ROFLUMILAST INCREASES BACTERIAL LOAD AND DISSEMINATION IN A MODEL OF *PSEUDOMONAS AERUGINOSA* AIRWAY INFECTION*


Respiratory Medicine & Allergology (G.K., R.B., A.E.) and Infection Medicine (P.P.), Department of Clinical Sciences Lund, Lund University, Skåne University Hospital, SE-221 85 Lund, Sweden.
Running title: *Roflumilast impairs airway host defense*

*Correspondence and request for reprints should be addressed to: Gopinath Kasetty, Ph.D., Biomedical Center, B14, Tornavägen 10, SE-221 84 Lund, Sweden. Phone: +46-46-2227118, E-mail: Gopinath.Kasetty@med.lu.se

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List of nonstandard abbreviations

ANOVA, analysis of variance; BAL, Bronchoalveolar lavage; COPD, Chronic Obstructive Pulmonary Disease; DAMP, Danger-Associated Molecular Patterns; Phosphodiesterase 4, PDE4.
ABSTRACT

Exacerbations present a major clinical problem in many patients suffering from COPD. Roflumilast, an inhibitor of phosphodiesterase 4, has beneficial effects in several clinical trials and is currently widely used to prevent exacerbations in severe COPD. Roflumilast has anti-inflammatory properties that may interfere with potentially important host defense functions, including cytotoxic properties of neutrophils at sites of inflammation. Since chronic bacterial infection are prevalent in severe COPD, *Pseudomonas aeruginosa* being a major pathogen, we hypothesized that this drug could impair host defense against *P. aeruginosa*. In this study, mice were pretreated with vehicle alone or roflumilast at doses of 5 mg/kg or 10 mg/kg followed by instillation of *P. aeruginosa* in the airways. Bacterial load and dissemination as well as inflammatory markers and immune cells present in the airways were followed. Roflumilast increased mortality, bacterial load and dissemination in mice infected with *P. aeruginosa*. In addition, roflumilast-treated mice had significantly lower number of neutrophils in the bronchi but not in the lung tissue airways compared with untreated mice. Several proinflammatory cytokines decreased in roflumilast-treated mice but neither the neutrophil-recruiting chemokine KC nor IL-6. The findings show that roflumilast-treatment impair host defense against *P. aeruginosa* in the airways. This may imply that patients suffering from chronic bacterial infection of the airways could benefit from being withheld treatment with roflumilast.
INTRODUCTION

Exacerbations promote disease progression in COPD and frequent exacerbations increase both morbidity and mortality in patients suffering from the disease (Soler-Cataluna et al., 2005). An exacerbation is defined by clinical signs including dyspnea, increased sputum volumes, and sputum purulence (Anthonisen et al., 1987). Approximately, half of the exacerbations are considered having an infectious origin where virus and bacteria are equally common (Sethi and Murphy, 2008). Corticosteroids, either alone or, in some cases combined with antibiotics are in most cases sufficient to treat exacerbations per se. However, the effect from antibiotics is most pronounced in the case of sputum purulence and in severe exacerbations, speaking for a high degree of heterogeneity and important roles for non-infectious triggers of inflammation (Walters et al., 2009; Vollenweider et al., 2012). A moderate preventive effect on the incidence of exacerbations has been demonstrated for inhaled corticosteroids in combination with long-acting beta agonists and the anticholinergic agent tiotropium (Calverley et al., 2007; Tashkin et al., 2008). As a consequence, there has been a longstanding and unmet need for novel treatment strategies to prevent exacerbations in COPD.

Increased intracellular levels of cyclic adenosine monophosphate (cAMP) can reduce the activation of a wide range of recruited immune and resident cells in the lung. Phosphodiesterase 4 (PDE4) is an intracellular enzyme that catalyses the hydrolysis of cAMP, thereby regulating its downstream signaling (Page and Spina, 2012). The PDE4 family consists of four genes and more than 20 splice variants (Houslay et al., 2007). PDE4 of the subtypes A-D are expressed to various extents in several cells that are of key importance in COPD including neutrophils, macrophages, dendritic cells, cytotoxic T cells, and airway epithelial cells (Page and Spina, 2011; Page and Spina, 2012). The benzamide derivatives, roflumilast (3-cyclo-propylmethoxy-4-difluoromethoxy-N-[3,5-di-chloropyrid-4-yl]-
benzamide) was identified as a potent and selective PDE4 inhibitor (Hatzelmann and Schudt, 2001). In clinical trials, roflumilast improves lung function and reduces the frequency of exacerbations in COPD patients with symptoms of chronic bronchitis and a history of frequent exacerbations (Calverley et al., 2009; Fabbri et al., 2009; Rennard et al., 2011).

Structural and immunologic changes in the airways of patients with severe COPD increase the risk of chronic bacterial colonization, in particular by \textit{Pseudomonas aeruginosa}, which in turn increases the risk of exacerbations and further decline in lung function (Wilkinson et al., 2003). Initially, infection with \textit{P. aeruginosa} is transient and lasts for a few months but as the disease progresses, the bacterial infection often become chronic and with appearance of mucoid and multiresistant strains, eradication becomes difficult (Murphy et al., 2008). In an epidemiologic study, a three-fold increase risk of \textit{P. aeruginosa} bacteremia was observed in COPD patients (Inghammar et al., 2014).

The anti-inflammatory properties of roflumilast may impair airway host defense. In the light of \textit{P. aeruginosa} being an important bacterial pathogen in severe COPD, both in stable disease and during exacerbations, this study set out to study a possible effect from roflumilast on the course of \textit{P. aeruginosa} airway infection.
METHODS

Animals

BALB/cJRj female mice (9–10 weeks old, weighing 18 to 22 g; Janvier-Labs, Le Genest-Saint-Isle, France) were maintained under specific pathogen-free conditions and had free access to commercial chow and water. All mouse experiments were conducted according to the institutional guidelines and were approved of by the Malmo-Lund Animal Care Ethics Committee (M90-15).

Inoculum preparation

Bioluminescent bacteria *Pseudomonas aeruginosa* (strain Xen 41, derived from the parental pleural isolate *P. aeruginosa* PA01; PerkinElmer, Waltham, MA) possesses a copy of the luxCDABE operon of *Photorhabdus luminescens* integrated at a single site on the chromosome. Bacteria were grown in Todd Hewitt Broth (TH) broth aerobically at 37°C.

Roflumilast treatment

Roflumilast (Selleckchem, Houston, TX) was suspended in 0.5% methylcellulose- 2.5% polyethylene glycol solution and sonicated to ensure a uniform suspension. Vehicle-treated control animals received an oral administration of polyethylene glycol-methylcellulose (0.2 ml), whereas the test group received an oral administration of roflumilast suspension (5 or 10 mg/kg). For the experiments measuring infection and inflammatory indices, the drug was administered 24 and 2 hours prior to inoculation of bacteria and animals were sacrificed 18 hour after inoculation.
**P. aeruginosa** inoculation and *in vivo* bioluminescence monitoring of infection

*P. aeruginosa* (strain Xen 41) was grown to logarithmic phase (OD$_{620}$~0.5), harvested, washed in PBS and diluted in the same buffer to $2 \times 10^8$ cfu/ml. Each animal was infected with intranasal instillation of 50 μl bacteria after pre-treatment with roflumilast. Non-infected control animals received only PBS. *In vivo* imaging was carried out immediately or 18 hour after bacterial infection. Mice were anaesthetized through inhalation of aerosolized isoflurane mixed with oxygen. The mice were then transferred to an *in vivo* imaging system chamber (IVIS Spectrum Preclinical In Vivo Imaging System; PerkinElmer) ventral side up, and luminescence was measured followed by analysis using a Spectrum three-dimensional imaging system with Living Image® (PerkinElmer). For evaluation of animal survival, mice showing the defined and approved end-point criteria (immobilization and shaking) were sacrificed by an overdose of isoflurane (Abbott Laboratories, North Chicago, IL) and counted as non-survivors.

**Bronchoalveolar lavage (BAL) and cell counts**

The mice were euthanized approximately 18 hours post-infection. BAL was performed after *in vivo* imaging. A total volume of 1 ml PBS containing 0.1 mM EDTA was used to lavage the lungs. When required, red blood cells were removed by resuspending the BAL cells in 100 μl lysis buffer (150 mM NH$_4$Cl, 10 mM KHCO$_3$, 0.1 mM EDTA, pH 7.2) for 2 min at room temperature (RT) followed by washing in 1 mL PBS. The total number of cells was then counted and adjusted to cells/mL BAL. For differential counts, cytospin preparation of cells were stained with Modified Wright-Giemsa stain (Sigma-Aldrich, St. Louis, MI) and a minimum of 300 cells were counted per BAL sample.

**Determination of bacterial colony forming units**
In order to study bacterial dissemination to organs, spleen, kidney and lungs were harvested from euthanized animals. These organs were mechanically homogenized and serial dilutions were subsequently plated on TH agar plates overnight at 37°C in order to enumerate the colony-forming units (cfu) present in each organ.

**Collection of lungs for immunohistochemistry and homogenization**

Post sacrifice, the left lung lobes were perfused with Histofix (Histolab, Göteborg, Sweden), submerged in 4% buffered formaldehyde. After dehydration and paraffin embedding, 3 µm sections were generated from the tissue blocks. After rehydration and antigen retrieval, sections were incubated with goat antibodies against murine myeloperoxidase (MPO) or preimmune goat IgG (R&D Systems, Abingdon, England), followed by rinsing, and bound antibodies were detected using horseradish peroxidase-conjugated secondary rabbit anti-goat antibodies (diluted 1:2,500) and visualized using 3,3-diaminobenzidine as a chromogen. Some sections were also used for staining with hematoxylin and eosin (H&E).

The right lung lobes were snap-frozen in liquid nitrogen and stored at -80°C until further analysis. The snap-frozen lungs were thawed and homogenized in T-PER solution (Thermo Scientific, Göteborg, Sweden) containing protease inhibitor (Pefabloc SC, Sigma-Aldrich) at a final concentration of 1 mM. Lung homogenates were centrifuged at 9,000 × g for 10 minutes at 4°C and the supernatants were collected. Total protein concentrations in the lung tissue homogenates were determined using a BCA kit (Sigma-Aldrich).

**Cytokine assay**

The cytokines IL-6, IL-10, MCP-1, INF-γ, and TNF-α were measured in plasma, BAL fluid (BALF), and lung tissue homogenates from mice after 18 hour post-infection with *P.*
*aeruginosa* using a cytometric bead array (Mouse Inflammation Kit; Becton Dickinson, Franklin Lakes, NJ) according to the manufacturer’s instructions. Levels of MPO and KC were measured using a colorimetric kit (Sigma-Aldrich) and ELISA kit (R&D systems) respectively.

**Statistical analyses**

For statistical evaluation of more than two experimental groups, one-way with Dunn’s multiple comparisons post-test or two-way ANOVA with Bonferroni post-test were used. All statistical evaluations were performed using the GraphPad Prism software 6.0 (GraphPad Software, La Jolla, CA) with ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001.
RESULTS

**Roflumilast impairs survival *Pseudomonas aeruginosa* airway infection**

A model of *P. aeruginosa* airway infection was used to investigate whether there was a difference in survival comparing vehicle-treated and roflumilast-treated mice respectively (Figure 1). Mice were pre-treated with vehicle or roflumilast at two different doses (5 and 10 mg/kg respectively), administered 24 hours and 2 hours before infection. At the time of infection, mice were subject to intranasal challenge with 2 x 10⁸ cfu/ml bacteria and followed for 7 days. Mice showing predefined end-point criteria (immobilization and shaking) were euthanized. Roflumilast at both doses, significantly decreased survival, occurring on day two and three, compared with infected mice treated with vehicle. Non-infected mice treated with roflumilast (10 mg/kg) did not show decreased survival during seven days, nor did mice infected mice treated with vehicle (data not shown). To investigate whether continued treatment with roflumilast during the course of infection had any effect on the outcome, mice were given an additional dose (5 mg/kg) or vehicle 24 hours after instillation of bacteria. Continued roflumilast treatment also resulted in decreased survival (Supplemental Figure 1).

**Roflumilast increases bacterial load and dissemination**

To investigate bacterial load in the airways and possible dissemination, quantitation by viable counts as well as *in vivo* imaging in real time (IVIS) using bioluminescent bacteria were used (Figure 2). Mice were pre-treated with vehicle or roflumilast (at doses of 5 mg/kg and 10 mg/kg respectively, 24 hours and 2 hours before infection) followed by challenge intranasally with *P. aeruginosa*. After 18 hours, the mice were euthanized followed by collection of bronchoalveolar lavage fluid (BALF) and organs (lungs, spleens, and kidneys were homogenized). Part of BALF and organ homogenates were plated on agar plates to determine
bacterial cfu (Figure 2A). In BALF, a significant increased bacterial load was observed in mice pre-treated with the higher dose of roflumilast (10 mg/kg). In lung tissues, both doses of roflumilast increased the number of bacteria, while in spleens and kidneys, only the higher dose of roflumilast (10 mg/kg) significantly increased the bacterial loads.

Using the semi-quantitative IVIS to follow dissemination of bioluminescent bacteria in live mice, similar patterns were seen (Figure 2B). Dissemination of bacteria to the lungs and internal organs of the abdomen were most pronounced at the higher dose of roflumilast (10 mg/kg) (Figure 2B). This was confirmed by quantitation of bioluminescence where only the higher dose of roflumilast (10 mg/kg) showed a significant increase of bacteria (Figure 2C).

**Roflumilast decreases the number of neutrophils in bronchoalveolar fluid**

Following pretreatment (vehicle or roflumilast at 5 mg/kg and 10 mg/kg respectively) and intranasal infection with *P. aeruginosa*, mice were euthanized and BALF was collected (Figure 3). Roflumilast-treatment at both doses caused a strong decrease in the total number of cells, comparing infected mice treated with vehicle with those of roflumilast-treated mice (Figure 3A). Differential counts showed that the number of neutrophils was strongly down-regulated in mice treated with roflumilast at both doses (Figure 3B).

Neutrophils are key effector cells in host defense against bacterial infection. To investigate whether the number of neutrophils were decreased also in lung tissue, the myeloperoxidase (MPO) activity, a neutrophil granule protein, was determined in lung tissue homogenates (Figure 3C). Interestingly, increased MPO activity was observed in roflumilast-treated with infection. The neutrophil-recruiting chemokine CXCL1/KC was also measured in BALF, 18 hours post infection. A significant increase was found comparing infected mice treated with vehicle and roflumilast at 10 mg/kg (Figure 3D).
Roflumilast increases the number of neutrophils in lung tissue

To determine whether the decreased number of neutrophils in the BALF, corresponded to decreased recruitment or accumulation in the lung tissue, immunohistochemistry (IHC) was performed (Figure 4). Following pretreatment (vehicle or roflumilast at 5 mg/kg and 10 mg/kg respectively) and intranasal infection with *P. aeruginosa*, lungs were collected, processed for immunohistochemistry followed by staining to detect myeloperoxidase (MPO) or stained with a combination of haematoxylin and eosin (H&E) (Figure 4A). Morphometric quantification of MPO-staining was performed and a significant increase was seen in infected mice treated with roflumilast at 10 mg/kg compared with infected mice treated with vehicle alone (Figure 4B).

Effects from roflumilast on cytokine levels

Cytokines are important during airway infection and the impact on their production from roflumilast is an important read-out (Strieter et al., 2002). Thus, the levels of the pro-inflammatory cytokines (IL-6, MCP-1, and TNF-α), a Th1 cytokine (INF-γ) and anti-inflammatory cytokine (IL-10) were measured in plasma, BALF, and lung tissue (homogenates) 18 hours after bacterial inoculation (Figure 5).

IL-6 showed significant increases in both plasma and BALF at the higher dose of roflumilast (10 mg/kg). Interestingly, the anti-inflammatory cytokine IL-10 showed a significant increase in both plasma (at both 5 and 10 mg/kg) and in BALF (at 10 mg/kg). MCP-1 decreased in both BALF and lung tissue with roflumilast (10 mg/kg). Similarly, both INF-γ and TNF-α decreased in BALF at both doses of roflumilast. In addition, roflumilast (10 mg/kg) decreased TNF-α in lung tissue. Taken together, roflumilast, in particular at a higher dose, increase IL-6 and the anti-inflammatory cytokine IL-10 while other pro-inflammatory key cytokines are down-regulated in infected mice.
DISCUSSION

This study shows that the PDE4-inhibitor roflumilast impairs survival in parallel with increasing both bacterial load and dissemination in a mice model of *P. aeruginosa* airway infection. A paradoxical decrease in the number of neutrophils in the bronchi was seen in parallel with an unchanged or, at a higher dose of roflumilast, even increased numbers of lung tissue-dwelling neutrophils. Roflumilast-treated mice showed a changed pattern of cytokines with an anti-inflammatory pattern except for an increase in IL-6 and the neutrophil-recruiting chemokine KC. The findings suggest that impaired host defense functions of neutrophils result in increased vulnerability to *P. aeruginosa* infection during treatment with roflumilast. The findings may have bearing on treatment with roflumilast of patients suffering from COPD and concomitant bacterial infection.

Why are neutrophil numbers decreased in BALF but not in lung-tissue of infected, roflumilast-treated mice? Using a model with LPS-induced inflammation, roflumilast decreased transepithelial migration, mediated by a strong inhibitory effect on cytokine and chemokine secretion by epithelial cells through inhibition of PDE4B. Additionally, LPS-induced stress fibers, required for directed migration of neutrophils into the alveolar space, were reduced by roflumilast (Konrad et al., 2015). Similarly to this study, a model with cigarette-smoke induced inflammation, showed that roflumilast-treated mice had decreased numbers of neutrophils in BALF but not in lung tissue (Wan et al., 2010). In COPD patients, roflumilast significantly reduced the absolute number of neutrophils in sputum compared with patients treated with placebo (Grootendorst et al., 2007).

Why is airway host defense compromised despite the unchanged or even increased number of neutrophils in the lung tissue? Most likely, this is due to inhibited cytotoxic functions of neutrophils, the main effector cell in host defense during bacterial infection. *In vitro*, roflumilast inhibits degranulation and generation of reactive oxygen species in neutrophils but
also inhibit proinflammatory functions of other important immune cells such as monocytes, CD4+ T cells, and dendritic cells (Hatzelmann and Schudt, 2001; Jacob et al., 2004; Jones et al., 2005). In vitro studies on human cells also show that roflumilast inhibits TNF-α production by LPS-stimulated human mononuclear cells and inhibits both IL-8 and LTB4-induced neutrophil chemotaxis (Suzuki et al., 2015). Similar to our findings, investigation of human lung tissue explants showed that roflumilast reduced the release of TNF-α and MCP-1/CCL2 from LPS-stimulated human lung explants, whereas levels of the neutrophil-recruiting chemokines CXCL1, CXCL5 and CXCL8 were not altered (Buenestado et al., 2013). Interestingly, roflumilast alone at a higher dose (100 mg/kg) caused a significant increase in plasma and lung tissue KC/CXCL1 levels and lung tissue neutrophils (McCluskie et al., 2006).

The dosing of roflumilast chosen in this study corresponds to those used in several other studies of murine models relevant to COPD, i.e. 1-100 mg/kg (Kwak et al., 2005; Martorana et al., 2005; McCluskie et al., 2006; Le Quement et al., 2008; Wan et al., 2010; Suzuki et al., 2013). However, one should bear in mind that in humans, roflumilast is used at a dose of 500 µg daily corresponding to 7-10 µg/kg (Calverley et al., 2009). Thus, the difference in dose used in this study compared to that used in humans differ two to three orders of magnitude. Thus, the immunosuppressive effect seen in the current study is likely to be more pronounced than that in humans.

Whether roflumilast increases the bacterial burden in the lower airways or promotes invasive bacterial disease in patients suffering from COPD that are infected with P. aeruginosa remain to be investigated. Other factors may affect the outcome such as lung tissue remodeling and the altered immune status of the airways in COPD, aspects that were not taken into account in this model where healthy mice were used.
The beneficial effects from roflumilast is obvious in patients with frequent exacerbations, chronic bronchitis, and severe COPD where it improves lung function and reduces the frequency of exacerbations (Calverley et al., 2009; Fabbri et al., 2009; Rennard et al., 2011).

To the best of our knowledge, neither the prevalence of *P. aeruginosa* infection nor airway bacterial loads during the course of these clinical trials were followed. Exacerbations in COPD are triggered to an equal extent by viruses and bacteria. The most common bacterial species being *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis* and *P. aeruginosa* (Sethi and Murphy, 2008). However, in approximately one third to half of cases, the exacerbations are triggered by non-infectious stimuli where molecules released from damaged tissue (so-called “Danger-Associated Molecular Patterns”; DAMPs) can result in an exacerbation (Pittman and Kubes, 2013). One hypothesis is that subgroups of COPD-patients suffering benefit from treatment with roflumilast to various extent, depending of etiology i.e. being mainly sterile (i.e. DAMP-induced), viral, or bacterial.

The findings of the current study suggest that an improved phenotyping of COPD patients receiving roflumilast may improve the therapeutical effect and decrease the risk of harmful bacterial airway infection.

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**Author Contribution**

Participated in research design: Kasetty and Egesten.

Conducted experiments: Kasetty, Bhongir, and Papareddy.

Performed data analysis: Kasetty, Bhogir, Papareddy, and Egesten.

Wrote or contributed to the writing of the manuscript: Kasetty and Egesten.
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FIGURE LEGENDS

Figure 1. Roflumilast impairs survival in *P. aeruginosa* airway infection.

The survival of vehicle-treated and roflumilast-treated mice infected with bacteria (*P. aeruginosa*, strain Xen 41) was monitored for 7 days (only the course during the first 96 hours is shown in the figure). The treatment was two oral doses of roflumilast (5 and 10 mg/kg respectively) or vehicle alone, administered 24 hours and 2 hours before infection. At the time of infection, mice were subject to intranasal challenge with 50 µl of PBS containing 2 x 10^8 cfu/ml bacteria (six animals in each group). Statistically significant differences were found comparing the groups pretreated with roflumilast followed by infection with the group treated with vehicle followed by infection. Roflumilast alone (10 mg/kg) did not affect survival. Statistical comparisons of survival curves was performed using the Mantel-Cox’s test. **P ≤ 0.01 and ***P ≤ 0.001.

Figure 2. Roflumilast increases bacterial load and dissemination in *P. aeruginosa* airway infection.

(A) Mice treated with vehicle alone (oral administration 24 hours and 2 hours before infection) or treated with roflumilast (oral administration at a dose of 5 mg/kg and 10 mg/kg respectively, 24 hours and 2 hours before infection) were challenged intranasally with *P. aeruginosa* (strain Xen 41 in 50 µl PBS containing 2 x 10^8 cfu/ml bacteria). After 18 h, the mice were sacrificed and bronchoalveolar lavage fluid (BALF) and organs (lungs, spleens, and kidneys were homogenized) were collected and plated on agar plates to determine bacterial cfu. The data shown represent mean and SD, each group consisted of ten mice.

(B) Dissemination of *P. aeruginosa* (strain Xen 41 in 50 µl PBS containing 2 x 10^8 cfu/ml bacteria) using the bioluminescent properties of the bacteria and an *in vivo* imaging system (IVIS Imaging System). Images of anesthetized mice were taken immediately after infection.
(0 hours) and at 18 hours post infection. Representative pictures of 4 of totally 10 mice in each group are shown. Bacterial load and dissemination appears as a bioluminescent spectrum where blue indicates a low number of bacteria whereas red represents a high number. Dissemination to regions corresponding to the lungs and kidneys are seen in some mice.

(C) Quantitation of the detected bioluminescence presented in (B), comparing mice treated with vehicle or roflumilast (5 mg/kg and 10 mg/kg respectively) followed by infection with *P. aeruginosa* (strain Xen41). The luminescence of each group (10 animals; mean and SD) at 0 hours and 18 hours are shown. Statistically significant differences between the values for the roflumilast untreated and treated mice were observed at 18 hours post-infection. One-way ANOVA with Dunn’s multiple comparisons post-test or two-way ANOVA with Bonferroni post-test was used for statistical analysis. \( *P \leq 0.05, **P \leq 0.01, \text{ and } ****P \leq 0.0001. \)

**Figure 3. Roflumilast decreases the number of neutrophils in bronchoalveolar fluid but increase the number in the lung tissue.**

(A) Following pretreatment (vehicle or roflumilast at 5 mg/kg and 10 mg/kg respectively) and intranasal infection with *P. aeruginosa*, broncoalveolar fluid (BALF) was collected and total number of cells was determined. Roflumilast-treatment caused a significant decrease in the total number of cells compared to untreated mice (ten mice in each group). (B) To determine the phenotype of cells, cytospin and Wright-Giemsa staining were performed. Significant decreases in the number of neutrophils were observed in roflumilast-treated animals. (C) The right lungs of mice sacrificed 18 hours after infection were homogenized and myeloperoxidase (MPO) activity was estimated. No significant differences were found. (D) The neutrophil-recruiting chemokine CXCL1/KC was measured in BALF, 18 hours post infection. A significant increase was found comparing infected mice treated with vehicle and roflumilast at 10 mg/kg.
One-way ANOVA with Dunn’s multiple comparisons post-test or two-way ANOVA with Bonferroni post-test was used for statistical analysis. \( *P \leq 0.05, **P \leq 0.01, \) and \( ****P \leq 0.0001.\)

**Figure 4. Roflumilast at a higher dose increase the number of neutrophils in lung tissue.**

(A) Following pretreatment (vehicle or roflumilast at 5 mg/kg and 10 mg/kg respectively) and intranasal infection with \( P. \) aeruginosa, lungs were collected, processed for immunohistochemistry followed by staining to detect myeloperoxidase (MPO) or stained with a combination of haematoxylin and eosin (H&E). MPO-containing cells and released MPO are detected by a peroxidase-reaction resulting in a brownish staining.

(B) Quantification of MPO-staining, expressed as pixels/mm\(^2\), was performed on lung tissue sections, ten animals in each group. A significant increase was seen in infected mice treated with roflumilast at 10 mg/kg compared with infected mice treated with vehicle, and total number of cells was determined. One-way ANOVA with Dunn’s multiple comparisons post-test was used for statistical analysis. \( *P \leq 0.05.\)

**Figure 5. Cytokine levels in plasma, BALF, and lung homogenates during infection.**

Measurement of cytokines 18 hours after bacterial infection in roflumilast-treated and untreated mice respectively. Cytokines were measured in plasma, BALF and lung tissue homogenates. The results are expressed as mean ±SEM (ten animals in each group). One-way ANOVA with Dunn’s multiple comparisons post-test was used for statistical analysis. \( *P \leq 0.05 \) and \( **P \leq 0.01.\)
Figure 1

The graph shows the percent survival over time (h) for different treatment groups. The x-axis represents time in hours, ranging from 0 to 96. The y-axis represents percent survival, ranging from 0 to 100.

- **Bacteria + Vehicle**
- **Bacteria + Roflumilast (5 mg/kg)**
- **Bacteria + Roflumilast (10 mg/kg)**
- **Roflumilast (10 mg/kg)**

**Statistical Significance:**
- **Bacteria + Roflumilast (5 mg/kg)** marked with **,** indicating a significant difference.
- **Bacteria + Roflumilast (10 mg/kg)** marked with **,** indicating a highly significant difference.
- **Roflumilast (10 mg/kg)** marked with **,** indicating a statistically significant difference.
Figure 2

A

Bacterial load (Cfu) in BALF, Lung, Spleen, and Kidney with different doses of roflumilast (mg/kg).

B

Luminescence of mice at 0 and 18 hours after treatment with bacteria and roflumilast (5 mg/kg and 10 mg/kg).

C

Total flux at 0 and 18 hours with varying doses of bacteria and roflumilast (mg/kg).
Figure 4

A

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B

![Bar graph showing MPO (pixels/m²) for different conditions.](chart.png)
Figure 5

IL-6 (pg/mL)

Plasma

BALF

Lung

IL-10 (pg/mL)

MCP-1 (pg/mL)

INF-γ (pg/mL)

TNF-α (pg/mL)

Bacteria

Rofamilast (mg/kg)

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