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In vivo and *in vitro* investigation of anti-inflammatory and mucus-regulatory activities of a fixed combination of thyme and primula extracts

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ABSTRACT

Hypersecretion of viscous mucus is one of the hallmark symptoms of acute and chronic bronchitis and typically develops secondary to an inflammation of the airway epithelium. Bronchipret® TP film-coated tablets (BRO), a herbal medicinal product containing a fixed combination of thyme herb and primula root extracts, has been successfully used clinically for the treatment of acute bronchitis for more than two decades. However, the underlying pharmacological mechanisms of action have not been fully understood so far.

We investigated the anti-inflammatory and mucus-regulatory effects of orally administered BRO in an animal model of pulmonary inflammation that was experimentally induced by intratracheal LPS instillation. BRO was administered once daily for up to three days following the induction of inflammation. Treatment with BRO effectively inhibited polymorphonuclear cell influx into the lung as well as the increase in mucin 5ac (MUC5AC) protein. Furthermore, the LPS-induced increase of goblet cell numbers was significantly attenuated by BRO treatment.

Subsequent *in vitro* investigations with IL-13 stimulated human primary respiratory epithelium and the Calu-3 respiratory epithelial cell line in air-liquid-interface culture confirmed the effects on mucus production and goblet cell numbers observed in the *in vivo* studies. They further suggest that the reduction of MUC5AC protein secretion by BRO is associated with reduced *MUC5AC* mRNA expression as assessed by quantitative Real-Time PCR.

Our studies provide evidence that BRO exerts both anti-inflammatory and mucus-regulatory activity and that BRO's effect on mucin production is partially independent from its anti-inflammatory activity. These results contribute to the understanding of the modes of action underlying the clinical efficacy of BRO in acute bronchitis patients.

1. Introduction

Acute bronchitis is a common clinical diagnosis defined by cough due to self-limited inflammation of the trachea and the larger airways without pneumonia [1,2]. Cough is the most frequent reason for visits to primary care physicians, accounting for around 8% of all consultations with about 20% of cases of unfitness for work and about 10% of the days off work due to illness. The annual prevalence of cough in the general population is reported as about 10–33% [3]. Non-influenza-related respiratory tract infections impose a greater economic burden on the public health system than many other clinical conditions [4]. In terms of pathophysiology, acute bronchitis usually develops in the course of infection with respiratory viruses that trigger an inflammatory reaction in the infected airways. This is often associated with the production of large quantities of tenacious and difficult-to-expectorate mucus.

Although data on mucus viscosity from acute bronchitis patients is missing, studies from chronic bronchitis phenotypes highlight the common pathophysiological finding of mucus hyperconcentration [5]. Viscosity of mucus is determined by its osmotic pressure which in term is affected by the relative content of mucins [5]. Mucins are large glycosylated proteins, of which the gel-forming mucin 5ac (MUC5AC) released from goblet cells is typically upregulated following inflammatory events in the airways via IL-4/IL-13 receptor activation and EGFR-signaling [6–8]. The excessive formation of viscous mucus along with a decreased ability of the ciliated epithelial cells of the lower

Abbreviations: BALF, bronchoalveolar lavage fluid; BRO, Bronchipret® TP film-coated tablets; DER, drug extract ratio; LPS, lipopolysaccharide; MUC, mucin; RT, room temperature

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airways to remove the sticky mucus is believed to contribute to disease pathogenesis in bronchitis patients [9].

Due to the usually viral nature of the infection in acute bronchitis no causal therapy is available and routine antibiotic use is not recommended in current guidelines [10]. The established therapeutic focus is on symptomatic treatment where well-tolerated therapeutic options are warranted due to the self-limited character of the disease. Given its key role in the pathophysiology of acute bronchitis, targeting inflammation-induced goblet cell metaplasia and viscous mucus hypersecretion represents a promising therapeutic approach to attenuate cough and to facilitate expectoration.

Bronchipret® film-coated tablets (BRO), a herbal medicinal product containing a fixed combination of dry extracts from thyme herb and primula roots, has been used as a treatment for cough with excessive mucus during infections/inflammations of the lower respiratory tract for more than 20 years. A clinical trial in patients with acute bronchitis and cough demonstrated therapeutic efficacy of BRO on symptom relieve and recovery time [11]. Although a number of pharmacological activities have been reported for thyme herb [12–14], the mechanism of action underlying the clinical efficacy of the thyme herb/primula root extract mixture BRO remained largely elusive.

Here, we report on studies aimed to identify potential anti-inflammatory and mucus-regulatory effects of BRO in different non-clinical experimental conditions. We employed a tiered approach of *in vivo* studies of orally administered BRO in a rat model of LPS-induced pulmonary inflammation complemented by *in vitro* experiments in air-liquid interface cultured human respiratory epithelial cells. The combination of these studies allowed us to identify different pharmacological activities of BRO that regulate mucus protein production under experimental inflammatory conditions and that likely contribute to the clinical efficacy of this herbal medicinal product in acute bronchitis.

2. Materials and methods

2.1. Test items of the herbal extracts in Bronchipret® TP film-coated tablets

For all experiments a mixture of the genuine dry extracts of thyme herb and primula root contained in Bronchipret® TP film-coated tablets (BRO) without excipients and with a final thyme/primula dry extract ratio of 2.67:1 was used. Both extracts are standardized to the content of specific marker compounds. The herbal extract mixture was provided by Bionorica SE, Neumarkt, Germany.

For the *in vivo* studies the dry extract mixture was suspended in 0.9% saline. For the *in vitro* experiments the mixture was dissolved with 50% ethanol ($v \cdot v^{-1}$) to a concentration of 100 $mg \cdot mL^{-1}$ and homogenized for 5 min by vortexing, followed by 30 min incubation in an ultrasonic bath at room temperature (RT). The resulting suspension was then centrifuged at 3000 × g for 10 min at RT and the corresponding supernatant was filtrated through a disposable syringe filter (PVDF; pore size, 0.22–0.45 µm; e.g. Millipore, Billerica, USA) and immediately used for the assays.

2.2. Animal housing

The animal studies are reported based on the ARRIVE guidelines [15]. Male Wistar rats weighing 160–260 g were obtained from a commercial breeder (Rappolovo, St. Petersburg, Russia) and were kept in polycarbonate cages (6 animals per cage) throughout the experiment at room conditions of 21 ± 1 °C and 61–75% humidity in a 12 h light/dark cycle. They had *ad libitum* access to water and complete pellet diet (Protein 19%; Aller Petfood, Kuzmolovskiy, Russia). Experiments were performed according to the recommendations and policies of the National Standard of Russian Federation GOST 33044-2014, under a protocol which had been previously approved by the Ethics Committee of the Institute of Experimental Pharmacology CJSC (Russia, Saint-Petersburg). All painful manipulations of the animals were conducted in accordance with regulatory standards (Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes).

2.3. Induction of bronchoalveolitis and animal treatment

Bronchoalveolitis was induced as previously described [16]. Two separate studies were performed (study A and study B).

In brief, 176 animals (study A: n = 10 to 16 per group; study B: n = 12) were randomly divided and allocated to the 5 treatment groups by means of a modified method of block randomization [17]. One additional group served as healthy control group (sham; study A: n = 10; study B: n = 12). Thus, in total 198 animals were used for the *in vivo* experiments described herein. In study B, 6 of the 12 animals per group and time point were used for bronchoalveolar lavage fluid and tissue analyses (MUC5AC production and myeloperoxidase activity). The remaining 6 animals were used for histological analyses (goblet cells). The animals were acclimatized for 14 days prior to the start of the experiment.

Bronchoalveolitis was induced in previously anesthetized animals (Zoletil® (tiletamine/zolazepam: Virbac, France) at 0.4 $mg \cdot kg^{-1}$ body weight (b.w.) i.v.) by intratracheal injection of 100 µg LPS from *Escherichia coli* (Sigma-Aldrich, USA) per animal 1 h prior to the first oral administration of the test compounds. The animals were then treated once daily by oral gavage with either vehicle (0.9% saline), Dexamethasone (Dex, 5 $mg \cdot kg^{-1}$ b.w.) or BRO (study A: 13.6, 68 or 339 $mg \cdot kg^{-1}$ b.w.; study B: 68, 203 or 678 $mg \cdot kg^{-1}$ b.w.) at an administration volume of 10 $mL \cdot kg^{-1}$ b.w.. The doses corresponded to the 0.2-, 1- and 5-fold (study A) and the 1-, 3- and 10-fold (study B) of the currently recommended human daily dose after allometric conversion to human equivalent doses [18].

2.4. Bronchoalveolar lavage fluid and tissue collection and analysis

Animals were sacrificed by terminal anesthesia with Zoletil® at 0.7 $mg \cdot kg^{-1}$ b.w. i.v. at 48 h (study A, 10–16 rats per group) and at 48 h and 72 h (study B, 6 rats per group) post LPS challenge, respectively for sampling of bronchoalveolar lavage fluid (BALF) and lung tissue as described previously [16]. In brief, BALF was collected after ligation of the right main bronchus and a catheter was inserted from the trachea into the left lung. Warm saline (37 °C) was repeatedly run through the catheter and the resulting BALF (ca. 200 µL) was passed through a mesh (200 µm; Pharmaceutical company Volga Manufactory, LLC) to remove mucus followed by centrifugation (1500 × g) at 4 °C for 15 min (centrifuge Z216 MK, Hermle Labortechnik GmbH, Germany).

The resulting pellets were resuspended in 2 mL PBS. To remove cell debris the cell suspension was centrifuged again and the pellet resuspended in 1 mL PBS. This suspension was employed for total cell count. BALF total cell numbers were counted using the Abacus Junior Vet veterinarian hematological analyzer (Diatron, Austria). The percentage of granulocytes was determined by light microscopy of May-Grünwald-Giemsa stained slides. At least 100 cells were counted for determination of the granulocyte population.

2.5. Analysis of lung tissue homogenates

Lung tissue samples were frozen and stored at −80 °C prior to analysis. For the preparation of lung homogenates right lung lobes were weighed, and equal parts by weight were rinsed in ice-cold PBS to remove excess blood after thawing. Tissue homogenization was performed in 5 mL of cold PBS using a biological tissue chopper (Polytron PT 1600E System, Kinematica, Switzerland). The homogenates were centrifuged at 5000 × g and 4 °C for 5 min. Supernatants were removed, immediately aliquoted and stored at −20 °C until analysis. Total protein content was measured spectrophotometrically [19].

Myeloperoxidase (MPO) activity was determined in lung tissue

homogenates by a colorimetric assay (Sigma-Aldrich, USA) that assesses enzyme activity based on the formation of hypochlorous acid. One unit of MPO activity was defined as the amount of the enzyme that hydrolyzes the substrate and generates taurine chloramine to consume 1.0 μmoles of 5-thio-2-nitrobenzoic acid per minute at 25 °C. MPO activity was calculated as $nmol \cdot min^{-1} \cdot mL^{-1} = mU \cdot mL^{-1}$. The results in the figures are given in $U \cdot mg^{-1}$ tissue.

MUC5AC protein was quantified in lung tissue homogenates with a rat-specific ELISA kit for mucin 5 subtype AC (USCN, Japan) according to the manufacturer's instructions. Optical density of the samples was evaluated at 412 nm using a plate spectrophotometer (xMark, BioRad, USA).

2.6. Histological quantification of goblet cells

In study B, tissue of the right lung was collected from the remaining 6 animals per group and per time point for histological evaluation of lung tissue sections. Samples were fixed in 10% neutral formalin for 24 h, dehydrated by immersion in ethanol series (70–95%) and xylene and then embedded in paraffin. Tissue sections of 3–5 μm were stained with hematoxylin and eosin (H&E) and with Alcian blue (pH 2.5) to assess goblet cell numbers (within 1 mm of the epithelial layer medium-sized bronchi, prepared from the central region of the lungs) and to identify glycosaminoglycans. Blinded morphological examination was performed using light-optical microscopy (Leica DC320; Leica Microsystems, Germany) at 200-fold magnification [20].

2.7. IL-13-induced MUC5AC protein and mRNA expression in Calu-3 cells

Calu-3 bronchial epithelial cells (American Type Culture Collection, Rockville, USA) derived from human lung adenoma [21] were maintained in DMEM/F12 culture media supplemented with 10% FBS, penicillin, streptomycin, L-glutamine, HEPES and non-essential amino acids. 1.5×10^6 cells were seeded into the apical side of the transwell insert (polyester, 6.5 mm diameter, 0.4 μm pore size; Corning, New York, USA) and cultured in 1.5 mL culture medium on the apical and 2.0 mL culture medium on the basolateral side. The medium was changed and transepithelial electrical resistance (TEER) was measured every other day until completion of the experiments. TEER measurements were performed using EVOM2 Epithelial Voltohmmeter and STX2 electrodes (World Precision Instrument, Inc., Sarasota, USA) according to the manufacturer's instructions. After the cells became confluent and TEER stabilized, the medium was aspired from the apical side and the cells were kept on ALI culture. Subsequently, cells were divided into 9 groups (Table 1) and were treated basolaterally every other day for 14 days. Every treatment batch consisted of 5 parallels.

After the treatment period, cell layers were washed twice on the apical side with 500 μL PBS to collect protein for the ELISA measurement of MUC5AC. The supernatant was concentrated to half the volume using a Speedvac (Thermo Fisher Scientific, Waltham, USA) (at 2000 rpm, 7 mbar, 1 h). To quantify the protein content, human MUC5AC ELISA kit (Elabscience, Bethesda, USA) was used according to the manufacturer's instructions.

Table 1
Treatment scheme for Calu-3 cell culture experiments.

Group	Stimulus	Treatment
1	none	none
2	vehicle (ethanol, 0.005% $v \cdot v^{-1}$)	vehicle (ethanol, 0.005% $v \cdot v^{-1}$)
3	vehicle (ethanol, 0.005% $v \cdot v^{-1}$)	N, N-dimethyl-sphingosine (5 μM)[a]
4	vehicle (ethanol, 0.005% $v \cdot v^{-1}$)	1 $\mu g \cdot mL^{-1}$ BRO
5	vehicle (ethanol, 0.005% $v \cdot v^{-1}$)	10 $\mu g \cdot mL^{-1}$ BRO
6	10 $\mu g \cdot mL^{-1}$ IL-13	vehicle (ethanol, 0.005% $v \cdot v^{-1}$)
7	10 $\mu g \cdot mL^{-1}$ IL-13	N, N-dimethyl-sphingosine (5 μM)[a]
8	10 $\mu g \cdot mL^{-1}$ IL-13	1 $\mu g \cdot mL^{-1}$ BRO
9	10 $\mu g \cdot mL^{-1}$ IL-13	10 $\mu g \cdot mL^{-1}$ BRO

[a] Sphingosine kinase inhibitor (reference control).

RNA was isolated with NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany) and cDNA was synthesized from the RNA templates using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, USA). All parallel samples from each treatment batch were handled individually. For the reverse transcription 1 μg total RNA was used. For quantitative Real-Time PCR reaction we used SensiFast SyberGreen (Bioline, London, UK) and the following primer sequences:

MUC5AC
Forward: 5′-CGACCTGTGCTGTGTACCAT-3′
Reverse: 5′-GTGCAGGGTCACATTCCTCA-3′
β-actin
Forward: 5′-GCGCGGCTACAGCTTCA-3′
Reverse: 5′-CTTAATGTCACGCACGATTTCC-3′
GAPDH
Forward: 5′-ATCCCTCCAAAATCAAGTGG-3′
Reverse: 5′-GGCTGTTGTCATACTTCTCA-3′
S18
Forward: 5′-ATGGCGGCGTCTGTATTAAAC-3′
Reverse: 5′-AGAACCATATCGCTCCTGGTAT-3′

All samples were run in triplicates. Three housekeeping genes (β-actin, GAPDH, S18) and one target gene (*MUC5AC*) were analyzed during the study. PCR conditions were set as follows: one cycle 95 °C for 2 min, 40 cycles at 95 °C for 5 s and 60 °C for 30 s. RQ values were calculated according to the $2^{-\Delta\Delta Ct}$ formula on the mean values of the three housekeeping genes.

2.8. IL-13-induced goblet cell hyperplasia and MUC5AC production in primary human respiratory epithelium

Human respiratory epithelium (MucilAir™; [22]) was sustained in ALI culture. IL-13 (10 $ng \cdot mL^{-1}$) and the test compounds BRO, PS1145 (IKK inhibitor) or vehicle (ethanol, 0.05% $v \cdot v^{-1}$) were added basolaterally for 14 consecutive days. The culture medium was renewed every two or three days.

Sections of epithelia were stained histologically by Alcian blue staining (goblet cells) or by immunohistochemistry (MUC5AC; Abcam, UK) and scanned. From 27 to 45 images from ×20 magnification per sample were used for quantification. The quantification was performed using the program Image Pro Plus (version 6.2; Media Cybernetics, USA).

To calculate the percentage of positive (stained) cells for each section relative to the total area of the section, the following calculation was applied:

% of positive cells = (area occupied by positive cells x 100)/total area of the section.

2.9. Statistics

For the *in vivo* studies all data are presented as mean + standard error of the mean (SEM). Statistical data evaluation versus (*vs*.) the LPS/vehicle control was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (of log-transformed data in case of heteroscedasticity). In case of unequal variances and for the histology analyses the non-parametric Kruskall-Wallis test, followed by Dunn's multiple comparison test *vs*. the LPS-vehicle group was used for statistical analysis. Comparison of the sham group *vs*. the LPS/vehicle control group was assessed by student's t-test featuring Welch's correction in case of unequal variances.

For *in vitro* experiments results are expressed as arithmetic means + SEM of n = 3 (primary human respiratory epithelium) or n = 5 replicates (Calu-3 cells), respectively. Statistical data evaluation *vs*. solvent controls and/or the cells treated with IL-13 only was

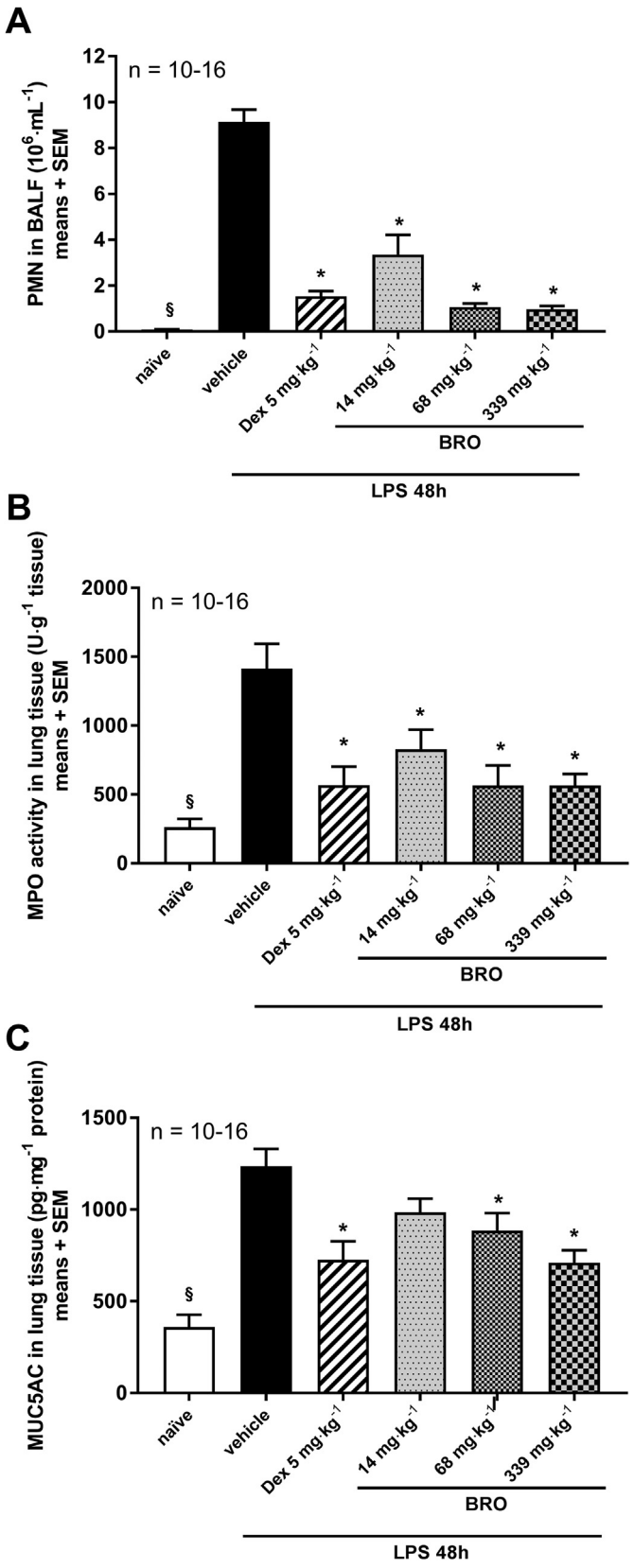

Fig. 1. Effects of BRO treatment on inflammatory and mucus-regulatory parameters in LPS-treated rats after 48 h (study A). Animals were treated twice with BRO, Dex or vehicle following intratracheal LPS instillation. PMN (A) were counted in BALF, MPO activity (B) and MUC5AC protein (C) were quantified in lung tissue homogenates. N = 10–16. $^{§}p < 0.05$; *t*-test *vs.* naïve. *p < 0.05; ANOVA + Dunnett's *vs.* vehicle.

performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

Statistical analysis was done with GraphPad Prism 7 software (GraphPad Software Inc., San Diego, CA). A p value < 0.05 was considered statistically significant.

3. Results

3.1. Anti-inflammatory and mucus-regulatory activities of BRO in the animal model of LPS-induced pulmonary inflammation

For our initial study (study A) we selected an observation period of 48 h and an oral dose range corresponding to the 0.2-, 1- and 5-fold equivalents of the currently recommended human daily dose of BRO by allometric scaling (14, 68 and 339 mg·kg^{-1}b.w.; [18]). In previous experiments this time point had proven to be suitable to study both inflammatory and mucus-regulatory parameters in this model. The administration of LPS led to a significant increase of inflammatory cells as measured by PMN cell counts in BALF ($9.1{\cdot}10^6 \pm 0.54$ *vs.* 0.1 ± 0.01 cells·mL^{-1}; Fig. 1A). Treatment with BRO at all administered dose levels and with the positive control Dex (5 mg·kg^{-1} b.w.) led to a prominent, significant and dose-dependent attenuation of the infiltration of inflammatory cells to the lung at 48 h *post* LPS.

The effects on MPO activity, an indirect marker of PMN infiltration into tissue, were similar (Fig. 1B) and demonstrate that BRO exerts its anti-inflammatory activity in this experimental setup at doses as low as 14 mg·kg^{-1} b.w. (0.2-fold equivalent of the recommended human daily dose).

The influx of PMN into the lung caused by LPS was accompanied by an increase in levels of MUC5AC protein, indicating that LPS had changed the basal conditions of mucus regulation. Similar to the inhibition of PMN influx, BRO treatment decreased MUC5AC protein levels with statistically significant effects starting at 68 mg·kg^{-1} b.w. (Fig. 1C).

In a second, confirmatory study (study B) we decided to include an additional 72 h observation time point to better characterize potential treatment effects on structural changes of the epithelium which we expected to have a delayed onset compared to inflammation based on previous experiments in this model [16]. In addition, the dose levels were increased to the 1-, 3- and 10-fold equivalents of the human daily dose (68, 203 and 678 mg·kg^{-1} b.w.) to assess the maximum efficacy of BRO treatment on MUC5AC protein production and structural changes indicative of tissue remodeling. Similarly to the first study BRO treatment dose-dependently ameliorated LPS-induced inflammation at 48 h as measured by tissue MPO activity with statistical significance reached at a dose of 678 mg·kg^{-1} b.w.. However, the effect of BRO was weaker than that of the positive control Dex in this study. After 72 h inflammation had generally decreased in all study groups due to the self-limiting character of the model. However, inflammation in BRO-treated animals was still less pronounced than in the vehicle-treated animals (Fig. 2A).

In terms of mucus parameters, LPS challenge led to a gradual increase over time in MUC5AC protein content with a maximum at 72 h (1420.0 ± 239.9 pg·mg^{-1} protein) when compared to the levels in the naïve controls (247.0 ± 42.5 pg·mg^{-1} protein) (Fig. 2B). This increase in mucus protein content was associated with a simultaneous increase in the number of mucus producing goblet cells (Fig. 2C), as assessed histologically after Alcian blue staining with 80.2 ± 1.3 cells·mm^{-1} when compared to the numbers in sham-treated controls (33.8 ± 0.5 cells·mm^{-1}). Treatment with BRO at all dose levels tested significantly attenuated MUC5AC protein increase to a similar degree as the positive control dexamethasone (Dex) (Fig. 2B). Although goblet cell metaplasia became already apparent at 48 h, the increase was more evident at 72 h *post* LPS challenge. Both BRO and Dex treatments partially inhibited the numerical increase in goblet cells (Fig. 2C) in bronchial epithelium with significant and most pronounced effects

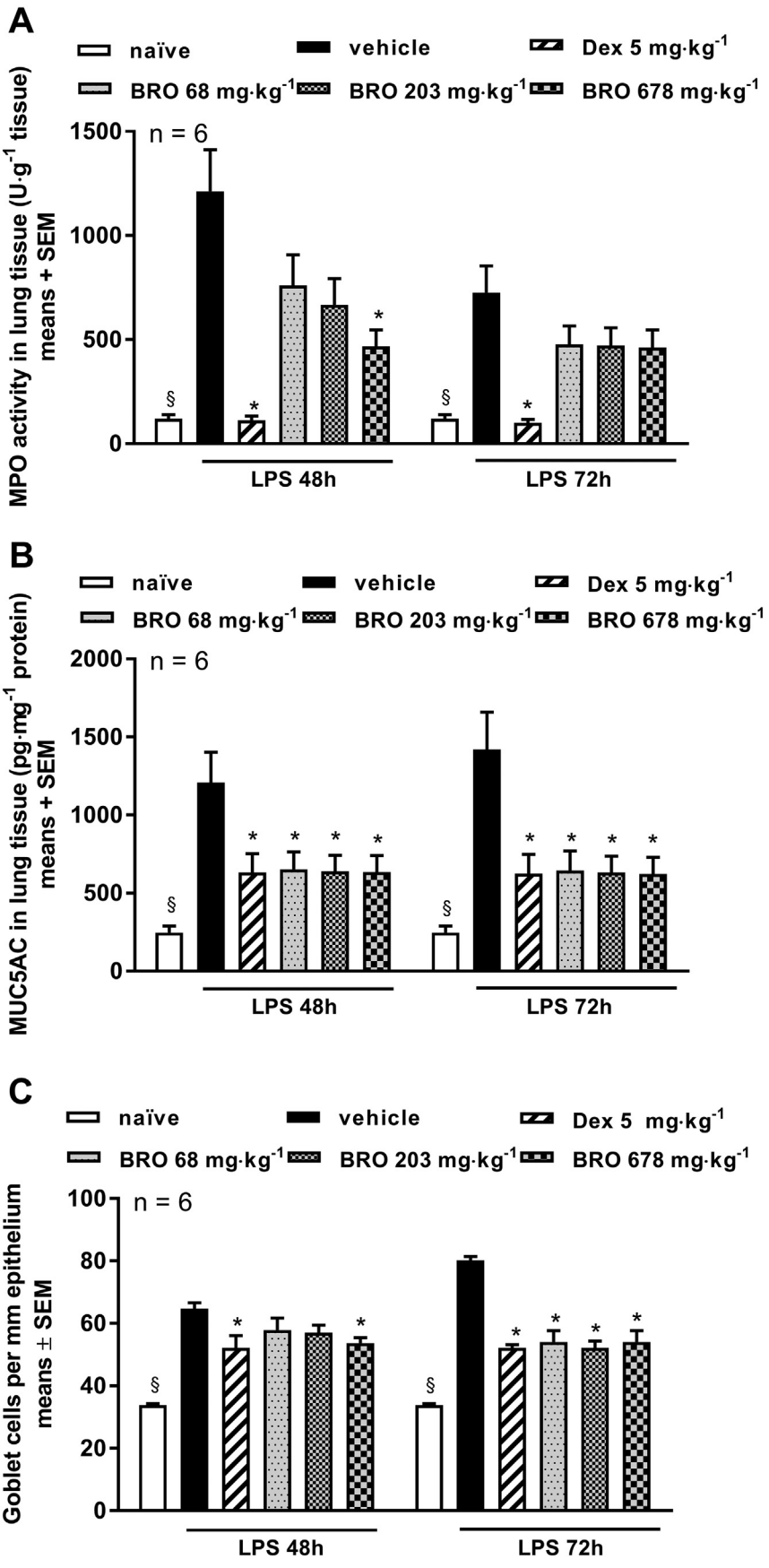

Fig. 2. Effects of BRO treatment on inflammatory parameters and goblet cell metaplasia in LPS-treated rats after 48 and 72 h (study B). Animals were treated up to three times with BRO, Dex or vehicle following intratracheal LPS instillation. MPO activity (A) and MUC5AC protein (B) were quantified in lung tissue homogenates. Goblet cell density (C) was determined in paraffin sections of lung tissue samples stained with hematoxylin/eosin and Alcian blue. Goblet cells were counted in medium-sized bronchi, taken at the central regions of the lungs within 1 mm of the epithelial layer. N = 6. $^{\S}p < 0.05$; *t*-test *vs.* naïve. $^{*}p < 0.05$; ANOVA + Dunnett's *vs.* vehicle.

observed in BRO-treated animals at 72 h.

3.2. In vitro effects of BRO on IL-13-induced goblet cell metaplasia and MUC5AC production/release in human respiratory epithelium

The therapeutic activity against indicators of dysregulated mucus protein production and bronchial epithelial remodeling in our animal studies is considered an important observation likely to translate into normalization of mucus consistency and attenuation of excessive production of difficult-to-expectorate phlegm in acute bronchitis patients. Due to the perceived high relevance of these findings for the identification of potential modes of action of BRO, we decided to conduct additional *in vitro* studies to further characterize the mucus-regulatory activity of BRO. The increases in MUC5AC protein levels in the *in vivo* model were induced by an inflammatory challenge (LPS) and BRO treatment clearly exerted anti-inflammatory effects. Hence, it is impossible to differentiate whether the amelioration of mucus parameters merely reflects the functional consequence of the inhibition of cell influx by BRO, or rather presents an inflammation-independent activity that directly affects mucus regulation. To assess whether BRO has the ability to exert direct effects on epithelial cells we investigated the influence of BRO on MUC5AC and goblet cell metaplasia in human epithelial cell ALI cultures stimulated with IL-13.

In a first setup we tested the effects of BRO on MUC5AC protein secretion and mRNA expression in the Calu-3 cell line on ALI culture. Fourteen days of IL-13 treatment led to a strong and significant increase of MUC5AC formation both on secreted protein (9.9 ± 0.71 *vs.* 1.7 ± 0.02 $pg \cdot mL^{-1}$; Fig. 3A) and mRNA (4.8 ± 0.17 *vs.* 1.0 ± 0.15 RQ; Fig. 3B) expression level. Basolateral treatment with the reference control DMS (sphingosine kinase inhibitor) or with BRO had no effect on otherwise non-stimulated cells but significantly reduced both protein secretion and mRNA expression in cells that had been stimulated with IL-13 in a concentration-dependent manner. BRO inhibited MUC5AC secretion by 37% and 57% (1 and 10 $\mu g \cdot mL^{-1}$, respectively) and *MUC5AC* mRNA expression by 48% and 56% (1 and 10 $\mu g \cdot mL^{-1}$, respectively). This indicates that BRO directly influences MUC5AC production by intervening already with the transcription of its mRNA.

To investigate whether also the inhibition of goblet cell metaplasia could be reproduced *in vitro* we performed additional experiments with primary human epithelium cultured under ALI conditions (MucilAir®).

As with Calu-3 cells, MUC5AC protein expression, as assessed by immunohistochemistry, was significantly induced by IL-13 treatment (25.5 ± 5.91% *vs.* 0.6 ± 0.07% MUC5AC-stained area; Fig. 4A). Treatment with BRO (10 $\mu g \cdot mL^{-1}$) attenuated this induction (16.4 ± 1.66% MUC-stained area and 37% inhibition). Although this effect was not statistically significant at the tested concentration it confirms the findings in the Calu-3 cell line.

IL-13 treatment furthermore led to a strong and statistically significant metaplasia of goblet cells (5.4 ± 0.74% vs. 0.7 ± 0.04% Alcian blue-stained area; Fig. 4B). Treatment with BRO inhibited metaplasia by 55% although the effect did not reach statistical significance (2.8 ± 0.44% Alcian blue-stained area).

Treatment with the reference compound PS1145 (IKK inhibitor) significantly inhibited both the induction of MUC5AC expression and goblet cell metaplasia.

4. Discussion

The herbal medicinal product Bronchipret® TP film-coated tablets (BRO) containing a fixed combination of thyme herb and primula root extracts has shown clinical efficacy in reducing the number of coughing fits and other bronchitis symptoms in patients suffering from acute bronchitis with difficulties in expectoration [11]. The clinical evidence of efficacy led to a recommendation of BRO in the S3 guideline of the German Respiratory Society on the diagnosis and treatment of cough in adults [10].

Starting from its demonstrated clinical efficacy we aimed to elucidate modes of action potentially underlying the therapeutic benefit of BRO in acute bronchitis. Replication of the condition of virally induced acute and self-limiting bronchitis in animal models is challenging due to differences in susceptibility to viral pathogens as compared to humans. We investigated the effects of orally administered BRO in a more mechanistic animal model of pulmonary inflammation induced by intratracheal instillation of LPS to study effects of BRO on parameters of inflammation and mucus production. This model, although lacking the viral etiology typical for acute bronchitis in humans, is characterized by a local infiltration of leukocytes, edema, production of inflammatory mediators and histological changes in the bronchi such as the metaplasia of mucus-producing goblet cells and thus appears suitable to study the pharmacological effects of treatments of self-limiting acute bronchitis [20]. In addition, there is no suitable virally-induced rodent model of acute bronchitis available that resembles the pathology found in humans. Most viral infection models either result in no or only slight bronchitis-like symptoms or in the death of the animals.

The results obtained in our *in vivo* studies demonstrate that BRO possesses potent anti-inflammatory and mucus-regulatory activity as shown by its effects on inflammatory cell influx, MUC5AC protein production and goblet cell metaplasia. They furthermore suggest that BRO treatment had only limited effects on earlier stages of inflammation-induced epithelial activation (48 h) but rather prevented later stage progressive goblet cell meta/hyperplasia *e.g.* by acting on resolution processes or by directly addressing the epithelial remodeling (72 h). The lack of a dose dependency in the confirmation study suggests that the maximum therapeutic efficacy attainable by BRO had already been achieved at the 1-fold equivalent of the human daily dose or that more frequent applications to simulate the clinically achieved exposure (in humans 3 times a day *vs.* once daily in our animal study) are required to further maximize the therapeutic effect of oral BRO application.

The present studies did not focus on identifying the mechanisms

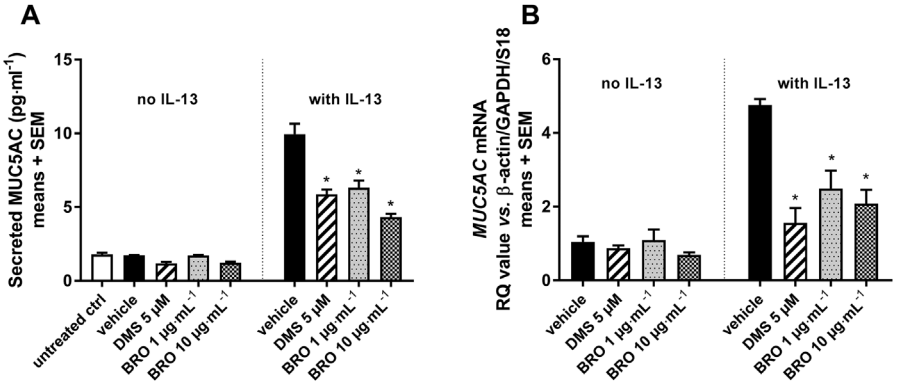

Fig. 3. Effects of BRO treatment on IL-13-induced MUC5AC protein secretion (A) and *MUC5AC* mRNA expression (B) in the human respiratory epithelium cell line Calu-3.
Differentiated cells were kept on air-liquid-interface (ALI) cell culture and were treated basolaterally for 14 d with BRO, DMS or vehicle either in the presence of IL-13 or without stimulus. Secreted MUC5AC was washed off the apical cell surface and quantified by ELISA, *MUC5AC* mRNA expression was evaluated by quantitative Real-Time PCR using the $2^{-\Delta\Delta CT}$ method against three housekeeping genes (β-actin, GAPDH and S18). *p < 0.05; ANOVA + Dunnett's test *vs.* vehicle (negative control).

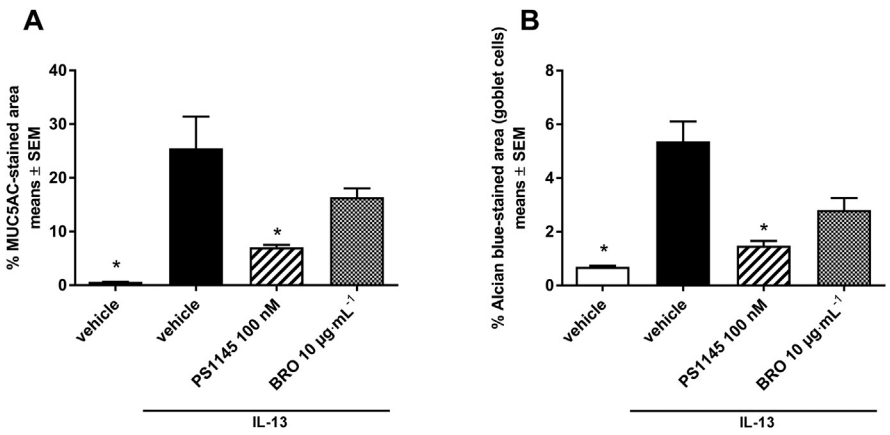

Fig. 4. Effects of BRO treatment on IL-13-induced MUC5AC protein expression (A) and goblet cell metaplasia (B) in MucilAir® primary human respiratory epithelium cell cultures.
MucilAir® ALI cultures were treated with IL-13 and the test compounds BRO and PS1145 for 14 consecutive days. Quantification of goblet cells and MUC5AC protein was performed microscopically on slides stained with Alcian blue or immunohistochemistry, respectively.
*p < 0.05; ANOVA + Dunnett's test *vs.* IL-13/vehicle (negative control).

underlying the inhibition of inflammatory cell influx by BRO treatment. However, recently conducted studies demonstrated attenuation of the LPS-induced increase in PGE_2 and leukotriene levels in BALF by BRO treatment. In addition, BRO and the single ingredient thyme herb extract were shown to inhibit 5-lipoxygenase activity and leukotriene release by human leukocytes *in vitro* [23]. Hence, interference with the release of pro-inflammatory mediators could contribute to the inhibition of inflammatory cell influx observed in animals treated with BRO. In a comparable experimental set-up [16], we demonstrated anti-inflammatory efficacy of a thyme herb/ivy leaf extract fixed combination, suggesting that thyme herb (supported by common ingredients as *e.g.* saponins from primula root or ivy leaf) is an important common driver of the observed inhibitory effect on cell influx exerted by the two herbal medicinal products. However, further studies on the relative contribution of either ingredient are required to definitely settle this point.

In order to elucidate whether BRO exerts direct mucus-regulatory effects on epithelial cells we subsequently performed cell culture experiments with human respiratory epithelium stimulated with IL-13. While IL-13 is also a pro-inflammatory stimulus any confounding effects by the inhibitory activity of BRO on immune cell influx or mediator production are excluded in this experimental environment. IL-13 is a cytokine produced by T-lymphocytes and plays a central role in the pathogenesis of asthma [24]. It induces MUC5AC production *via* activation of the STAT6 and subsequently TGF-β/SMAD pathways [8,25] and goblet cell metaplasia involving ERK, p38 MAPK and PI3-kinase [26] in respiratory epithelial cell cultures. The effects of BRO treatment in both Calu-3 cells as well as primary human bronchial epithelium cells reflected the findings observed in the *in vivo* studies and suggest BRO to intervene directly with MUC5AC production on a transcriptional level.

Our results from the primary epithelium culture furthermore confirmed BRO's activity on goblet cells observed *in vivo* by inhibiting the metaplasia of goblet cells following IL-13 stimulation.

This in turn suggests that BRO can affect both MUC5AC expression and goblet cell metaplasia by directly acting on epithelial cells, independent of its inhibitory activity on inflammatory cell influx. However, multiple modes of action might be simultaneously involved in the *in vivo* and clinical situations: it has been described that the cysteinyl-leukotriene LTE_4 is involved in the upregulation of MUC5AC [27]. The above-mentioned work in the model of LPS-induced pulmonary inflammation as well as in human neutrophils showed that BRO has also an inhibiting effect on leukotriene biosynthesis [23]. In addition, neutrophil derived proteases such as neutrophil elastase have been linked with increased mucin expression [28]. Since BRO administration reduced the number of granulocytes in lung tissue either by inhibition of cell influx or acceleration of resolution, BRO treatment is likely to result in an attenuated release of neutrophil derived drivers of goblet cell metaplasia and MUC5AC induction. Therefore, it appears feasible that under *in vivo* conditions BRO treatment probably exerts its mucus regulatory effects by both anti-inflammatory and direct mucus-regulatory activities on epithelial cells.

We are aware that a transfer of data and conclusions obtained in our animal model of LPS-induced pulmonary inflammation and cell cultures with human bronchial epithelial cells to the clinical situation in acute bronchitis patients has to be attempted with caution. For herbal medicinal products such as BRO extrapolation of data is further complicated by the inherent difficulty to clearly ascribe efficacy to specific individual ingredients and to compare exposure levels in animals and humans. For thyme extract the ingredients thymol, carvacrol and thymoquinone have been suggested to play a role in its pharmaceutical activity. Indeed, it has been reported that thymol increases mucociliary transport in the mouse [12] and exerts anti-inflammatory effects possibly by interfering with the NFκB signaling pathway and by down-regulation of mediator production and release [13,29,30] whereas thymoquinone has been shown to intervene with inflammatory events relevant for the respiratory tract *in vitro* and *in vivo* [31–33]. In line with these data, oral administration of carvacrol reduced cytokine expression and markers of oxidative stress in LPS challenged mice [34], suggesting a potential contribution of monoterpene ingredients in the efficacy conferred by thyme herb extract. Indeed, this was corroborated by our own work with these compounds on leukotriene release from A23187-stimulated leukocytes [23]. Nevertheless, further research on pharmacological properties and pharmacokinetics with ingredients of both thyme herb and primula root extracts will be required to identify the most relevant active compounds responsible for the efficacy of BRO in the treatment of patients suffering from acute bronchitis.

Finally, the findings reported here open up new vistas on a possible benefit of BRO in chronic inflammatory airway conditions such as chronic bronchitis, COPD and cystic fibrosis as these are characterized by a mucus hypersecretory phenotype and severe goblet cell metaplasia [25]. Actually, it has been shown for chemical-synthetic agents used to treat COPD and chronic bronchitis such as the phosphodiesterase-4 inhibitor roflumilast to also act on the properties of mucus [35,36]. Further research should substantiate this potential capacity.

5. Conclusions

Taken together, our data suggest that direct effects on mucin production and goblet cell metaplasia as well as indirect effects *via* anti-inflammatory mechanisms potentially underlie the mucus-regulatory activity of Bronchipret® TP film-coated tablets observed in *in vivo* studies of experimentally induced pulmonary inflammation. These effects might contribute essentially to the product's therapeutic efficacy in patients suffering from acute bronchitis with viscous mucus and cough and might also form the basis for its application in chronic inflammatory airway diseases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pupt.2018.04.009.

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