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Role of histamine H_4 receptor ligands in bleomycin-induced pulmonary fibrosis

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ABSTRACT

Fibrosis of lung tissue is a disease where a chronic inflammatory process determines a pathological remodelling of lung parenchyma. The animal model obtained by intra-tracheal administration of bleomycin in C57BL/6 mice is one of the most validated murine model. Bleomycin stimulates oxidative stress and the production of pro-inflammatory mediators. Histamine H_4R have recently been implicated in inflammation and immune diseases. This study was focused to investigate the effects of H_4R ligands in the modulation of inflammation and in the reduction of lung fibrosis in C57BL/6 mice treated with bleomycin.

C57BL/6 mice were treated with vehicle, JNJ7777120 (JNJ, selective H_4R antagonist) or ST-1006 (partial H_4R agonist), ST-994 (H_4R neutral antagonist) and ST-1012 (inverse H_4R agonist) at equimolar doses, released by micro-osmotic pumps for 21 days. Airway resistance to inflation was assayed and lung samples were processed to measure malondialdehyde (TBARS); 8-hydroxy-2′-deoxyguanosine (8OH*d*G); myeloperoxidase (MPO); COX-2 expression and activity as markers of oxidative stress and inflammation. Fibrosis and airway remodelling were evaluated throughout transforming growth factor-β (TGF-β), percentage of positive Goblet cells, smooth muscle layer thickness determination.

Our results indicated that JNJ, ST-994 and ST-1012 decreased inflammation and oxidative stress markers, *i.e.* the number of infiltrating leukocytes evaluated as lung tissue MPO, COX-2 expression and activity, TBARS and 8OH*d*G production. They also reduced the level of TGF-β, a pro-fibrotic cytokine, collagen deposition, thickness of smooth muscle layer, Goblet cells hyperplasia; resulting in a decrease of airway functional impairment.

The results here reported clearly demonstrated that H_4R ligands have a beneficial effect in a model of lung fibrosis in the mouse, thus indicating that H_4R antagonists or inverse agonists could be a novel therapeutic strategy for lung inflammatory diseases.

Abbreviations: COX-2, cyclooxygenase-2; H_4R, histamine H_4 receptor; 8OH*d*G, 8-hydroxy-2′-deoxyguanosine; JNJ7777120, 1-[(5-chloro-1H-indol-2yl)carbonyl]-4methylpiperazine; MPO, myeloperoxidase; PGE_2, prostaglandin E_2; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substances; TGFβ, transforming growth factor β; ST-994, N^4-(4-methylbenzyl)-6-(4-methylpiperazin-1-yl) pyrimidine-2,4-diamine; ST-1006, N^4-(2,6-dichlorobenzyl)-6-(methylpiperazin-1-yl) pyrimidine-2,4-diamine; ST-1012, N^4-(1,3-dihydro-2H-isoindol-2-yl)-6-(4-methylpiperazin-1-ylo) pyrimidine-2-amine.

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1. Introduction

Lung fibrosis is a pathological response to chronic inflammation, which determines damage of epithelial cells, vascular exudation and infiltration of leukocytes in the alveolar spaces.

The vascular exudate participates in the organization of extracellular tissue together with the proliferation of fibroblasts and their activation into myofibroblast and with the transdifferentiation of epithelial cells or the differentiation of circulating fibrocytes. Myofibroblasts, organized into aggregation of cells known as fibroblastic *foci,* are activated fibroblasts, secreting extra amount of connective tissue matrix and collagen, establishing the fibrotic process [1]. This process determines a progressive airway stiffening, making breathing difficult and leading to respiratory failure. Among the fibrotic diseases of the lung, idiopathic pulmonary fibrosis (IPF) is the most common and severe disease with a prevalence increasing every year [2,3] with a high mortality rate and a median survival of ~3 years [4]. At the moment, the available pharmacological treatments of IPF are usually symptomatic and not effective [5,6] and some patients require lung transplantation [5]. Anti-inflammatory agents, such as glucocorticoids or immunosuppressive drugs, have been the conventional pharmacological treatment, although current reviews suggest that there is no therapeutic benefit with these drugs in comparison with their significant side effects [7]. Therefore, novel treatment options, oriented towards novel substances and/or based on new therapeutic targets, are urgently required. Oxidative stress is thought to play an important role in the pathogenesis of lung inflammation, increasing neutrophil sequestration in the pulmonary microvasculature and gene expression of proinflammatory mediators [8]. Moreover, several lines of evidence confirm the involvement of mast cells in the pathogenetic mechanism of lung fibrosis [9,10]. The number of mast cells was found significantly increased both in lung biopsies from fibrotic patients [11] and in animal samples of bleomycin-induced pulmonary fibrosis [12], suggesting that mast cell mediators play an important role in inflammation and in smooth muscle cell hypertrophy, by secreting histamine, tryptase and growth factors [13]. Although the relationship between mast cells and tissue remodelling remains not yet defined, it has been reported that the histamine released by mast cells contributes to the stimulation of other profibrotic stimuli, thus playing a role in the control of fibrotic process. Histamine is a pleiotropic mediator which exerts its biological effects through the activation of four different G-protein-coupled receptor subtypes (H_1-$_4$R) [14]. The H_1R is involved in allergic and immune responses, the H_2R regulates gastric acid secretion, the H_3R, a presynaptic receptor mainly present in the Central Nervous System, controls neurotransmitter release [15], and the H_4R, expressed in hematopoietic cells, activates mast cells, eosinophil and neutrophil migration [16,17]. Moreover, Kohyama and coworkers [18] demonstrated that the H_4R mediates *in vitro* the profibrotic effects of histamine on human foetal lung fibroblasts. In fact, the histamine effect on potentiating fibronectin-induced lung fibroblast migration was blocked by the selective H_4 antagonist JNJ7777120 (JNJ). These data suggest that the histamine H_4R could be a novel target for the development of new drugs for the treatment of lung inflammatory and fibrotic disease [18].

The aim of the present study was to evaluate whether H_4R ligands could have a therapeutic effect in lung fibrosis [19,20]. To support the rationale of our hypothesis, we investigated the effects of different, novel, H_4R ligands in controlling inflammation and fibrosis in an *in vivo* mouse model of bleomycin-induced pulmonary fibrosis.

2. Methods

2.1. Characterization of histamine H_4 receptor ligands in different screening models

The affinity for histamine H_4 receptors (H_4R) was evaluated on ^{3}[H]-histamine displacement assay on membrane preparation from Chinese hamster ovary (CHO)-K1 cells which stably express the human H_4R and the efficacy was evaluated on functional binding assay using ^{35}[S] GTPγS on membrane preparation from stable CHO-hH_4R. Functional gene reporter assay was performed in CHO-dukx cells stably expressed both the mouse H_4R and luciferase gene under the control of MRE/CRE responsive elements [21] and stimulated by forskolin 0.3 μM. Reference full agonist for efficacy was imetit in the mouse H_4R gene reporter assay. pKi for histamine H_1 receptors (H_1R) was determined with ^{3}[H]-pyrilamine displacement assay on membrane preparation from CHO-K1 cells stably expressing the human H_4R [22] and on Sf9 cell membranes co-expressing *h*H1R and RGS4 [23]; pKi for histamine H_2 receptors (H_2R) was determined with ^{3}[H]-tiotidine displacement assay on Sf9 cell membranes with *h*H_2R-$Gs_{\alpha s}$ fusion protein [23]; pKi for histamine H_3 receptors (H_3R) was performed with ^{3}[H]*Nα*-methylhistamine displacement on Sf9 cell membranes co-expressing *h*H_3R, $G_{\alpha i2}$, $G_{\beta 1\gamma 2}$ and RGS4 [23]. Ki(s) were calculated according to Cheng and Prusoff [24].

2.2. Animals

Male C57BL/6 mice, approximately 2 months old and weighing 25–30 g, were used for the experiments. They were purchased from a commercial source (Harlan, Udine, Italy), fed a standard diet, and housed for at least 48 h under a 12-h light/dark photoperiod before the experiments. The study protocol complied with the Declaration of Helsinki and the recommendations of the European Economic Community (86/609/CEE) on animal experimentation and was approved by the animal care committee of the University of Florence (Florence, Italy). Experiments were carried out at the Centre for Laboratory Animal Housing and Experimentation, University of Florence. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [25,26].

2.3. Surgery and treatments

Fifty six mice were anesthetized with zolazepam/tiletamine (Zoletil, Virbac Srl, Milan, Italy; 50 μg/g i.p. in 100 μl of saline); 50 of them were treated with bleomycin (0.05 IU in 100 μl of saline), and the other six were treated with 100 μl of saline (referred to as non fibrotic negative controls, Saline), both delivered by intra-tracheal injection. Six mice did not undergo to surgery and used as control (Naïve).

The bleomycin treated mice, ten per group, were treated with continuous infusion of H_4R ligands by osmotic micropumps (Alzet, Cupertino, CA, USA) filled with 100 μl of PBS pH 7.4, containing the H_4R ligands at the reported concentrations: JNJ7777120 40 mg/kg; ST-994 53 mg/kg; ST-1006 60 mg/kg; ST-1012 53 mg/kg. Ten mice were treated only with PBS and referred to as fibrotic positive controls (Vehicle). The micropumps were implanted subcutaneously into a dorsal poach at day 0 and maintained for 21 days. They released 1.55 μl per day.

2.4. Functional assay of fibrosis

At day 21 after surgery, the mice were subjected to measurement of airway resistance to inflation, a functional parameter related to fibrosis-induced lung stiffness, by using a constant