Phytomedicine 22 (2015) 1172–1177

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed

Bronchipret® syrup containing thyme and ivy extracts suppresses bronchoalveolar inflammation and goblet cell hyperplasia in experimental bronchoalveolitis

Jan Seibel[a,*], Carlo Pergola[b], Oliver Werz[b], Kirill Kryshen[c], Katja Wosikowski[a], Martin D. Lehner[a], Jutta Haunschild[a]

[a] Preclinical R&D, Bionorica SE, Kerschensteinerstr. 11-15, D-92318 Neumarkt, Germany
[b] Chair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich-Schiller-University Jena, Philosophenweg 14, D-07743 Jena, Germany
[c] Saint-Petersburg Institute of Pharmacy, Leningrad Region, Vsevolozhsky District, 188663, Kuzmolovo P 245, Russia

ARTICLE INFO

Article history:
Received 12 January 2015
Revised 12 August 2015
Accepted 2 September 2015

Keywords:
Acute bronchitis
Thyme
Ivy
Anti-inflammatory
Goblet cells
5-lipoxygenase

ABSTRACT

Background/purpose: Acute bronchitis (AB) is a common lung condition characterized by inflammation of the large bronchi in response to infection. Bronchipret® syrup (BRO), a fixed combination of thyme and ivy extracts has been effectively used for the treatment of AB. Combining *in vivo* and mechanistic *in vitro* studies we aimed to provide a better understanding of the therapeutic potential of BRO on key aspects of AB and to identify potential mechanisms of action.
Methods: Bronchoalveolitis in rats was induced by intratracheal LPS instillation. BRO was administered p.o. once daily at 1- to 10-fold equivalents of the human daily dose. Animals were sacrificed 24–72 h *post* LPS challenge to analyze leukocyte numbers in lung tissue, bronchoalveolar lavage fluid (BALF) and blood as well as goblet cells in bronchial epithelium. Inhibitory effects of BRO analogue on leukotriene (LT) production were determined in human neutrophils and monocytes as well as on isolated 5-lipoxygenase (5-LO).
Results: BRO significantly reversed the LPS-induced increase in leukocyte numbers in lung tissue, BALF and blood as well as goblet cell numbers in bronchial epithelium. *In vitro*, BRO analogue suppressed cellular release of LTB_4 ($IC_{50} = 36\ \mu g \cdot ml^{-1}$) and cysLT ($IC_{50} = 10\ \mu g \cdot ml^{-1}$) and inhibited the activity of isolated 5-LO ($IC_{50} = 19\ \mu g \cdot ml^{-1}$).
Conclusion: BRO exerts significant anti-inflammatory effects and attenuates goblet cell metaplasia in LPS-induced bronchoalveolitis *in vivo* potentially *via* interference with 5-LO/LT signaling. These effects may contribute to its observed clinical efficacy in AB.

Introduction

Acute bronchitis (AB) is a predominantly self-limited respiratory disease comprising several different symptoms amongst which inflammation of the bronchi, increased production of viscous mucus and consequently cough are the most incriminating for the patient (Verheij et al. 1989). Cough is the most frequent reason for visits to primary care physicians and imposes a significant economic burden on the public health system (Holzinger et al. 2014). In addition to symptomatic treatment with anti- and protussive agents, mucolytics and adrenergic beta-2 agonists, antibiotics have commonly been used for AB therapy (Tackett and Atkins 2012) albeit recent publications indicate the lack of efficacy of antibiotic treatment in AB (Smith et al. 2014). Consequently, antibiotics should only be used to prevent an exacerbation of chronic bronchitis or in patients under the risk of developing pneumonia (Martinez 2004). Therefore, other effective and well-tolerated treatment options without the potential for increasing antimicrobial resistance are imperative.

Several clinical trials have demonstrated positive effects of Bronchipret® Syrup (BRO), a fixed combination of thyme herb and ivy leaf fluid extracts, on symptom relieve and recovery time in patients with AB and cough (Kemmerich et al. 2006; Marzian 2007). Based on these data BRO was included into the Guidelines of the German Respiratory Society for Diagnosis and Treatment of Adults Suffering from Acute or Chronic Cough (Kardos et al. 2010).

Abbreviations: AA, arachidonic acid; AB, acute bronchitis; 5-LO, 5-lipoxygenase; BALF, bronchoalveolar lavage fluid; BLT, LTB_4 receptor; BRO, Bronchipret® syrup; LT, leukotriene; COX, cyclooxygenase; DER, drug extract ratio; EtOH, ethanol; I_{max}, maximal inhibition; IC_{50}, calculated concentration of half-maximal inhibition; LPS, lipopolysaccharide; RT, room temperature.

* Corresponding author. Tel.: +49 (0) 9181/231 309; fax: +49 (0) 9181/231 6309.
E-mail address: jan.seibel@bionorica.de (J. Seibel).

http://dx.doi.org/10.1016/j.phymed.2015.09.001
0944-7113/

Although for both thyme and ivy single extracts anti-inflammatory, smooth muscle relaxing and/or secretolytic activities have been reported (Landa et al. 2009; Wienkotter et al. 2007; Wolf et al. 2011) the mechanisms underlying the clinical efficacy of BRO have not been identified, yet.

In the present study we applied a dual experimental approach to study the anti-inflammatory efficacy of BRO in experimentally induced AB *in vivo* and to identify potential mechanisms of action *in vitro*. Intratracheal LPS instillation in rats was used as a model system for AB to study the effects of BRO on three aspects of the disease *in vivo*: (i) bronchial inflammation *via* mucosal and submucosal granulocyte infiltration, (ii) mobilization of granulocytes into the bronchoalveolar fluid (BALF) and blood and (iii) the number of bronchial goblet cells as an indirect measure of tissue remodeling and mucus production. Due to the important role of leukotriene B_4 (LTB_4) in neutrophil chemotaxis and of cysteinyl leukotrienes (cysLTs) in bronchoconstriction we additionally investigated the effects of a BRO analogue on 5-LO product formation *in vitro*.

Methods

Test items of the herbal extracts

Bronchipret® syrup was provided by Bionorica SE, Neumarkt, Germany. For *in vivo* studies, rats were administered commercially available Bronchipret® syrup (BRO) with a final thyme/ivy extract ratio of 10:1 and an ethanol content of 7% $v{\cdot}v^{-1}$. Specifically, a 10 g preparation (equivalent to 8.85 ml) of BRO contains a mixture of 1.5 g thyme extract (1:2–2.5 drug extract ratio (DER) in ammonia solution 10% ($wt{\cdot}wt^{-1}$), glycerol 85% ($wt{\cdot}wt^{-1}$), ethanol 90% ($wt{\cdot}wt^{-1}$) and water (1:20:70:109)) and 0.15 g fluid extract derived from ivy leaves (DER: 1:1; extracting agent: ethanol 70% ($v{\cdot}v^{-1}$)). Both extracts are standardized to the content of specific marker compounds.

Due to the ethanol content of the fluid extracts contained in BRO, a mixture of genuine thyme herb dry extract and genuine ivy leaf liquid extract lyophilisate with identical composition (calculated as the ratio of the herbal substances) as contained in BRO (termed "BRO analogue") was used as a surrogate for studying 5-LO product synthesis *in vitro*. This mixture was diluted with 50% ethanol ($v{\cdot}v^{-1}$) to a concentration of 100 $mg{\cdot}ml^{-1}$, homogenized by vortexing and then incubated in an ultrasonic bath at room temperature (RT). The suspension was centrifuged (3000 × g, 10 min, RT) and the supernatant was filtrated through a disposable syringe filter (PVDF; pore size, 0.22–0.45 μm; Millipore, Billerica, MA) and immediately used for the assays.

Bronchoalveolitis in vivo model

Animal housing

Male Wistar rats weighing 250–300 g (Rappolovo, St. Petersburg, Russia) were kept in polycarbonate cages (6 animals/cage) at 21 ± 1 °C and 61–75% humidity in a 12 h light/dark cycle, with *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 R-53434-2009 and were approved by the Ethics Committee of the Saint-Petersburg Institute of Pharmacy JSC (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).

Induction of bronchoalveolitis and animal treatment

Prior to experimentation 180 animals (n = 36/group, 12 animals/time point) were randomly allocated to five treatment groups by a modified method of the block randomization (Altman and Bland 1999). One additional group served as healthy control group (sham; n = 12). Animals were acclimatized for 14 days prior to start of experiments.

Bronchoalveolitis was induced by intratracheal injection of 100 μg LPS from *Escherichia coli* 0111:B4 (Sigma–Aldrich, St. Louis, MO) in 200 μl 0.9% NaCl per animal 1 h prior to the first oral administration of the test compounds as described (Blackwell et al. 1999). The animals were then treated by oral gavage with either vehicle (7% $v{\cdot}v^{-1}$ ethanol) or BRO (1.7, 5.0, or 16.7 $ml{\cdot}kg^{-1}$). The doses corresponded to the 1-, 3- and 10-fold of the currently recommended human daily dose after allometric conversion to human equivalent doses (U.S. Food and Drug Administration 2005). Treatment comprised up to 3 administrations at 1, 25 and 49 h *post* LPS injection. Dexamethasone (Dex, 5 $mg{\cdot}kg^{-1}$) was also administered at 1, 25 and 49 h *post* LPS into the femoral muscle as a positive control to demonstrate an anti-inflammatory effect. All compounds (including vehicle) were diluted to a final ethanol content of 7% $v{\cdot}v^{-1}$ to assure equal ethanol uptake.

BALF, blood and tissue collection and analysis

For the analysis of BALF 6 rats/group were sampled at 24, 48 and 72 h *post* intratracheal LPS-instillation. Rats were anesthetized by intravenous injection of Zoletil® (tiletamine/zolazepam; Virbac, France) at 0.7 $mg{\cdot}kg^{-1}$. The right main bronchus was ligated 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 (Pharmaceutical company Volga Manufactory, LLC) to remove mucus, followed by centrifugation (1500 × g, 15 min, 4 °C).

Resulting supernatants were stored in liquid nitrogen until analysis. Pellets were resuspended and total leukocyte counts were measured with a veterinarian hematological analyzer (Abacus Junior Vet, Diatron, Hungary). Differential leukocyte counts of the BALF were performed in May-Grünwald/Giemsa stained slides using light microscopy. A minimum of 100 cells/slide were counted.

For collecting blood leukocytes, venous blood was withdrawn from the tail vein (6 animals/group/time point) into a plastic tube containing heparin (20 $ED{\cdot}ml^{-1}$; FGUP "Moscow endocrine factory", Russia) and leukocytes were counted in an Abacus Junior Vet hematological analyzer (Diatron, Hungary).

Lung histology was performed in hematoxylin and eosin stained formalin-fixated tissue slices from the right lung. Inflammation in the bronchial tissue was evaluated by semi-quantitative analysis of mucosal and submucosal granulocyte infiltration using a scoring system ranging from normal = 0 to severe = 3. For histochemical calculation of goblet cell numbers samples were taken from medium-sized bronchi obtained from central regions of the lungs. Three slides from each animal were counter-stained with Alcian blue at pH 2.5 and goblet cells within 1 mm of the epithelial layer were counted. All goblet cells in medium-sized bronchi per slide were counted. Morphological examination was performed using light-optical microscopy (Leica DC320 (Leica Microsystems, Germany)) at 200-fold magnification (Harris et al. 2007).

Blood cells and cell isolation

Neutrophils and monocytes were isolated from buffy coats from healthy adult volunteers obtained at the Institute of Transfusion Medicine, University Hospital Jena. Neutrophils were immediately isolated by dextran sedimentation and centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria) and hypotonic lysis of erythrocytes as described (Pergola et al. 2008). Monocytes were separated from peripheral blood mononuclear cells by adherence to culture flasks as described (Pergola et al. 2011). Cells were finally resuspended in PBS pH 7.4 containing 1 $mg{\cdot}ml^{-1}$ glucose and 1 mM $CaCl_2$ (PGC buffer).