European Journal of Pharmacology 768 (2015) 41–48

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Pulmonary, gastrointestinal and urogenital pharmacology

An inhaled phosphodiesterase 4 inhibitor E6005 suppresses pulmonary inflammation in mice

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ARTICLE INFO

Article history:
Received 22 June 2015
Received in revised form
2 October 2015
Accepted 7 October 2015
Available online 8 October 2015

Keywords:
Inhalation
Phosphodiesterase 4 inhibitor
E6005
Pulmonary inflammation

ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a progressive lung disease associated with significant morbidity and mortality. Although several oral phosphodiesterase 4 (PDE4) inhibitors have been developed for the treatment of COPD, their use has been restricted because of side effects including nausea and emesis. We hypothesized that delivery of a dry powdered PDE4 inhibitor by inhalation would minimize systemic absorption and enable local PDE4 inhibition to suppress inflammation within the lung. Neutrophilic pulmonary inflammation was induced in mice by intratracheal administration of lipopolysaccharide. Mice were treated intratracheally with a new dry powder PDE4 inhibitor, E6005 (methyl 4-[({3-[6,7-dimethoxy-2-(methylamino)quinazolin-4-yl]phenyl}amino) carbonyl] benzoate). The pharmacokinetics, cell profiles and levels of cytokines, chemokines, and lipid mediators in bronchoalveolar lavage fluid (BALF), and lung histology were assessed. Intratracheal administration of E6005 to mice resulted in high concentrations of the compound in the lungs. Histological analysis of E6005-treated mice demonstrated reduced inflammation of lung tissue that correlated with a decrease in BALF levels of neutrophils, proinflammatory cytokines, chemokines, and cysteinyl leukotrienes. Thus, intratracheal administration of E6005 effectively suppresses neutrophilic pulmonary inflammation, suggesting that the new inhaled dry powder PDE4 inhibitor represents an alternative to the conventional oral formulation for treating COPD.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by persistent airflow limitation that results from an enhanced inflammatory response in the lungs. It is a progressive lung disease associated with significant morbidity and mortality. Neutrophils are the most abundant inflammatory cells in the lung parenchyma and peripheral airways and neutrophil-derived reagents are strongly implicated in tissue injury in COPD (Hoenderdos and Condliffe, 2013).

Cyclic nucleotide phosphodiesterases (PDEs) are a family of enzymes that catalyze the breakdown of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate to their inactive forms. PDE4 is highly expressed in human immune cells, including neutrophils, eosinophils, monocytes/macrophages, lymphocytes, and airway epithelial cells that play an important role in the pathogenesis of inflammatory lung diseases (Page and Spina, 2012). Inhibition of PDE4 reduces most of the proinflammatory activities of these cells.

Oral PDE4 inhibitors have demonstrated some efficacy against COPD (Beghe et al., 2013); roflumilast has received approval from the European Medicines Agency and the U.S. Food and Drug Administration for the maintenance treatment of COPD. However, although roflumilast is considered safe, gastrointestinal adverse effects such as diarrhea, weight loss, and nausea result in the discontinuation of treatment (Chong et al., 2013). Although the definitive mechanism mediating these adverse effects is not completely understood, these may be induced by brain penetration of the compound, which occurs through systemic exposure of the absorbed drug from its application site (Robichaud et al., 2001, 2002).

PDE4 inhibitors exhibit a variety of anti-inflammatory effects. PDE4 inhibition results in intracellular accumulation of cAMP,

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http://dx.doi.org/10.1016/j.ejphar.2015.10.013
0014-2999/

causing the activation of protein kinase A (PKA), and subsequently resulting in the phosphorylation of the transcription factor cAMP response element binding protein (CREB). Activation of this cAMP/PKA/CREB signaling pathway suppresses the expression of proinflammatory cytokines, chemokines, and lipid mediators (Serezani et al., 2008). However, the precise mechanism by which PDE4 inhibitors exert their anti-inflammatory activities is unclear.

With the goal of developing an alternative platform for delivering PDE4 inhibitor to the lungs, we hypothesized that local administration of a new dry powder PDE4 inhibitor, E6005 (methyl 4-[({3-[6,7-dimethoxy-2-(methylamino)quinazolin-4-yl]phenyl}amino)carbonyl]benzoate), to the airways minimizes systemic absorption and enables local PDE4 inhibition within the lungs, which subsequently suppresses neutrophilic pulmonary inflammation.

Therefore, this study aimed to assess the pharmacokinetics of inhaled E6005 and to evaluate the therapeutic effectiveness of the compound in reducing neutrophilic pulmonary inflammation caused by lipopolysaccharide (LPS). Finally, to investigate the mechanism of anti-neutrophilic inflammatory properties of E6005, we examined its effects on the release of cytokines, chemokines, and lipid mediators in the lungs after LPS treatment.

2. Materials and methods

2.1. E6005 levels in lungs and serum after administration by different routes

Female C57BL/6 mice, aged 8–10 weeks, were obtained from CLEA Japan, Inc. (Tokyo, Japan) and housed under pathogen-free conditions. All studies were approved by the Division of Laboratory Animal Science Natural Science Center for Research and Education of Kagoshima University. E6005 was synthesized at Eisai Laboratories (Tokyo, Japan) (Ishii et al., 2013). Lactose was used as a negative control for E6005. To determine the route of E6005 administration that would achieve the highest concentration of E6005 in mice lungs, E6005 (100 μg) was administered intratracheally using a dry-powder insufflator (Model DP-4M, PennCentury, Wyndmoor, PA, USA) or by subcutaneous injection of an aqueous suspension. Oral route was not evaluated because E6005 is easily hydrolyzed by carboxyl esterase in the small intestine. After 1–7 h, bronchoalveolar lavage fluid (BALF) and serum were collected. BALF was collected by cannulating the trachea with a 24-gauge angiocatheter and flushing and aspirating three times with 1 ml of phosphate buffered saline, pH 7.4 (PBS), and then centrifuged at 750 g for 5 min. Blood was collected via the tail vein, allowed to clot for 60 min, and centrifuged at 10,000*g* for 10 min (Kibe et al., 2003). E6005 concentrations in BALF and serum were assessed using liquid chromatography–tandem mass spectrometry and normalized to total protein using a bicinchoninic acid protein assay kit (Thermo Scientific, Waltham, MA, USA).

2.2. Neutrophilic pulmonary inflammation therapy model

LPS (Sigma-Aldrich, St. Louis, MO, USA), 25 ng in 100 μL of PBS, was administered intratracheally to mice to induce neutrophilic pulmonary inflammation. PBS was a negative control. E6005 was administered intratracheally (1, 10 and 100 μg) or subcutaneously (100 μg) at 1 h before LPS administration. To determine the effects of delayed administration of E6005 on this model, E6005 (100 μg) was administered intratracheally at the same time as LPS, or 1 h after LPS administration. At 6 h after LPS injection, the total and differential BALF cell counts were calculated. Total cell counts were determined by light microscopy, using a standard hemacytometer. BALF was centrifuged at 50 g for 5 min. The cell pellet was resuspended in saline, and cytospin preparations (Cytospin 4, Thermo Scientific, Waltham, MA, USA) were made. Differential counts on 200 cells were performed by light microscopy, using a single-blind method after application of a modified Wright–Giemsa stain (Kibe et al., 2003).

2.3. Lung histopathology

To assess lung histopathology, mice were exsanguinated, and 2 ml of PBS was injected via the jugular vein to perfuse the lungs. Lungs were inflated to total lung capacity with 4% paraformaldehyde injection via an angiocatheter placed in the trachea and tied with sutures. The inflated lungs were removed *en bloc* and fixed in 4% paraformaldehyde followed by paraffin embedding. Standard hematoxylin and eosin staining was performed using the lung sections (5 μm) (Kibe et al., 2003).

2.4. Assessment of cytokines, chemokines, and lipid mediators in the lungs

Cytokine [tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), keratinocyte chemoattractant (KC)] and chemokine [macrophage inflammatory protein 1 alpha (MIP-1α), macrophage inflammatory protein 1 beta (MIP-1β), macrophage inflammatory protein 2 (MIP-2), interferon gamma-induced protein 10 (IP-10)] levels in BALF were measured by suspension array (Bio-Rad, Hercules, CA, USA). Using liquid chromatography–tandem mass spectrometry (Matsunaga et al., 2013; Matsunobu et al., 2013), we measured 17 lipid mediators, including leukotriene (LT)s, prostaglandins, thromboxanes, hydroxyeicosatetraenoic acid (HETE)s, and platelet-activating factor (PAF). In an *ex vivo* assay, mouse alveolar macrophages were isolated from BALF (Noda et al., 2013). Cells were resuspended in Dulbecco's modified Eagle's medium supplemented with 1% penicillin, and streptomycin. After allowing the cells to adhere to the plates at 37 °C for 2 h, nonadherent cells were removed by washing three times. The adhesive cells were incubated with E6005 (125 nM) or lactose dissolved in dimethyl sulfoxide for 1 h, and then incubated with LPS (10 μg/ml) for 6 h. Mouse TNF-α, MIP-1α, and MIP-1β levels in supernatants were measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA).

2.5. Statistical analysis

All data are shown as mean ± standard error. Statistical comparison was made by ANOVA, followed by Dunnett's test. A *P*-value of < 0.05 was accepted as indicating statistical significance.

3. Results

3.1. BALF and serum E6005 levels after administration by different routes

To determine which route of administration would achieve the highest lung concentration of E6005, we assessed BALF and serum E6005 levels after intratracheal or subcutaneous administration of the same dose of the compound. Intratracheal administration resulted in higher E6005 concentrations in BALF and reduced serum levels when compared with subcutaneous administration (Fig. 1). BALF levels of E6005 peaked at 1 h and gradually decreased over time. When assessing total protein levels in BALF as a marker of lung inflammation, their was no marked elevation after intratracheal administration of E6005 compared to subcutaneous administration throughout the experimental period (maximum level at 7 h: intratracheal, 339.6 ± 21.0 μg/ml vs. subcutaneous,