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Role of Histamine H_4 Receptor ligands in Bleomycin-induced pulmonary fibrosis

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Running title: Histamine H_4R ligands and pulmonary fibrosis

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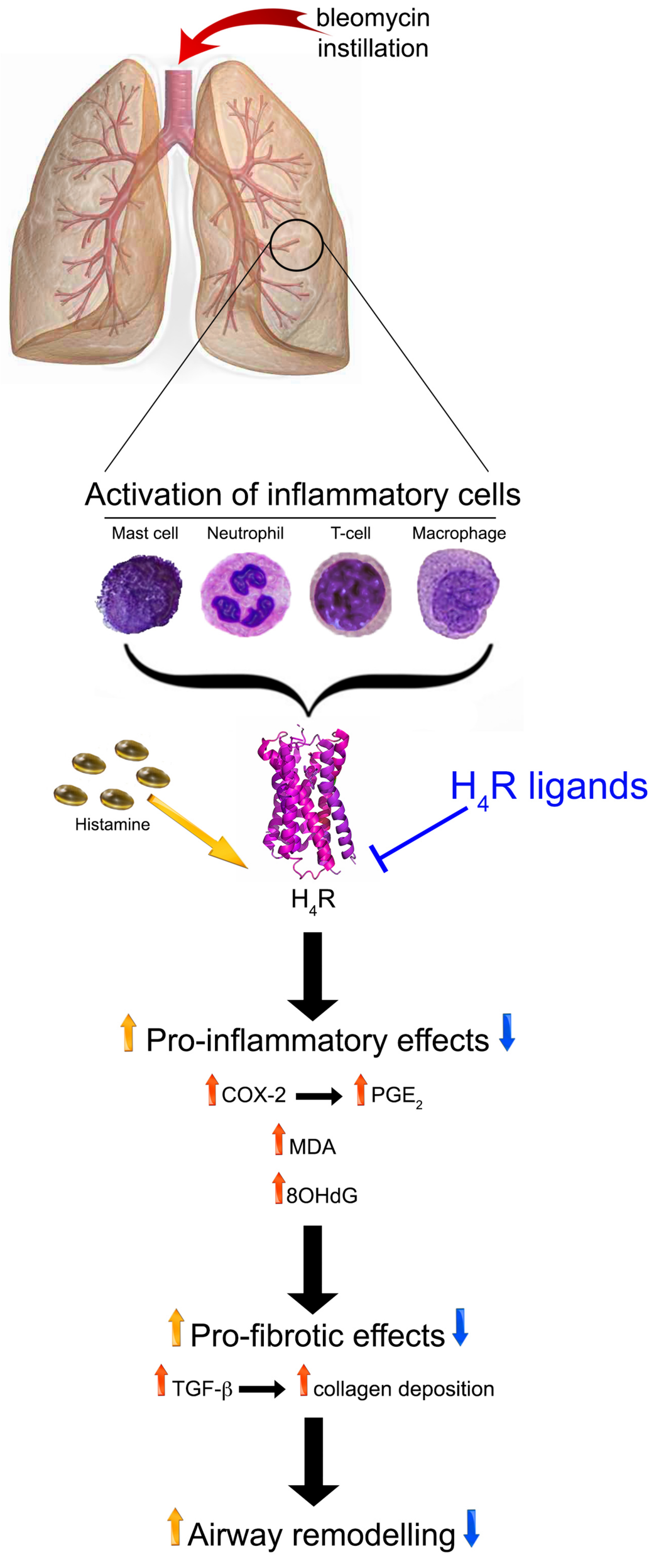
Graphical Abstract


bleomycin
instillation
Activation of inflammatory cells
Mast cell
Neutrophil
T-cell
Macrophage
Histamine
H4R ligands
H4R
Pro-inflammatory effects
COX-2
PGE2
MDA
8OHdG
Pro-fibrotic effects
TGF-β
collagen deposition
Airway remodelling

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.

Keywords: Histamine H_4 receptors, Histamine H_4 ligands, Bleomycin, Inflammation, Lung fibrosis, TGF-β.

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.

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 ($H_{1\text{-}4}R$) [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] GTPyS 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-G$s\alpha_s$ fusion protein [23]; pKi for histamine H_3 receptors (H_3R) was performed with ^{3}[H]N^{α}-methylhistamine displacement on Sf9 cell membranes co-expressing *h*H_3R, Gα_{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 to 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 volume mechanical ventilation method [27,28]. In brief, upon anaesthesia, the mice were operated on to insert a 22-gauge cannula (Venflon 2; Viggo Spectramed, Windlesham, UK, 0.8 mm diameter) into the trachea and then ventilated with a small-animal respirator (Ugo Basile, Comerio, Italy), adjusted to deliver a tidal volume of 0.8 ml at a rate of 20 strokes/min. Changes in lung resistance to inflation, defined as pressure at the airway opening (PAO), were registered by a high-sensitivity pressure transducer (P75 type 379; Harvard Apparatus Inc., Holliston, MA) connected to a polygraph (Harvard Apparatus Inc. Edenbridge, UK; settings: gain 1, chart speed 25 mm/s).

Changes in lung resistance to inflation (PAO), measured for at least 3 min and expressed as millimetres, were carried out on at least 40 consecutive tracings of respiratory strokes and then averaged.

2.5. Lung tissue sampling

After the functional assay, the animals were killed with an overdose of anesthetic, and the whole left lungs were excised and fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline for histological analysis. The right lungs were weighed, quickly frozen, and stored at −80°C. When needed for the biochemical assays, these samples were thawed at 4°C, homogenized on ice in 50 mM Tris-HCl buffer containing 180 mM KCl and 10 mM EDTA, pH 7.4, and then centrifuged at 10,000g, 4°C, for 30 min, unless otherwise reported. The supernatants and the pellets were collected and used for separate assays as detailed below.

2.6. Histology and assessment of collagen deposition, Goblet cell hyperplasia, and smooth muscle layer thickness

Histological sections, 6 μm thick, were cut from the paraffin-embedded lung samples. All sections were stained in a single session to minimize artifactual differences in the staining. Photomicrographs of the histological slides were randomly taken with a digital camera connected to a light microscope equipped with a x10 or x40 objective. Quantitative assessment of the stained sections was performed by computer-aided densitometry. Measurements of optical density (OD) and surface area were carried out using the free-share ImageJ 1.33 image analysis program (http://rsb.info.nih.gov/ij).

For assessment of lung collagen, the sections were stained with a simplified Azan method for collagen fibers according to Smolle *et al.*, [29] with minor modifications, in which azocarminium and orange G were omitted to reduce parenchymal tissue background. OD measurements of the aniline blue-stained collagen fibers were carried out upon selection of an

appropriate threshold to exclude aerial air spaces and bronchial/alveolar epithelium, according to Formigli *et al*. [30]. Values are means ± SEM of the OD measurements (arbitrary units) of individual mouse (five images each) from the different experimental groups.

For morphometry of smooth muscle layer thickness and bronchial Goblet cell number, both key markers of airway remodelling, lung tissue sections were stained with hematoxylin and eosin or with periodic acid-Schiff (PAS) staining for mucins, respectively. Digital photomicrographs of medium- and small-sized bronchi were taken at random. Measurements of the thickness of the bronchial smooth muscle layer were carried out on the digitized images using the above-mentioned software. PAS-stained Goblet cells and total bronchial epithelial cells were counted on bronchial cross-section profiles, and the percentage of Goblet cells was calculated. For both parameters, values are means ± SEM of individual mouse (five images each) from the different experimental groups (tested blind).

2.7. Western blot analysis for H_4R and COX-2 protein expression

The total protein for the lung samples were obtained as described previously [31]. Lung tissues were lysed with buffer containing 0.9% NaCl, 20 mmol/L Tris-HCl (pH 7.6), 0.1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.01% leupeptin, and homogenized. The total proteins (80 μg) as evaluated with the use of a bicinchoninic acid protein assay were subjected to Western blot and immunoblotting analysis as previously described [32]. The loading transfer of equal amounts of proteins were ascertained by either reblotting the membrane with an anti-tubulin antibody and/or staining the membrane with Ponceau S. The primary antibodies used were anti-H_4R polyclonal antibody (1,5 μg/mL, ab97997, Abcam, Cambridge, UK), anti-COX-2 polyclonal antibody (1:500, sc7951, Santa Cruz, Dallas, Texas, USA) and anti-β actin goat polyclonal antibody (1:500; ab8229, Abcam, Cambridge, UK). The binding of each primary antibody was determined by the addition of suitable peroxidase-conjugated secondary antibodies (anti-goat and anti-rabbit antibodies 1:15000). Densitometric analysis was performed with the ImageJ software.

2.8. Determination of myeloperoxidase (MPO)

This tissue indicator of leukocyte recruitment was determined in aliquots (100 µl) of lung homogenate supernatants, using a commercial ELISA kit (CardioMPO; Prognostix Inc., Cleveland, OH, USA), according to the manufacturer's instructions. The values are expressed as mU/mg of lung tissue (wet weight).

2.9. Determination of Prostaglandin E_2 (PGE_2)

The levels of PGE_2, the major cyclooxygenase product generated by activated inflammatory cells, were measured in aliquots (100 µl) of lung homogenate supernatants by using commercial ELISA kits (Cayman Chemical, Ann Arbor, MI), following the protocol provided by the manufacturer. The values are expressed as ng/mg of proteins, the latter determined with the Bradford method [32].

2.10. Determination of thiobarbituric acid-reactive substances (TBARS)

TBARS, such as malondialdehyde, are end-products of cell membrane lipid peroxidation by reactive oxygen species (ROS) and are considered reliable markers of oxidative tissue injury. They were determined by the measurement of the chromogen obtained from reaction of TBARS with 2-thiobarbituric acid [33]. In brief, 0.5 ml of 2-thiobarbituric acid (1% w/v) in 50 mM NaOH and 0.5 ml of HCl (25% w/v in water) were added to the lung tissue pellets. The mixture was placed in test tubes, sealed with screw caps, and heated in boiling water for 10 min. After cooling, the chromogen was extracted in 3 ml of 1-butanol, and the organic phase was separated by centrifugation at 2,000 *g* for 10 min. The absorbance of the organic phase was read spectrophotometrically at 532 nm wavelength over a standard curve of 1,1,3,3-tetramethoxypropane. The values are expressed as ng/mg of proteins, the latter determined as previously described [32].

2.11. Determination of 8-hydroxy-2'-deoxyguanosine (8OHdG)

Frozen lung samples were thawed at room temperature, and cell DNA isolation was performed as previously described [34] with minor modifications. The samples were homogenized in 1 ml of 10 mM PBS, pH 7.4, sonicated on ice for 1 min, added to 1 ml of 10 mM Tris-HCl buffer, pH 8, containing 10 mM EDTA, 10 mM NaCl, and 0.5% SDS, and incubated for 1 h at 37°C with 20 μg/ml RNase. Samples were incubated overnight at 37°C in the presence of 100 μg/ml proteinase K. Afterwards, the mixture was extracted with chloroform/isoamyl alcohol (10:2 v/v). DNA was precipitated from the aqueous phase with 0.2 volume of 10 M ammonium acetate, solubilized in 200 μl of 20 mM acetate buffer, pH 5.3, and denaturated at 90°C for 3 min. The extract was then supplemented with 10 IU of P1 nuclease in 10 μl and incubated for 1 h at 37°C with 5 IU of alkaline phosphatase in 0.4 M phosphate buffer, pH 8.8. All the procedures were performed in the dark. The mixture was filtered by an Amicon Micropure-EZ filter (Millipore Corporation, Billerica, MA), and 100 μl of each sample were used for 8OH*d*G determination by using an ELISA kit (JalCA, Shizuoka, Japan), following the instructions provided by the manufacturer. The values are expressed as ng/mg of proteins, the latter determined as previously reported [32].

2.12. Determination of Transforming Growth Factor-β (TGF-β)

The levels of TGF-β, the major profibrotic cytokine involved in fibroblast activation [35], were measured on aliquots (100 μl) of lung homogenate supernatants by using the Flow Cytomix assay (Bender Medsystems GmbH, Vienna, Austria), following the protocol provided by the manufacturer. In brief, suspensions of anti-TGF-β-coated beads were incubated with the samples and with a TGF-β standard curve, and then with biotin-conjugated secondary antibodies and streptavidin-phycoerythrin. Fluorescence was read with a cytofluorimeter (Epics XL; Beckman Coulter, Milan, Italy). Values are expressed as pg/μg of protein, determined as previously reported [32].

2.13. Statistical Analysis

For each assay, data were reported as mean values (± SEM) of individual average measures of the different animals per group. Significance of differences among the groups was assessed by one-way ANOVA followed by Newman-Keuls *post hoc* test for multiple comparisons, or, when only two groups had to be compared, by Student's *t* test for unpaired values. Calculations were made with Prism 4.03 statistical software (GraphPad Software, Inc., San Diego, CA,USA).

3. Results

3.1. Functional assay of fibrosis

Intra-tracheal bleomycin caused a statistically significant increase in airway stiffness, as showed by the clear-cut elevation in the pressure at airway opening (PAO) in the fibrotic positive controls given vehicle (14.67 ± 0.08 mm) compared with the non-fibrotic negative controls (12.43 ± 0.40 mm). Both compounds JNJ and ST-994, two selective antagonists of histamine H_4R, and ST-1012, an inverse agonist, caused a statistically significant reduction of airway stiffness (13.16 ± 0.38, 13.11 ± 0.42 mm and 12.5 ± 0.49, respectively). The results of the functional assay are reported in Figure 1. No effects were shown with ST-1006, a partial agonist (14,36 ± 0,37) (Fig. 1).

3.2. Expression of histamine H_4 receptor in lung tissue

The administration of bleomycin caused a statistically significant increase in the H_4R protein expression in lung homogenates. The increased expression of H_4R protein was significantly reduced in lung samples obtained from JNJ-treated animals. All the other studied ligands determined a decrease in H_4R protein expression although did not reach statistical significance (Fig. 2, panel A and B).

3.3. Effects of H_4 Receptor ligands on inflammation and oxidative stress markers

Intra-tracheal bleomycin injection was found to cause lung inflammation. The extent of inflammatory infiltrate in the lung, mainly macrophages and neutrophils, was significantly increased in positive control animals after bleomycin administration as shown by the increase in MPO activity (Fig. 3). The treatment of the animals with all the selected H_4R ligands caused a statistically significant reduction of MPO activity.

To gain further insight into the effects of the different H_4R ligands on inflammation, COX-2 expression and activity were evaluated. As expected, all the H_4R ligands studied reduced bleomycin-induced COX-2 expression (Fig. 4, panel A). This effect resulted also in a significant

reduction of PGE_2 production, the major COX-2 product generated by activated inflammatory cells (Fig. 4, panel B), obtained with the two antagonists (JNJ and ST-994) and with the inverse agonist (ST-1006). The production of MDA was evaluated as TBARS (Fig. 5, panel A), end-products of cell membrane lipid peroxidation by ROS and reliable markers of oxidative tissue injury. The production of TBARS was markedly increased in the fibrotic positive control compared with non-fibrotic negative one. As shown in Fig. 5, panel A, only the antagonists JNJ and ST-994 and the inverse agonist ST-1012 significantly reduced TBARS production.

Determination of 8OH*d*G (Fig. 5 panel B), an indicator of oxidative DNA damage, showed a similar trend as TBARS, markedly increased in the fibrotic positive control compared with the non-fibrotic one. The administration of JNJ, ST-994 and ST-1012 significantly reduced 8OH*d*G production. ST-1006 treatment did not affect TBARS and 8OH*d*G level.

3.4. Effects of H_4R ligands on fibrosis

The assay of TGF-β (Fig. 6), a major pro-fibrotic cytokine, showed that this molecule significantly increased in the bleomycin-treated animals as compared with the controls. Administration of all the selected histamine H_4R ligands caused a statistically significant reduction of TGF-β production. Morphological observation and computer-aided densitometry on Azan-stained sections (Fig. 7), which allows the determination of the optical density (OD) of collagen fibers, revealed a significant increase in collagen deposition in the lungs of the bleomycin-treated animals compared with the non-fibrotic negative controls. The treatment with JNJ, ST-994 and ST-1012 caused a robust, near complete, reduction of the amount of lung collagen fibers (Fig. 7, panel B). No effects were shown with ST-1006.

Bronchial remodeling was evaluated by measuring the relative number of PAS-positive Goblet cells (Fig. 8, panel A) and thickness of the smooth muscle layer (Fig. 8, panel B), key histological parameters of inflammation-induced adverse bronchial remodeling [36]. As expected, both these parameters were significantly increased in the bleomycin-treated mice. Notably, all the

tested compounds were able to significantly reduce the percentage of PAS-positive Goblet cells *versus* total bronchial epithelial cells (Fig. 8, panel A), as well as the thickness of the airway smooth muscle layer (Fig. 8, panel B). Although, the compound ST-1006 was less effective.

4. Discussion

The results here reported clearly demonstrated that H_4R ligands have a beneficial effect in a model of lung fibrosis in the mouse. In our experiments, the H_4R ligands were administered in a preventive mode. Compound JNJ, a selective H_4R antagonist, ST-994, a H_4R neutral antagonist and ST-1012, an inverse H_4R agonist, displayed robust anti-inflammatory and anti-fibrotic properties: in fact they consistently decreased markers of inflammation and oxidative stress, 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 resistance to inflation (PAO). The involvement of histamine in human lung fibrosis is indicated by the increased number of mast cells in the lung of patients affected by lung fibrosis in comparison to control subjects and their correlation with the severity of the disease [37]. Histamine concentration in the BAL fluid of IPF patients is 10-fold increased respect to the healthy subjects [38,39], and histamine stimulates human lung fibroblast migration through H_4R, [18]. Taken together, these evidences suggest that histamine and histaminergic H_4R receptor are involved in lung inflammation and fibrosis development. On the other hand, in bleomycin-induced animal model of lung fibrosis, naproxen and NO-donating naproxen (CINOD), two classic anti-inflammatory drugs, determined a significant reduction of lung inflammation and consequent fibrosis [28].

It is well known that chronic inflammation in the lung determines permanent alteration of lung structure characterized by airway epithelium modification and fibrosis. Indeed, the treatment with bleomycin activates the inflammatory cascade, as clearly underlined by the increase in MPO activity, histamine H_4R and COX-2 protein expression and activity (Fig. 2, 3 and 4), indicating an increase in the number of inflammatory cells migrated to the lung parenchyma. Histamine is implicated in the physiopathology of several inflammatory and immunological mechanisms, acting in both the acute and chronic phase. The function of histamine in cellular immunity, through the

control of cytokine and chemokine production and migration of inflammatory cells, beyond its traditional role in mediating fast airway hyper-responsiveness, has been defined [40]. In a mouse model of zymosan-induced peritonitis, histamine H_4R ligands exerted anti-inflammatory activity [41,42]. In this model, intra-peritoneal treatment of mice with zymosan induces neutrophil migration, which is blocked by an H_4R antagonist [43]. Accumulating experimental evidence indicates the role of H_4R in modulating lung inflammation, mainly through its effects on Th2 cell induction [44].

In a model of dermal inflammation, pro-inflammatory cytokines, such as IL-4 and chemokines, were increased in ear tissue on fluorescein isothiocyanate (FITC) challenge, an effect significantly reduced with the treatment with H_4R antagonists [45]. The administration of JNJ dose-dependently reduced the ear edema and the scratching response [46]. In a rat model of carrageenan-induced acute inflammation, H_4R antagonists decreased the formation of edema [28] and, in the early phase, significantly reduced the hyperalgesia consequent to thermal stimulation, controlling peripheral nociceptive pathway [47]. Histamine H_4R is functionally expressed in cells of immune system [48] including NK cells, dendritic cells (DCs) and monocytes, although the expression of this receptor in monocytes is still controversial. Zhu *et al.*,[49] demonstrated a very low expression of H_4R in resting $CD14^+$ cells, other authors only in activated monocytes [50] or in THP-1 clone 15 cells, which highly secret TNFα and IL-1β, which in turn might induce the expression of H_4R [48]. The presence of H_4R protein in cells of the innate immunity could explain how these cells localize at inflammation sites and justify the anti-inflammatory activity of H_4R antagonists. Indeed histamine is a robust chemotactic factor for these cells [48].

Our results highlighted that H_4R antagonists or inverse agonists inhibit the production of TGF-β, a pro-fibrotic cytokine and a recent identified target for the development of novel anti-fibrotic agents. Drugs controlling the formation and activity of TGF-β, such as anti-TGF-β monoclonal antibodies and pirfenidone, a drug which inhibit fibroblast activation, hold great

promise and were subject to clinical development [51]; however, their therapeutic efficacy on patients with lung fibrosis, especially IPF, still remains to be clearly defined [52]. On the other hand, the strategy of targeting prostaglandin synthesis by COX inhibitors is still controversial. In fact, patients with pulmonary fibrosis have decreased PGE_2 levels and may benefit from prostanoid administration [53]. There is still an unmet need for selective, potent, avoided of side-effects and safe anti-fibrotic drugs. The results here reported suggest H_4R ligands as novel therapeutic agents endowed with anti-inflammatory and anti-fibrotic activities, when administered in the inflammatory phase of the fibrotic onset. ST-1006, a partial agonist of H_4R, exerted only some beneficial effect. This result could be explained considering the intrinsic activity of all histaminergic receptors and in particular H_4R [54]

In conclusion, the dual effect of H_4R ligands on inflammation and fibrosis pathways, indicates their therapeutic potential for inflammatory and immune diseases, including idiopatic pulmonary fibrosis.

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Conflict of interest

The authors declare that they have no conflicts of interest to disclose. Part of the results results have been presented in EHRS meeting in 2012.

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Legends

Fig. 1
Spirometric evaluation. Bar graph and statistical analysis of PAO values (means ± SEM.) among the different experimental groups. Significance of differences (one-way ANOVA and Newman–Keuls post hoc test): *p < *0.05* *vs* Bleomycin + Vehicle.

Fig. 2
Western blot analysis of H_4R expression in the lung specimens from the different experimental groups (Panel A). The densitometric analysis of the bands was normalized to β-actin (Panel B). Data are representative of at least three independent experiments. Significance of differences (one-way ANOVA and Newman–Keuls post hoc test): **p<0.01 *vs* Bleomycin + Vehicle.

Fig. 3
Evaluation of leukocyte infiltration. Bar graph shows MPO levels in the lung tissue (means ± SEM) of the different experimental groups. Significance of differences (one-way ANOVA and Newman–Keuls post hoc test): **p<0.01 and ***p<0.001 *vs* Bleomycin + Vehicle.

Fig. 4
Western blot analysis of COX-2 expression in the lung specimens from the different experimental groups (Panel A). The densitometric analysis of the bands was normalized to β-actin (Panel B). Data are representative of at least three independent experiments. Prostaglandin production: bar graph shows the lung tissue levels of PGE_2 (means ± SEM) of the different experimental groups (Panel C). Significance of differences (one-way ANOVA and Newman–Keuls post hoc test): *p<0.05, **p<0.01 and ***p<0.001 *vs* bleomycin + vehicle.

Fig. 5
Evaluation of oxidative stress parameters. Bar graphs shows the lung tissue levels of TBARS (Panel A) and 8OH*d*G (Panel B) of the different experimental groups (means ± SEM). Significance of differences (one-way ANOVA and Newman–Keuls post hoc test): *p<0.05, **p<0.01 and ***p<0.001 *vs* Bleomycin + Vehicle.

Fig. 6
Evaluation of TGF-β. Bar graph shows the lung tissue levels of the profibrotic cytokine (means ± SEM) of the different experimental groups. Significance of differences (one-way ANOVA and Newman–Keuls post hoc test): ***p<0.001 *vs* Bleomycin + Vehicle.

Fig. 7
Evaluation of lung fibrosis. Representative micrographs of Azan-stained lung tissue sections from mice of the different experimental groups. Collagen fibers are deep blue stained. The lung from a fibrotic positive control shows marked fibrosis, which is absent in the lung from a non-fibrotic negative control and reduced by all the treatments. Scale bars, 50 μm. Densitometric analysis of Azan-stained sections is shown in bar graph as arbitrary OD units (means ± SEM). Significance of differences (one-way ANOVA and Newman–Keuls post hoc test): ***p<0.001 *vs* Bleomycin + Vehicle.

Fig. 8
Evaluation of Goblet cell hyperplasia (Panel A) and bronchial smooth muscle layer thickness (Panel B), shown as both the representative micrographs of the stained section and the relative densitometric analyses. The Goblet cells hyperplasia was measured by PAS staining and computer-aided morphometry in lung specimens of the different experimental group. The PAS-positive cells

are indicated by arrows. The histograms show the percentage of PAS-positive Goblet cells over total epithelial cells (means ± SEM) (Panel A). The smooth muscle thickness was assessed by computer-aided morphometry on hematoxylin and eosin-stained lung sections in lung specimens of the different experimental groups and indicated by double arrows. The histograms show the thickness of the smooth muscle (means ± SEM) (Panel B). Significance of differences (one-way ANOVA and Newman–Keuls post hoc test): *$p<0.05$ and **$p<0.01$ *vs* Bleomycin + Vehicle. Scale bar, 50 μm.

Fig. 1

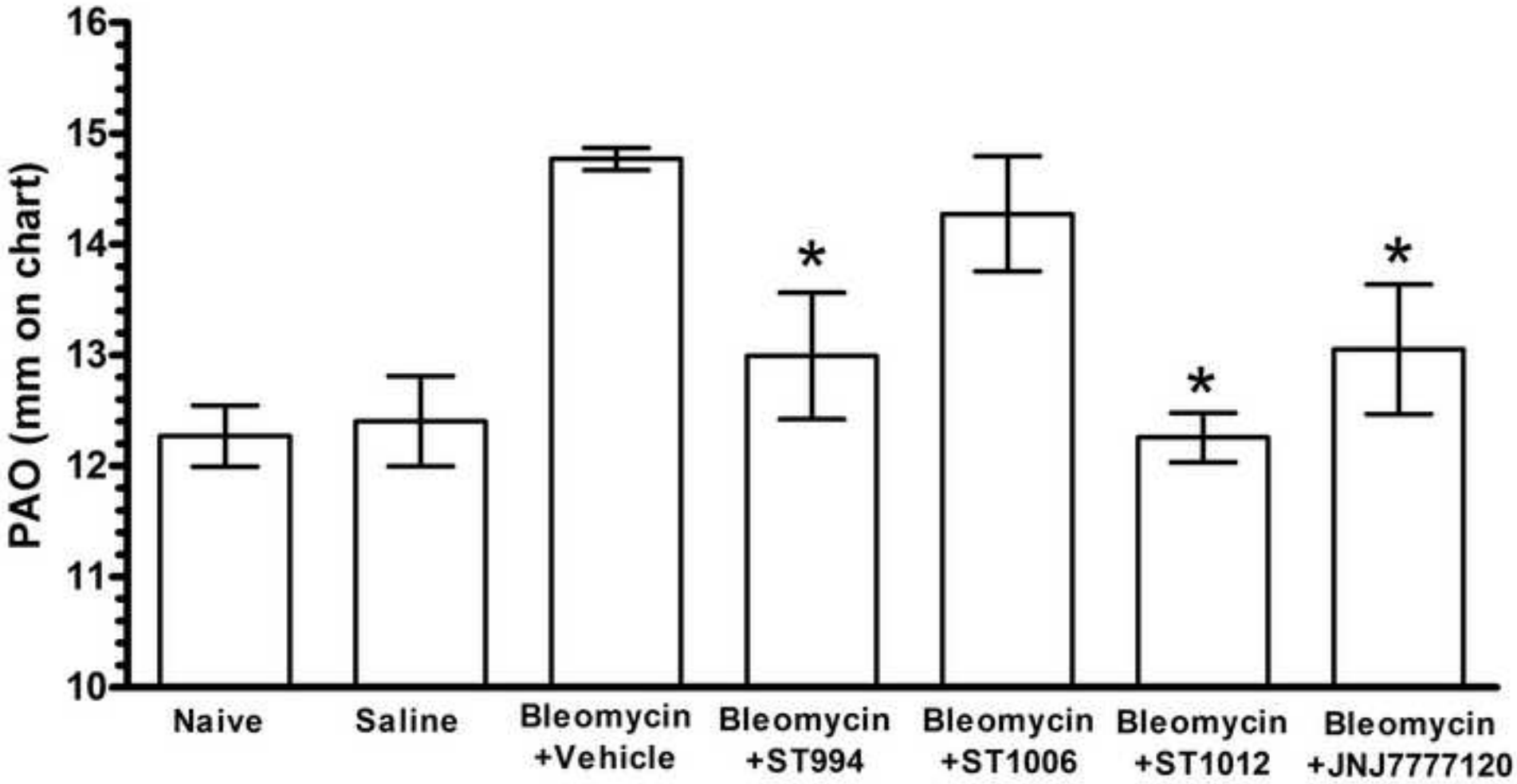
PAO (mm on chart)
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Fig. 2

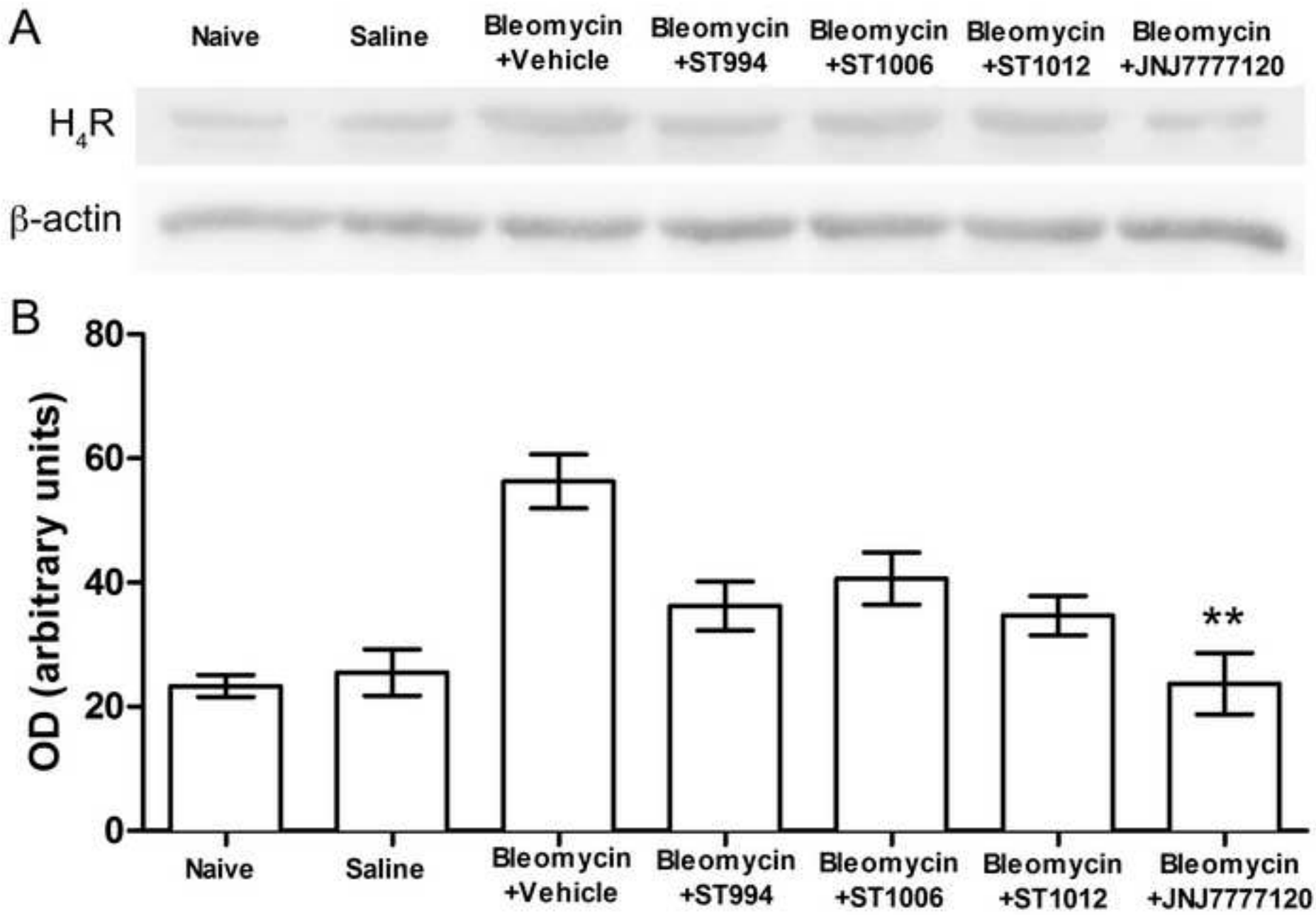
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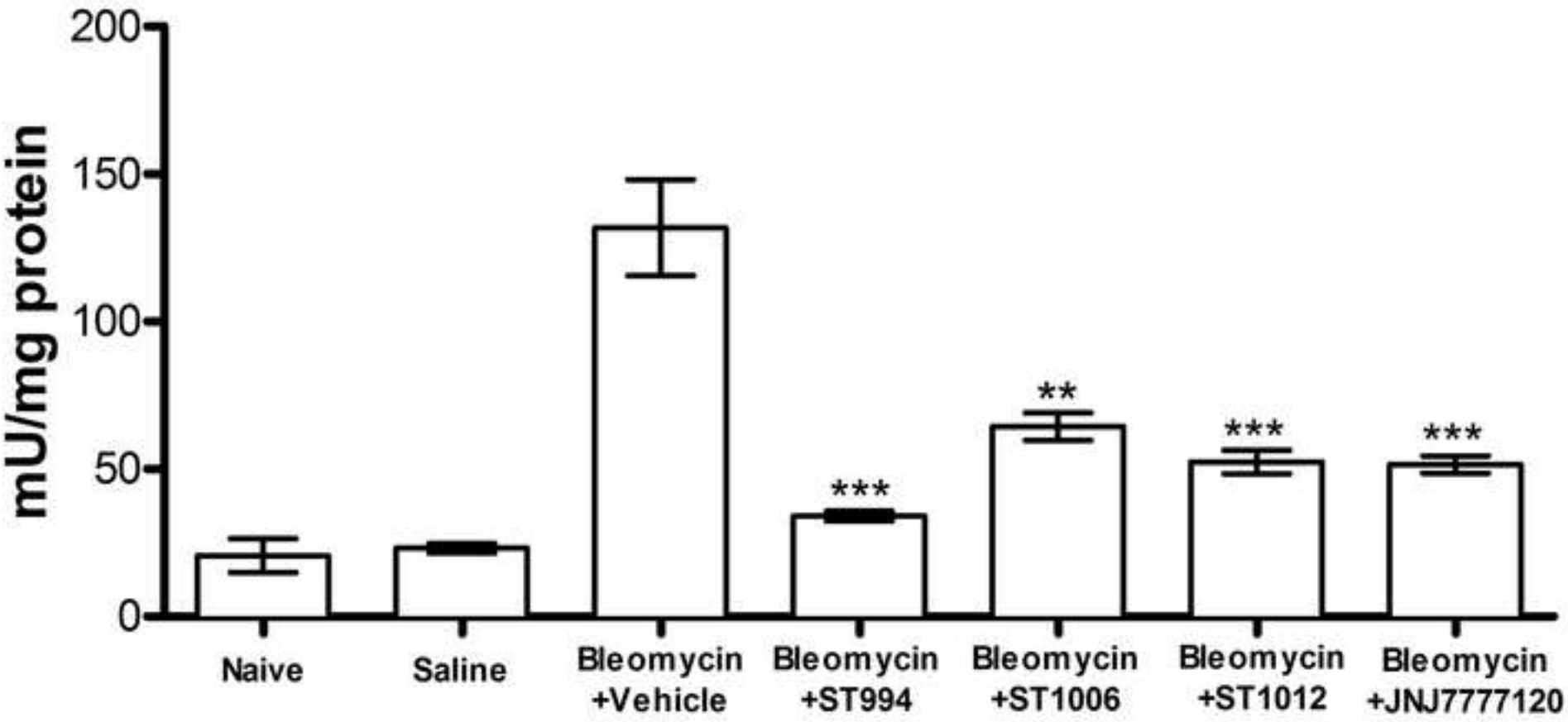
Fig. 3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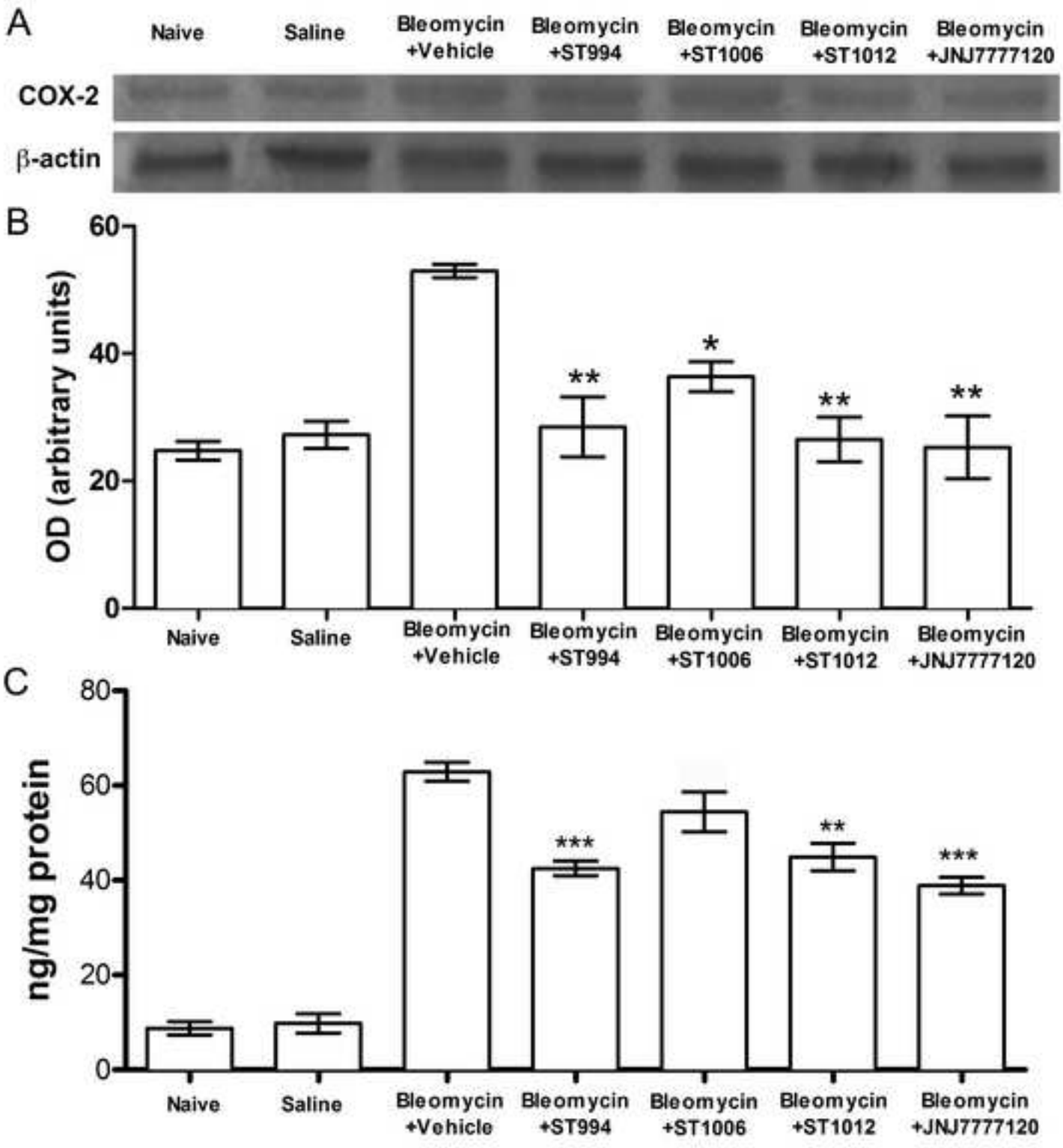

Fig. 5

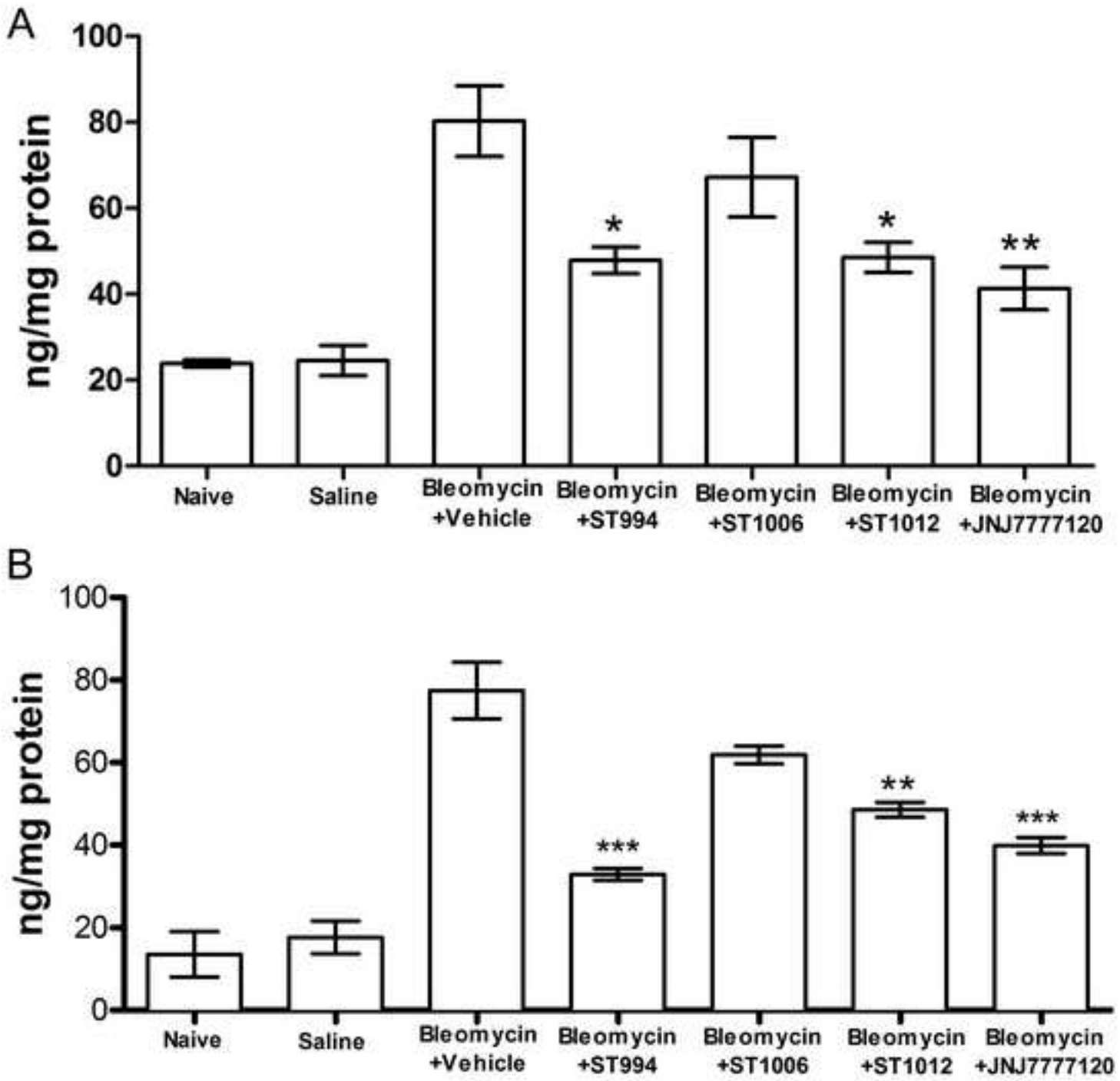
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Fig. 6

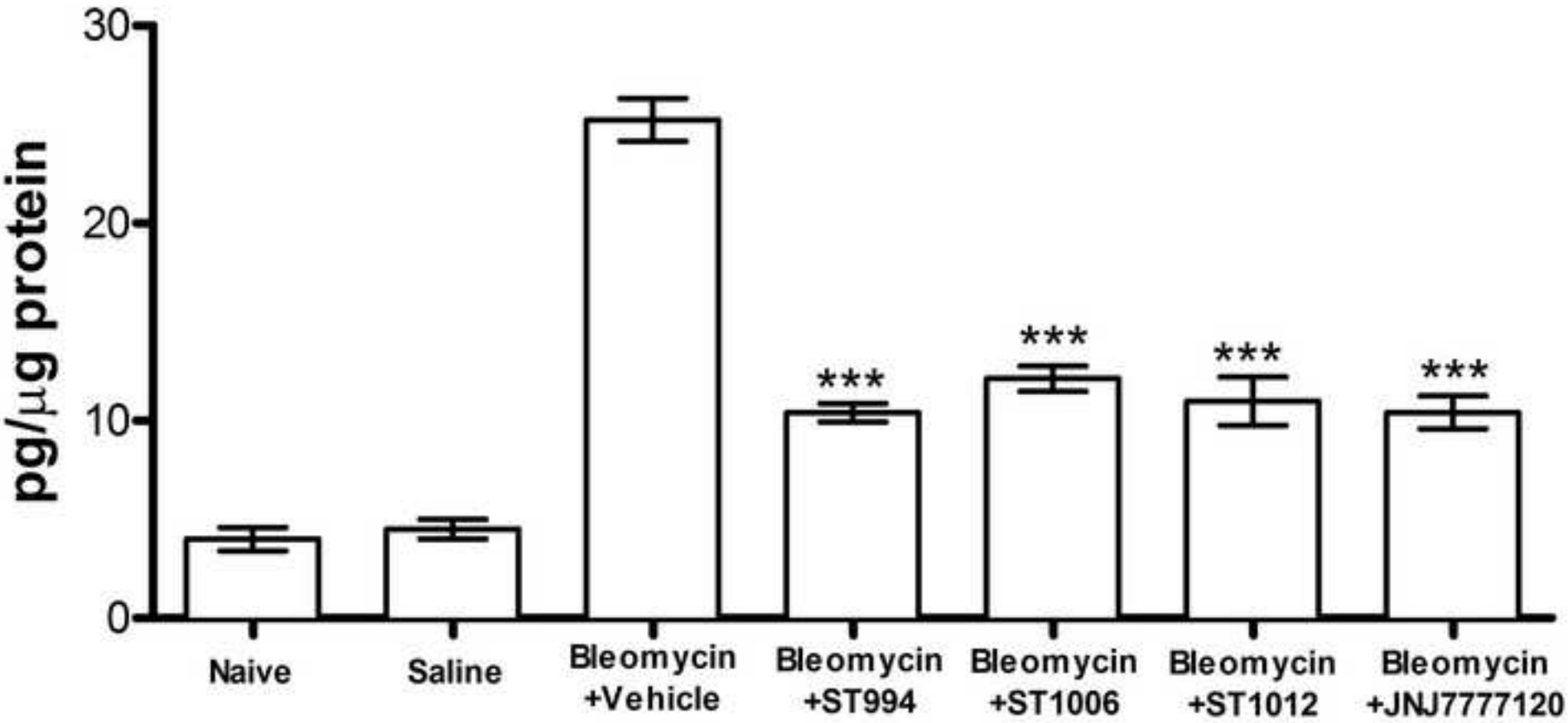
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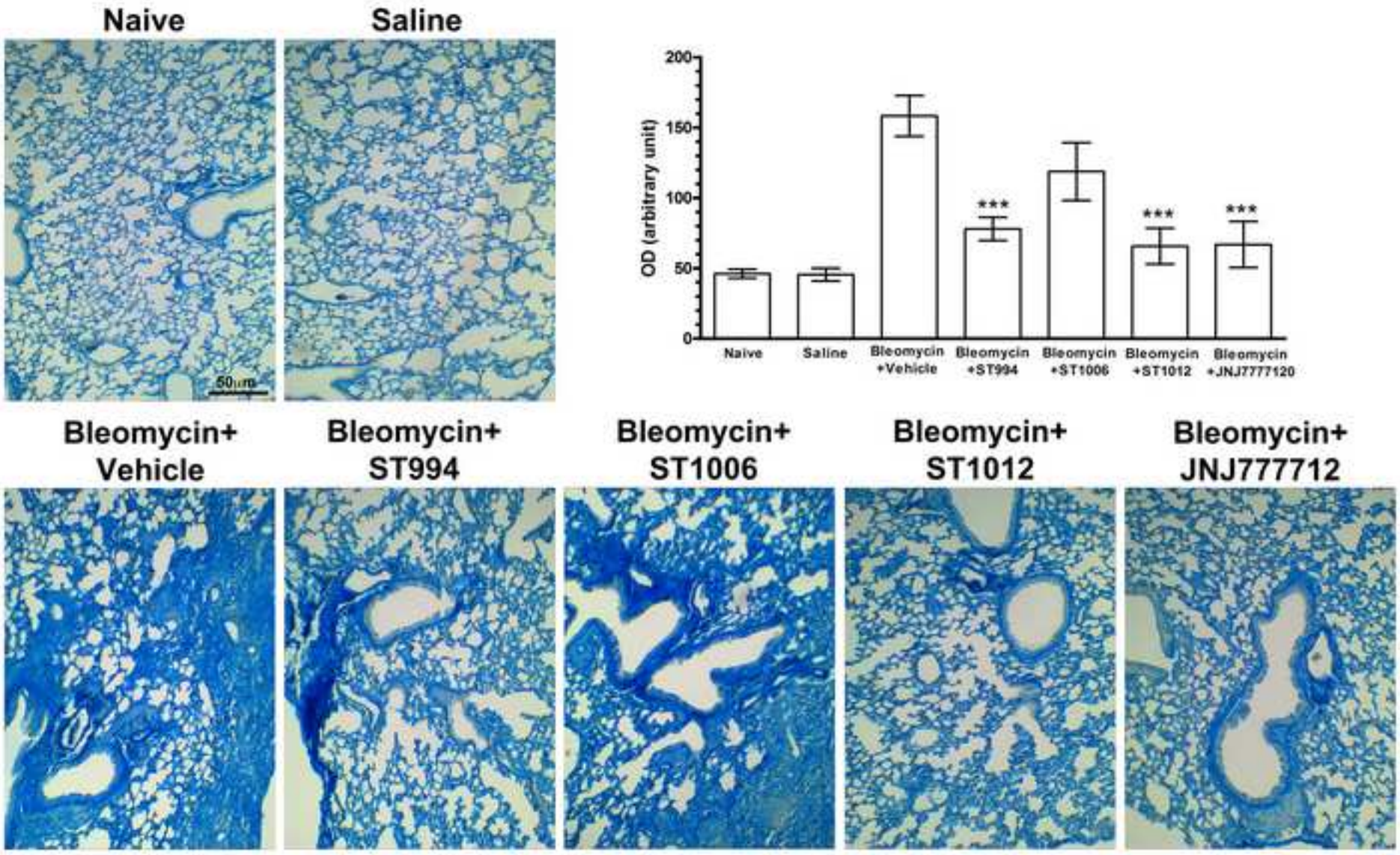
Fig. 7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