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Soluble epoxide hydrolase inhibitor AUDA decreases bleomycin-induced pulmonary toxicity in mice by inhibiting the p38/Smad3 pathways

Xin-wei Dong[a,1], Yong-liang Jia[a,1], ling-tian Ge[a,b], Bo Jiang[a], Jun-xia Jiang[a], Jian Shen[a], Ya-chao Jin[a], Yan Guan[a], Yun Sun[b,*], Qiang-min Xie[a,*]

[a] Key Laboratory of Respiratory Drugs Research, Zhejiang University School of Medicine, Hangzhou 310058, China
[b] Medical College of Yangzhou University, 11 Huaihai Road, Yangzhou 225001, China

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ABSTRACT

Bleomycin (BLM) has potent tumor cell-killing properties that have given it an important place in cancer chemotherapy, but pulmonary toxicity is its major adverse effect. Soluble epoxide hydrolase (sEH) inhibitors have been reported to have protective effects in fibrosis models, but the effects of AUDA, an sEH inhibitor of BLM-induced pulmonary toxicity and fibrosis, remain to be researched. In this study, we assessed the effects of AUDA on the BLM-induced pulmonary fibrosis in a mouse model, and transforming growth factor (TGF)-β_1-induced epithelial proliferation and epithelial-mesenchymal transition (EMT) in vitro by monitoring changes in pulmonary function, inflammatory response, fibrotic remodeling, and signaling pathways. AUDA was administered by intragastric administration (i.g) daily for three weeks, starting at seven days after intratracheal instillation of BLM. All examinations were performed 24 h after the last i.g. In vivo, AUDA significantly improved BLM-induced decline in lung function and body weight, and inhibited inflammatory cell accumulation and the mRNA and protein expression of interleukin (IL)-1β, TGF-β_1, and matrix metalloproteinase 9 (MMP-9) in lung tissue. Moreover, AUDA attenuated BLM-induced deposition of collagen fibers, destruction of alveolar structures, and pulmonary parenchyma. Additionally, AUDA regulated the expression of α-smooth muscle actin (α-SMA) and E-cadherin by inhibiting the Smad3/p38 signaling pathway. In vitro, AUDA significantly inhibited TGF-β_1-induced epithelial cells and fibroblast proliferation, reduced sEH expression and α-SMA expression, and increased epoxyeicosatrienoic acid (EET) levels and E-cadherin expression in epithelial cells. These effects were blocked by AUDA by downregulating the Smad3 and p38 signaling pathways. Taken together, these data indicate that treatment with sEH inhibitors may improve BLM-induced pulmonary toxicity.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a pathological condition in which chronic inflammation and changes to the extracellular matrix lead to alterations in lung tissue architecture and functional degradation of the lung (Gifford et al., 2012). Its pathophysiology is currently thought to involve epithelial injury with abnormal wound healing (Chambers and Mercer, 2015). However, repeated injury, such as those caused by chronic cigarette smoking, exposure to environmental toxins, and viral infection, can lead to a cascade of inflammatory system-related changes in the lung that result in abnormal deposition and composition of collagens and other elements of the extracellular matrix (ECM) as well as extensive damage to the tissue (Camelo et al., 2014). This pathological condition is called fibrosis. Bleomycin (BLM) is an effective chemotherapeutic agent mainly used in patients with Hodgkin's lymphoma or testicular cancer. However, the lung fibrosis as a negative consequence of BLM pharmacotherapy with cancers has limited its use in clinical settings (van der Schoot et al., 2016). BLM can cause alveolar cell damage and subsequent pulmonary inflammation independent from its effect on DNA by inducing lipid peroxidation. The

Abbreviations: AUDA, a soluble epoxide hydrolase inhibitor; BALF, bronchoalveolar lavage fluid; BLM, bleomycin; DMSO, dimethyl sulfoxide; DXM, dexamethasone; EMT, epithelial-mesenchymal transition; ECM, extracellular matrix; EET, epoxyeicosatrienoic acid; ELISA, enzyme-linked immunosorbent assay; EpFAs, epoxy fatty acids; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 16HBE, a human bronchial epithelial cell; H & E, stained with hematoxylin and eosin; HFL-1, a human lung fibroblast; HYP, hydroxyproline; IL, interleukin; IPF, idiopathic pulmonary fibrosis; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; MTT, methyl thiazolyl tetrazolium; PBS, phosphate-buffered saline; Penh, enhanced pause; qPCR, quantitative polymerase chain reaction; SB203580, a p38-specific inhibitor; SDS, sodium dodecyl sulfate; sEH, soluble epoxide hydrolase; siRNA, small interfering RNA; SIS3, a Smad3-specific inhibitor; α-SMA, α-smooth muscle actin; TGF, transforming growth factor; WBP, whole-body plethysmography

* Corresponding authors.
E-mail addresses: jgz7718@sina.com (Y. Sun), xieqm@zju.edu.cn (Q.-m. Xie).
[1] These authors contributed equally to this work.

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lung injury observed following BLM comprises an interstitial edema with an influx of inflammatory and immune cells, which lead to the development of pulmonary fibrosis, characterized by enhanced production and deposition of collagen and other matrix components (Hay et al., 1991; Azambuja et al., 2005). Hence, BLM-induced lung fibrosis has become a useful experimental model in animals for interstitial pneumonitis and pulmonary fibrosis (Della Latta et al., 2015).

There is growing evidence of the importance of lipid signaling in lung fibrosis, in addition to those commonly studied protein inflammatory mediators, bioactive lipids play an important role (Bennett and Gilroy, 2016). The eicosanoids are a class of lipid mediators that include leukotrienes, prostaglandins, and epoxyeicosatrienoic acids (EETs), produced through the activity of lipoxygenase (LOX), cyclooxygenase (COX) and cytochrome P450 enzymes, respectively (Huwiler and Pfeilschifter, 2009). The leukotrienes and prostaglandins have been implicated in the progression of lung fibrosis in studies employing leukotriene receptor antagonists and COX inhibitors. A leukotriene receptor antagonist, montelukast, attenuates serum and sputum levels of eosinophil cationic protein and interleukin-8 (IL-8), decreases sputum levels of myeloperoxidase, and increases serum and sputum levels of IL-10 in children with cystic fibrosis compared with placebo (Schmitt-Grohé and Zielen, 2005). The COX inhibitor, high-dose ibuprofen, can slow the progression of disease in people with cystic fibrosis, especially in children, which suggests that strategies to modulate lung inflammation can be beneficial for people with cystic fibrosis (Lands and Stanojevic, 2016).

A third class of arachidonic acid-derived mediators, the EETs, exhibits anti-inflammatory and anti-fibrotic properties (Bennett and Gilroy, 2016). The EETs are metabolized by soluble epoxide hydrolase (sEH), producing dihydroxy molecules that are less lipophilic and more readily conjugated, leading to their removal from the site of action (Morisseau and Hammock, 2013). sEH inhibitors modulate the levels of epoxy fatty acids (EpFAs) in tissues and dramatically reduce acute lung inflammation in a lipopolysaccharide (LPS)-induced model (Tao et al., 2016) as well as attenuate fibrosis and inflammation in the liver, lungs, heart, kidney, and pancreas (Harris et al., 2015; Zhou et al., 2016; Li et al., 2014; Kim et al., 2014; Morgan et al., 2013). 12-(3-adamantan-1-yl-ureido) dodecanoic acid (AUDA), a sEH inhibitor, has not been previously reported to treat pulmonary fibrosis, despite of its ability to inhibit the cellular mechanisms of fibrogenesis in a salt-sensitive hypertension model (Li et al., 2008). Here, we evaluated the effects and mechanisms of AUDA in a murine model of lung fibrosis. To evaluate a lung fibrosis model and compare the effects of AUDA with dexamethasone, a potent synthetic anti-inflammatory drug which is used as a positive control drug. This work might provide evidences that monotherapy of sEH inhibitors or sEH inhibitors in combination with other agents can potentially be used as therapeutic agents for pulmonary fibrosis and reducing the lung toxicity caused by chemotherapy of BLM.

2. Materials and methods

2.1. Animal care and experimental procedures

ICR mice (weighing 18–22 g) were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The certification number: NO. SCXK 2012–0002. In the study, we chose female mouse as a model of BLM-induced pulmonary toxicity referenced to the previous studies (Xia et al., 2016; Yara et al., 2001). The animals were housed in isolated ventilated cages (4–5 mice/cage) under a 12-h light/12-h dark cycle and received a normal chow diet (SLAC Laboratory Animal Co., Ltd. Shanghai, China) and purified water. All the animal experiments performed in this study were approved by an independent Animal Care and Use Committee of Zhejiang University (Hangzhou, China). All animal experimental procedures were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" and were in compliance with European Community specifications regarding the use of laboratory animals. After 5 days of acclimatization, a total of 50 mice were randomly allocated into 5 groups of 10 mice/group: control group (only phosphate-buffered saline [PBS]), model group (BLM 2 mg/kg + Saline), BLM 2 mg/kg + AUDA 3 mg/kg group, BLM 2 mg/kg + AUDA 10 mg/kg, and BLM 2 mg/kg + dexamethasone (DXM) 0.5 mg/kg, as a positive control. The mice were anesthetized by an intraperitoneal injection of 3% pentobarbital sodium (10 ml/kg). To reduce systemic drug delivery of BLM-caused other adverse reactions or other organ fibrosis, and maintain higher drug concentration pulmonary in the local lung tissues, in most of the studies, BLM was given by a single intratracheal instillation in weight-adjusted dosages (Mouratis and Aidinis, 2011). In this study, the pulmonary fibrosis model was developed by intratracheal instillation of BLM (volume 20 μl/20 g BW, Bleomycin Hydrochloride for Injection, Nippon Kayaku Co. Ltd) dissolved in sterile PBS according to our previous report (Liu et al., 2017). Animals in the control group were administered the same amount of PBS without BLM. The model group and treatment groups were administered saline, DXM (Tianyao Pharmaceutical Co., Wuhan, Hubei Province, China) at 0.5 mg/kg, or 12-(3-adamantan-1-ylureido)-dodecanoic acid (AUDA; Cayman Chemical Company, Ann Arbor MI) at 3 or 10 mg/kg by intragastric route daily for 3 weeks at 7 days after intratracheal instillation of BLM. The AUDA dose for the intragastric administration was referenced from Tao et al. report (Tao et al., 2016) and pharmacokinetic parameters of intragastric administration from Liu et al. (Liu et al., 2009). They reported the area under the concentration–time curve to terminal time (AUC_t) and maximum blood concentration (C_{max}) of AUDA is 18 ± 2 (μM min) and 66 ± 31 (nM) after 5 mg/kg intragastric administration in mice, respectively. The body weight of each mouse was measured and recorded every 7 days. Mice were euthanized through an intraperitoneal injection of 6 g/kg urethane 24 h after the last drug treatment. The left lung was ligatured with nylon wire, and the right lung tissues were lavaged with PBS 3 × 0.5 ml. Bronchial alveolar lavage fluid (BALF) was collected and centrifuged at 500 × *g* for 10 min at 4 °C. The pelleted BALF cells were resuspended in PBS, and the total number of leukocytes was counted in a Neubauer chamber. Two hundred cells from BALF stained with Wright–Giemsa were counted under a light microscope. The total number of each cell type was determined by multiplying the percentage of each cell type by the total number of cells. The upper side of the left lung lobe was cut for the measurement of hydroxyproline (HYP), proinflammatory cytokines, and fibrotic factor levels. The bottom of the left lung lobe was preserved in 10% formalin for immunohistochemistry and histologic examination (Liu et al., 2017).

2.2. Determination of pulmonary function

A commercially available plethysmograph, acquisition software, and mouse-sized plethymograph chambers (Buxco Electronics, Troy, NY) were used for total pulmonary airflow analysis in unrestrained conscious mice. This system allows measurement of the differential pressure within the chambers caused by the animal's breathing. Pressure differences between the chambers containing individual animals and a reference chamber were used to extrapolate minute volume, tidal volume, breathing frequency, and enhanced pause (Penh) (Hoymann, 2007). Penh is a function of total pulmonary airflow during the respiratory cycle and is described by the following equation: Penh = [(Te/Tr-1) × (PEF/PIF)], where PEP is the peak expiratory pressure, PIP is the peak inspiratory pressure, and pause is a component of expiration time (Te/Tr-1). This parameter is dependent on the breathing pattern and correlates with airway resistance, as measured by traditional invasive techniques in ventilated mice (Hoymann, 2007). According to the methods for pulmonary function described in our previous paper (Qin et al., 2012), 24 h after the last drug treatment, a mouse was acclimatized to the plethysmograph chambers for 10 min prior to the evaluations. The Penh values of spontaneously breathing