8/9 (89%)	0/5 (0%)	4/5 (80%)	0/5 (0%)
Spleen:					
Minimal to moderate lymphoid depletion	0/10 (0%)	3/9 (33%)	0/5 (0%)	2/5 (40%)	1/5 (20%)
Marked lymphoid depletion	0/10 (0%)	6/9 (67%)	0/5 (0%)	2/5 (40%)	0/5 (0%)
Mesenteric lymph node:					
Minimal to moderate cellular depletion	0/10 (0%)	6/9 (67%)	0/5 (0%)	2/5 (40%)	0/5 (0%)
Marked cellular depletion	0/10 (0%)	0/9 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Mesentery:					
Minimal to moderate focal/multifocal hemorrhages	10/10 (100%)	9/9 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
Minimal to moderate multifocal perivascular mononuclear/polymorphonuclear infiltration with plasma exudates and fibroblast proliferation	0/10 (0%)	1/9 (11%)	0/5 (0%)	2/5 (40%)	0/5 (0%)
Marked multifocal perivascular mononuclear/polymorphonuclear infiltration with plasma exudates and fibroblast proliferation	0/10 (0%)	8/9 (89%)	0/5 (0%)	2/5 (40%)	0/5 (0%)

In an initial experiment, six male Wistar rats were treated for 4 consecutive days with roflumilast ± concomitant administration of the COX-inhibitor diclofenac. In a follow-up experiment, six male Wistar rats were treated for 4 consecutive days with roflumilast ± concomitant administration of the COX-1 selective inhibitor SC-560 or ± concomitant administration of the COX-2 selective inhibitor lumiracoxib respectively, as detailed in Materials and Methods. Data shown are accumulated from these two independent experiments; total animal numbers $n = 10–12$ (control group), $n = 9–12$ (roflumilast group), $n = 5–6$ (other groups respectively). One animal in the roflumilast group died early during the night of day 4 and no samples were taken from this animal.

proliferation in the mesentery. Only one animal in the roflumilast + lumiracoxib group showed slight lymphoid depletion of the spleen. In summary, lumiracoxib but not SC-560 could efficiently inhibit or even blunt roflumilast-mediated effects in this setting, while SC-560 and lumiracoxib alone had no effects on the observed ante- and post-mortem parameters.

The measured plasma concentration at the estimated peak time of lumiracoxib 1 h after administration was 5.4 μM, suggesting full and selective blockade of COX-2 (Esser *et al.*, 2005). Although SC-560 may have an estimated peak time of 3–4 h (Teng *et al.*, 2003; Teng and Davies, 2004), we measured plasma concentrations of SC-560 parallel to lumiracoxib at 1 h and found 115 nM plasma SC-560. Considering published *in vitro* and *in vivo* data, dosing and plasma levels of SC-560 applied in our experimental setting suggests full and selective blockade of COX-1 (Smith *et al.*, 1998; Sud'ina *et al.*, 2008). The plasma concentrations of roflumilast measured at the end of the experiment at day 5 in the roflumilast + SC-560/lumiracoxib groups were lower than in the roflumilast group, but, as indicated before, were in a range that provoked toxicological effects in other short-term tolerability experiments (data not shown).

COX-2 inhibition does not alter the efficacy of roflumilast in LPS-driven lung inflammation

As we found that concomitant COX-2 inhibition could prevent roflumilast-mediated adverse effects, we were interested to know whether inhibition of COX-2 may have any effect (beneficial or detrimental) on the efficacy of roflumilast in our previously

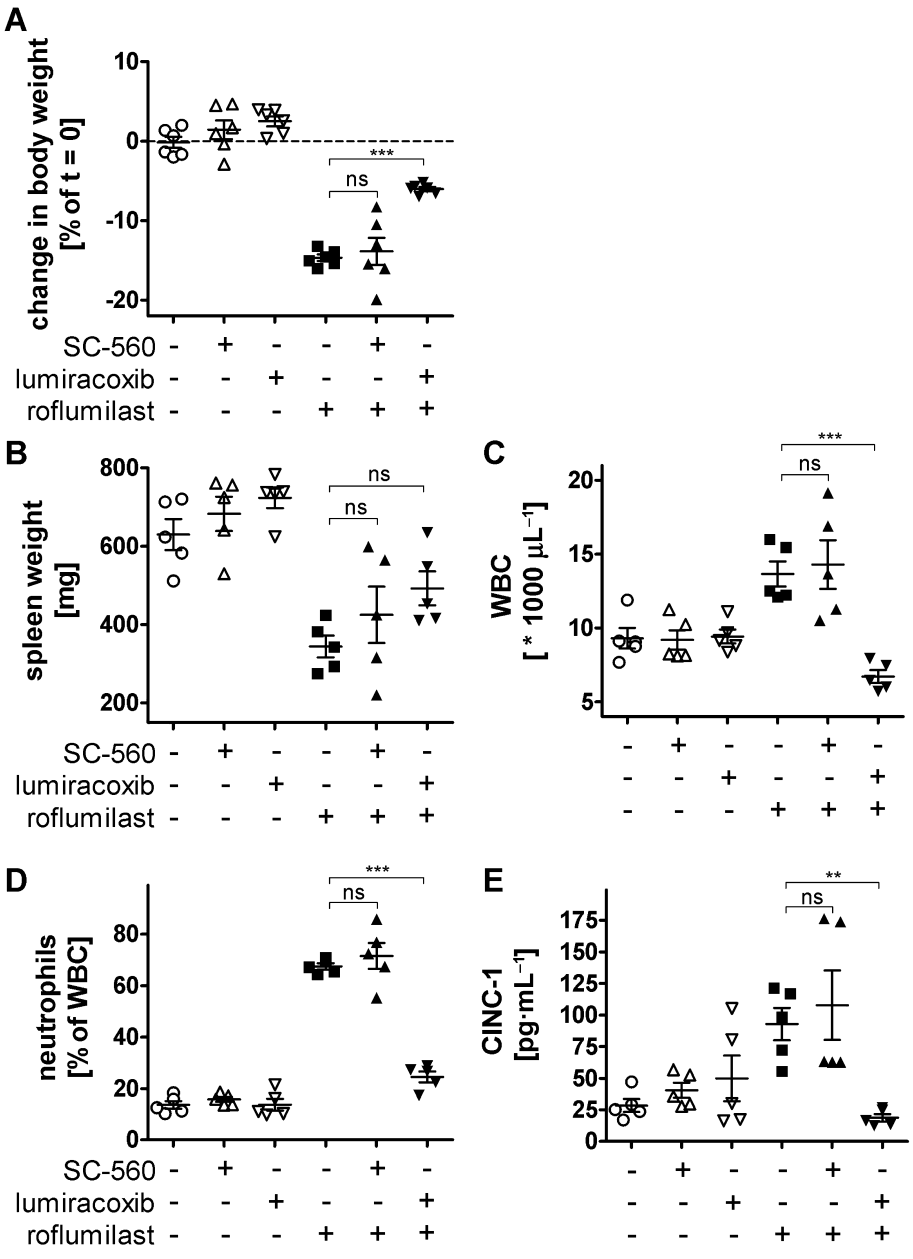

Figure 4

The COX-2 selective inhibitor lumiracoxib, but not the COX-1 selective inhibitor SC-560 prevents roflumilast-mediated effects. Rats were treated with vehicle (group 'control'), with SC-560 alone (4 mg·kg^{-1}·day^{-1}; group 'SC-560'), with lumiracoxib alone (4 mg·kg^{-1}·day^{-1}; group 'lumiracoxib'), with roflumilast alone (10 mg·kg^{-1}·day^{-1}; group 'roflumilast'), with roflumilast and SC-560 (10 mg·kg^{-1}·day^{-1} roflumilast and 4 mg·kg^{-1}·day^{-1} SC-560; group 'roflumilast + SC-560'), or with roflumilast and lumiracoxib (10 mg·kg^{-1}·day^{-1} roflumilast and 4 mg·kg^{-1}·day^{-1} lumiracoxib; group 'roflumilast + lumiracoxib') for 4 consecutive days. (A) Body weight at the end of the experiment (day 4 for one animal per group; day 5 for five animals per group respectively) is shown as % change from the corresponding pretreatment value at the start of the experiment on day 1 at 8 a.m. ($t = 0$) and is given as mean ± SEM (scatter dot plot). The initial mean absolute body weight was 306 ± 5 g. At day 5, animals were killed for (B) macroscopic analysis (spleen weight), for (C, D) haematological analysis [absolute white blood cells (WBC) and % blood neutrophils] and for (E) ELISA measurement of plasma cytokine-induced neutrophil chemoattractant-1 (CINC-1). Values are expressed as mean ± SEM (scatter dot plot) of one experiment with five animals per group. Statistical analysis was performed using two-way ANOVA followed by a Bonferroni's post test; $^{**}P < 0.01$, $^{***}P < 0.001$. ns, not significant.

applied acute LPS-driven lung inflammation model. Lumiracoxib (at the dose of 2 mg·kg^{-1} that was used in the short-term tolerability experiment) administered 1 h after administration of roflumilast had no effect on the anti-inflammatory efficacy and potency of roflumilast (Figure 5). In this setting, lumiracoxib alone showed no significant effects up to 10 mg·kg^{-1}.

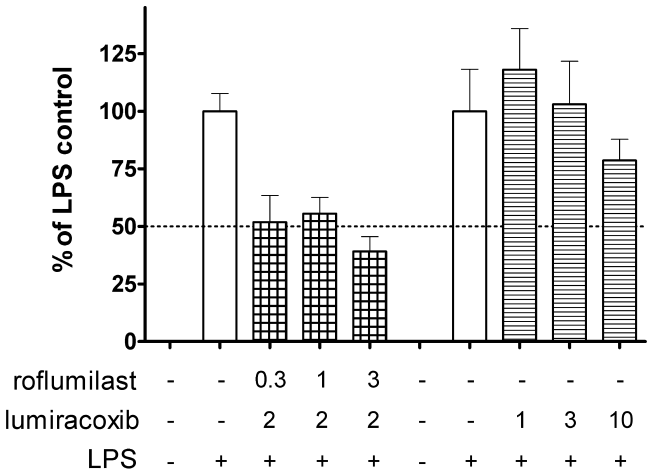

Figure 5

The COX-2 selective inhibitor lumiracoxib has no effect on the efficacy of roflumilast in the lipopolysaccharide (LPS)-induced acute lung injury model. Two hours prior to challenge, rats were treated with vehicle or with roflumilast (0.3, 1, or 3 mg·kg^{-1} respectively). Lumiracoxib or vehicle was administered 1 h before challenge (1, 2, 3 or 10 mg·kg^{-1} respectively). Neutrophil influx into the bronchoalveolar lavage was measured 4 h after challenging with aerosolized LPS (3 mg·mL^{-1} for 30 min). Values are in % of LPS control (set to 100%) and are expressed as mean + SEM using eight animals per treatment.

Discussion and conclusions

The objective of this study was to assess directly anti- and pro-inflammatory effects of the PDE4 inhibitor roflumilast in Wistar rats and to elucidate whether roflumilast-mediated adverse effects induced in a toxicological setting could be therapeutically prevented.

Firstly, we confirmed that roflumilast, a highly potent and selective PDE4 inhibitor (Hatzelmann and Schudt, 2001), showed *in vivo* efficacy by inhibiting LPS-aerosol induced influx of neutrophils into the BAL of Wistar rats. The half-maximal inhibitory dosis of orally administered roflumilast that we determined in our model (ID_{50} = 1 mg·kg^{-1}) agrees well with previously published preclinical data showing similar anti-inflammatory efficacy of roflumilast in other rat animal models of pulmonary inflammation (Bundschuh *et al.*, 2001; Wollin *et al.*, 2006; Cortijo *et al.*, 2009). Likewise, s.c. administration of roflumilast was reported to suppress LPS aerosol-induced neutrophil influx into the BAL of BALB/c mice with 62% inhibition at 3 mg·kg^{-1} (McCluskie *et al.*, 2006). Paradoxically, the latter report also revealed unexpected pro-inflammatory effects of roflumilast at higher concentrations (100 mg·kg^{-1}), such as an increase in lung tissue neutrophils and plasma and lung tissue KC levels (McCluskie *et al.*, 2006).

These findings, together with other reported pro-inflammatory and adverse effects of high doses of PDE4 inhibitors in rats (Larson *et al.*, 1996; Slim *et al.*, 2002; Dietsch *et al.*, 2006; Mecklenburg *et al.*, 2006), prompted us to investigate the consequences of roflumilast treatment in Wistar rats by performing a 5-day short-term tolerability experiment with daily oral doses of 10 mg·kg^{-1} of roflumilast respectively, for 4 consecutive days followed by histopathological evaluation at day 5. In this model, submicromolar plasma concentrations of roflumilast and the major, similarly active N-oxide metabolite induced several pertinent changes, such as a significant body weight loss (up to 14% at day 5), spleen weight loss (1.7-fold decrease), leukocytosis (1.7-fold increase in white blood cells) and blood neutrophilia (4.3-fold increase in percent blood neutrophils). Because McCluskie *et al.* proposed KC and IL-8 as potential biomarkers of the pro-inflammatory potential of PDE4 inhibitors (McCluskie *et al.*, 2006), we measured the rat KC/IL-8 homologue CINC-1 in the rat plasma samples and found 2.2-fold elevated plasma CINC-1 levels in roflumilast-treated animals compared to control animals. This confirms the potential prognostic value of IL-8 family members to assess PDE4 inhibitor-mediated effects. Whereas McCluskie *et al.* (2006) proposed endothelial cells as a source for IL-8, our own unpublished data identified IL-8 induction in response to treatment with roflumilast in the human gut epithelial cell line T84 (data not shown), pointing to gut epithelial cells as a potential source for IL-8. In contrast to a previous report (Dietsch *et al.*, 2006), rat plasma IL-6 levels were not affected by roflumilast treatment in our study (data not shown). This discrepancy with the latter study may be explained by the use of different PDE4 inhibitors and different animal strains.

In addition to body weight loss, animals treated with roflumilast showed an increased incidence of diarrhea and secretion of harderian glands in the present study. Furthermore, several histopathological changes were observed, such as cellular depletion of the thymus, the spleen and the mesenteric lymph nodes, and multifocal perivascular mononuclear/polymorphonuclear infiltration with plasma exudates and fibroblast proliferation in the mesentery. In summary, we conclude that the 5-day short-term tolerability model that we used closely resembles major pathological changes that occur in longer exploratory studies and partly confirms and expands previous studies using different PDE4 inhibitors in different toxicological settings (Larson *et al.*, 1996; Slim *et al.*, 2002; Dietsch *et al.*, 2006; Mecklenburg *et al.*, 2006). Thus, we provide further evidence that, although experimental setting,

dosage, animal strain and/or PDE4 inhibitor used may have a differential impact, the observed effects are overall target-related. In contrast to some other toxicological studies with higher doses of PDE4 inhibitors, roflumilast provoked adverse effects at the comparatively moderate dose of 10 $mg \cdot kg^{-1}$ in our study protocol. Some evidence from literature suggests that the rat is a very PDE4 inhibitor-sensitive species (Bian *et al.*, 2004; Giembycz, 2006), but it is also possible that some specific characteristics of roflumilast (such as potency, tissue distribution, or other yet undefined characteristics) may be responsible for the observed effects at the dose of 10 $mg \cdot kg^{-1}$.

Although a variety of toxicological effects have been linked to PDE4 inhibition in rats, the causal pathophysiological mechanisms of these effects are mainly undefined. There is some evidence suggesting that the profound adverse effects of rolipram may be attributed to lack of potency (requiring higher dosing) and/or selectivity, but also newer, second-generation PDE4 inhibitors of different compound classes have been found to cause pertinent changes in rodent species, mainly of a general pro-inflammatory nature (Slim *et al.*, 2002; 2003; Dietsch *et al.*, 2006; Dagues *et al.*, 2007a; Korkmaz *et al.*, 2009). Because COX-2 enzymes may be induced by cAMP elevating agents such as PDE4 inhibitors (Klein *et al.*, 2007), we investigated whether the roflumilast-mediated effects may be triggered via the COX pathway. For the therapeutic modulation of COX, we used the NSAIDs diclofenac (an almost equipotent inhibitor of the two isoenyzmes of COX), SC-560 (a COX-1 selective inhibitor) and lumiracoxib (a COX-2 selective inhibitor) (Esser *et al.*, 2005; Sud'ina *et al.*, 2008). Remarkably, the observed roflumilast-triggered adverse effects in our rat model could be either substantially reduced (body weight loss, blood neutrophilia, spleen weight loss) or even completely abolished (diarrhea, secretion of harderian glands, white blood cell increase, CINC-1 induction, almost all histopathological findings) by coadministration of therapeutic doses of diclofenac and lumiracoxib, whereas SC-560 had overall no protective effects.

COX-2 is involved in the complex arachidonic acid network that regulates a wide range of cellular actions, including vasoconstriction and vasodilation, pain, inflammation, cytoprotection, aggregation and many more (Simmons *et al.*, 2004). In the present study, we have demonstrated that COX-2 substantially contributed to the pathophysiological effects of roflumilast. Further studies are needed to determine COX-2 activity before and after PDE4 inhibition, but there are several possibilities how cAMP modulation via PDE4 inhibition may trigger increased COX-2 activity on a molecular basis (Klein *et al.*, 2007). cAMP-responsive elements have been recognized as one of the central regulatory elements in the COX-2 promoter region and – although strongly dependent on the cell type and setting – the cAMP pathway was shown to induce COX-2 expression under various conditions (Tanabe and Tohnai, 2002; Bradbury *et al.*, 2003; Klein *et al.*, 2007; Park *et al.*, 2010). One could speculate that the preventive effect of therapeutic inhibition of COX-2 is based on increased signalling events upstream of COX-2 (e.g. COX-2 inhibition may increase potentially protective lipoxin signalling) and/or on suppressed signalling events downstream of COX-2 (e.g. COX-2 inhibition may decrease potentially detrimental prostaglandin E_2, D_2 or $F_{2\alpha}$, or thromboxane effects). For example, the addition of a PDE4 inhibitor may potentiate the tonic low level of prostaglandin production present under normal conditions and may drive some of the observed adverse effects via the upregulation of prostaglandins such as prostaglandin E_2 that has been shown to have pro-inflammatory effects (Clarke *et al.*, 2005; Sugimoto *et al.*, 2005; Dey *et al.*, 2009). Further studies are needed that address which eicosanoid/prostanoid mediator(s) is (are) involved in exerting PDE4 inhibitor-mediated adverse effects.

Concomitant application of the corticosteroid dexamethasone was also shown to reduce PDE4 inhibitor-mediated adverse effects (Slim *et al.*, 2002; 2003; Dietsch *et al.*, 2006). However, as glucocorticoids affect many molecular pathways and exert broad anti-inflammatory actions, therapeutic intervention with dexamethasone may not be useful to elucidate the distinct molecular pathways triggering PDE4-mediated toxicologies. Interestingly, corticosteroids have been shown to reduce COX-2 mRNA stability (Lasa *et al.*, 2001) and some of the protective actions of dexamethasone may thus be mediated via suppression of COX-2.

Although COX-2 inhibition fully prevented the majority of roflumilast-mediated adverse effects in our experiments, diclofenac and lumiracoxib could not fully prevent body weight loss, blood neutrophilia and spleen weight loss (the latter finding only in the case of lumiracoxib). This observation could be explained by different doses and by different kinetics of the compounds that were used; however, we cannot rule out that other pathways than the COX-2 pathway are additionally involved in promoting the roflumilast-mediated effects. In this regard, we asked whether the elevation in CINC-1 could be causal to roflumilast-mediated pathophysiology, but concomitant administration of the CXCR2 inhibitor SB-656933 (GSK) in our rat short-term tolerability experiment failed to prevent

roflumilast-mediated toxicity (data not shown). Similarly, blockade of the adenosine A_{2B} receptor with the A_{2B} receptor antagonist MRS1754 had no protective effects in roflumilast-treated rats, although preliminary data demonstrated that roflumilast-induced IL-8 release from human T84 cells could be fully blocked by concomitant administration of MRS1754 *in vitro* (data not shown).

As indicated before, there are several lines of evidence that the rat is a very PDE4-sensitive species and that pathophysiological effects observed in the rat are overall less pronounced in other animal species, such as pig, dog, or non-human primates, or in humans (Bian *et al*., 2004; Giembycz, 2006). How the protective effects of COX-2 inhibition on PDE4 inhibitor-mediated adverse effects may translate into clinical relevance is not yet clear. However, considering diarrhea as one key PDE4 inhibitor-related adverse effect, we demonstrated full protection in our rat model by concomitant administration of COX-2 inhibiting NSAIDs. Due to the fact that rats are not able to display an emetic response, the rat model used in the present study is not an appropriate model to address the question whether COX-2 inhibition may also decrease the emetic potential of PDE4 inhibitors, another adverse effect frequently reported in humans (Lipworth, 2005; Spina, 2008).

Because lumiracoxib had no effect on the efficacy of roflumilast in our acute LPS-driven lung inflammation model, we conclude that coadministration of NSAIDs inhibiting COX-2 with PDE4 inhibitors would not interfere with the efficacy of PDE4 inhibitors, but may be beneficial by increasing the therapeutic window of PDE4 inhibitors. Further studies will be necessary to evaluate whether concomitant administration of NSAIDs and PDE4 inhibitors may be of therapeutic relevance in humans.

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Conflicts of interest

None to declare.

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