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Title: **Modulation of bleomycin induced lung fibrosis by serotonin receptor antagonists in mice** *(88 characters)*

***Shoryt title*: Serotonin receptors in lung fibrosis** (*37 characters*)

Authors: Aurélie Fabre[*,†], Joëlle Marchal-Somme[*], Sylvain Marchand-Adam[*,‡], Christophe Quesnel[*], Raphaël Borie[*], Monique Dehoux[*,§], Claude Ruffié[*], Jacques Callebert[¶], Jean-Marie Launay[¶], Dominique Hénin[†], Paul Soler[*], Bruno Crestani[*,‡]

[*]Inserm, unit 700 ; Université Paris 7, Faculté de Médecine Denis Diderot ;

Assistance Publique Hôpitaux de Paris, Hôpital Bichat, [§]Laboratoire de Biochimie A, [†]Service d'Anatomie Pathologique, et [‡]Service de Pneumologie ; [¶]Hôpital Lariboisière Service de Biochimie.

Corresponding author :

Dr Aurélie FABRE, Inserm U700, Faculté Xavier Bichat, 16 rue Henri Huchard, 75018 Paris, France.

Phone : +33 1 44 85 62 53

Fax : +331 40 25 88 18

e-mail : aurelie_fabre@yahoo.fr

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Abstract (*198 words*)

Serotonin (5 hydroxytryptamine, 5-HT) is known to increase proliferation and collagen synthesis by fibroblasts. Two receptor subtypes (5-HT2A and 5-HT2B) have been shown to play the most important role in the lung.

We investigated the role of serotonin in lung fibrosis using the bleomycin mouse model of lung fibrosis.

Serotonin concentrations in lung homogenates significantly increased over the time course of bleomycin induced fibrosis, with a maximum at day 7. The expression of serotonin receptors 5-HT2A and 5-HT2B increased in the lung after bleomycin, as assessed by PCR, specific binding and immunohistochemistry. Blockage of 5-HT2A receptors (by ketanserin) and 5-HT2B receptors (by SB215505) reduced bleomycin-induced lung fibrosis as assessed by reduced lung collagen contents and procollagen 1 and procollagen 3 mRNA expression. Serotonin antagonists promoted an anti-fibrotic environment through the decrease of lung TGF-beta1, CTGF and PAI-1 mRNA but they had minimal effects on lung inflammation as assessed by BAL cytology analysis. Interestingly, the 5-HT2B receptor was strongly expressed by fibroblasts in the fibroblastic foci in human idiopathic pulmonary fibrosis samples.

We conclude that serotonin is involved in the pathophysiology of bleomycin-induced lung fibrosis in mice and may be identified as a therapeutic target in lung fibrotic disorders.

Keywords: bleomycin; fibrosis lung; receptors; serotonin;

Introduction

Pulmonary fibrosis is a chronic interstitial lung disease which responds poorly to available medical therapies and carries a potentially fatal prognosis. The course is usually indolent but inexorable. Most patients die of progressive respiratory failure within 3-5 years of the onset of symptoms, and current therapies are of unproven benefit (1) and a huge effort is being developed worldwide to identify new therapeutics.

Serotonin (5 hydroxytryptamine, 5-HT), is a vasoactive peptide synthesised from tryptophan by enterochromaffin cells in the gut and by endothelial cells (2, 3) . Very low levels of circulating free serotonin are present in the blood as most of 5-HT is pooled in platelets. In physiological conditions, the lung is exposed to low levels of circulating serotonin. In pathological conditions release of serotonin stored by platelet and endothelial cells may increase serotonin concentration both locally and in the circulation. To date, fourteen 5-HT receptors have been identified (4), most are G coupled receptors, and 5HT2A and 5HT2B receptor subtypes have been shown to play the most important role in the lung where serotonin participates in the control of vasoreactivity and bronchoreactivity (5, 6).

Recent i*n vitro* studies have demonstrated the mitogenic and pro-fibrotic role of 5-HT on different types of mesenchymal cells. Serotonin enhanced the proliferation of fibroblasts cultured from pulmonary arteries from hypoxic rats, and its effect was co-mitogenic with the addition of serum (7). Outside the lung, 5-HT has been shown to play a critical role in modulating the characteristic phenotypic changes of the hepatic stellate cells in response to liver injury (8). In renal mesangial cells, 5-HT potently activated the extracellular signal-regulated kinase (ERK), induces transforming growth factor beta 1 (TGF- beta1) and increased cell proliferation in mesangial cells via the 5-HT2A receptor (9) Similarly, the addition of 5-HT on aortic valve interstitial cells induced increased collagen synthesis as well as TGF- beta1 mRNA expression and activity (10) through the activation of the 5-HT2A

receptor (11). Stimulation of the 5-HT2B receptor may also contribute to the remodelling properties of serotonin as demonstrated in a mouse model of pulmonary hypertension (12). Interestingly, the 5-HT2B receptor has been shown to regulate cell cycle progression in concert with the platelet derived growth factor receptor (PDGFR) pathway (12).

In view of these data, we hypothesised that 5-HT could play a role in the pathophysiology of lung fibrosis through its pro-proliferative and pro-fibrotic properties, via its 5-HT2A and 5-HT2B receptors. The aims of this study were (a) to characterise the expression of serotonin receptors in the murine and human fibrotic lung, (b) to determine whether modulation of the serotonin pathway using specific pharmacological antagonists of 5-HT2A and 5-HT2B receptors could attenuate the development of lung fibrosis and alter BAL inflammatory components in the murine model of bleomycin-induced lung fibrosis, and (c) to characterise the pathways involved in this effect.

Materials and methods

Bleomycin lung fibrosis

All experiments were performed using adult 6-7-week-old male C57BL/6 mice weighing 20-24g (Janvier, Le Genest Saint Isle, France), approved by the local animal committee and according to institutional guidelines that comply with national and international regulations. The mice had free access to water and food *ad libitum* prior and during pharmacological treatments.

On day 0, the mice were anesthetised intramuscularly with ketamine hydrochloride (45 mg/Kg) and xylazine 2% (9mg/Kg) and then were administered a unique dose of intra-tracheal bleomycin hydrochloride (Bleomycine Bellon, Aventis, Paris, France), 80µg, suspended in 50 microliters of 0.9% sterile saline. Naive mice were used as controls.

A group of animals was used for the evaluation of serotonin concentrations and serotonin receptors expression in the lung over the time course of lung fibrosis development. At day 3, 7 and 14 post-instillation, animals were euthanized with intra-peritoneal ketamine (60 mg/Kg) and xylazine (8 mg/kg). To minimise platelet activation, mice received 50 I.U. of heparin intra-peritoneally 15 min prior to sacrifice.

A second group of animals was used to assess the effect of 5-HT2A and 5-HT2B receptors antagonists in the bleomycin mouse model. Mice were administered intra-tracheal bleomycin on day 0, then treated with a daily intra-peritoneal injection of ketanserin (Sigma, 2mg/Kg/day, diluted in 200µl of sterile saline, a specific antagonist of the 5-HT2A receptor), or SB215505 (0.5mg/Kg/day, diluted in 200 microliters of sterile saline, a specific antagonist of the 5-HT2B receptor kindly provided by GlaxoSmithKline). Both these molecules were LPS-free. Mice were treated from day 0 to day 13. Control animals received the vehicle only.

For bronchoalveolar lavage (BAL) and histological studies, mice were administered either

bleomycin or the same volume of saline intra-tracheally, and then treated with ketanserin and SB215505 over the 14 day-period.

Animals were sacrificed on day 3, day 7 and day 14.

Serotonin assay in lung homogenates

The caudate and apical lobes of the right lung were snap-frozen for serotonin dosage. Lungs were homogenized in ice cold 0.1 M acetic acid containing metabisulfite sodium (10 microM), EDTA (10 microM) and ascorbic acid (10 microM). After centrifugation (15min, 17500g at 4°C), the supernatant was passed through a 10.000 MW filter (Nanosep 10kD, Pall, VWR, Fontenay-sous-Bois, France) by centrifugation (30 min, 12500g at 4°C). Then, a 20 microliters aliquot of sample was analyzed for serotonin by isocratic elution and electrochemical detection on a serial electrode array of coulometric flow-through graphite electrodes (Coularray, ESA, EUROSEP, Cergy St Christophe, France). Serotonin was then identified based on his retention time as well as his electrochemical behavior across the arrays. The analysis, data reduction and peak identification were fully automated. The results were expressed in pmol/ caudate and apical lobes.

5-HT2A and 5-HT2B receptor binding on lung tissue

Quantification of the 5-HT2A and 5-HT2B receptors in lung tissue was performed by binding experiments using selective tritiated radioligands (MDL 100907 – 745 GBq/mmol - for 5-HT2AR and LY-266097 - 925 GBq/mmol -for 5-HT2BR) provided by Dr J. Würch (Roche, Basel, Switzerland). Briefly, cell membranes were prepared by four cycles of homogenization and centrifugation (48,000 x g for 15 min) at 4°C. Assays were established to achieve steady state conditions and to optimize specific bindings. Fifty micrograms of membrane proteins were incubated with 1 nM ^{3}[H]-MDL 100907 or ^{3}[H]-LY-266097 at 4°C for 60 min. Non-

specific bindings were determined using 1µM ketanserin (5-HT2A receptor) or RS-127445 (5-HT2B receptor). Assays were terminated by vacuum filtration through glass fibre filters (GF/B grade), which had been pretreated with 0.1% polyethyleneimine. Total and bound radioactivities were determined by liquid scintillation counting. Greater than 80% specific binding was achieved in these assays.

Bronchoalveolar lavage

BAL was obtained by cannulating the trachea, and washing the lungs four times with 250 microliters aliquots of sterile saline at D14. Total cell counts were made using a Malassez glass counter. Differential cell counts were estimated from cytospin preparations by counting 200 cells stained with Diff-Quick (Baxter Dade AG, Dudingen, Germany). BAL supernatant was then centrifuged, aliquoted with aprotinin, and stored at –20°C until analysis.

TGF -beta1assay

Activated TGF-beta1 concentration in BAL were determined by ELISA (Quantikine® mouse/rat/porcine TGF-β1, R&D System Europe, Lille, France) according to the manufacturer's instructions.

Determination of lung inositol triphosphate levels.

In order to check that ketanserin and SB215505 at the doses of 2mg/Kg/day and 0.5mg/Kg/day respectively, were active *in vivo* in the lung, we measured lung inositol 1,4,5 triphosphate (IP3) concentration in mice treated with ketanserin and SB215505 at increasing doses. Mice (5 mice per group) received daily intraperitoneal injections of ketanserin or SB215505. On day 7, mice were euthanized and their lung sampled and homogenised. 2,5-

dimethoxy-4-iodoamphetamine (DOI) (1 µM) was added to the homogenate. After centrifugation, IP3 was quantified in the supernatant with use of a radioimmunoassay (Amersham TRK1000 IP3 kit, Les Ulis, Paris, France) as previously described (6,13). Each dosage was repeated 3 times. The results are expressed as picomoles/mg protein.

Lung morphology

The left lung was fixed by inflation with a buffered 4% paraformaldehyde solution for 24h then embedded in paraffin and stained with haematoxylin-eosin saffron (HPS), Masson's trichrome (MT) and PicroSirius (PS).

Immunohistochemistry

Paraformaldehyde-fixed paraffin-embedded 3 micrometers sections of mouse left lung, were deparaffined in xylene and alcohols, and pre-treated in citrate buffer pH 6 for 45min for antigen retrieval. Serial sections were pretreated with peroxidase blocking reagent (ARK (Animal Research Kit) detection kit Dako, Trappes, France) and then incubated with anti 5-HT2A or anti 5-HT2B receptor antibodies (BD Pharmingen, clone G186-1117 and A72-1 respectively) for 15min at room temperature. Staining was revealed by diaminobenzidine using the ARK detection kit. All immunostainings were performed at the same time in a given animal to limit variability in levels of expression. Negative controls were incubated with mouse immunoglobulin G1 (IgG1, Dako, Trappes, France).

Total soluble collagen assay

Frozen non-lavaged right lung was homogenized in 1ml of ice cold sample buffer (10 mM Tris- HCl-buffered solution (pH 7.4) containing 2 M NaCl, 1 mM PMSF, 1 mM EDTA, and 0.01% Tween 20). The lung homogenates was shaken for 24h at 4°C and centrifuged at 15000

rpm for 60 min to remove debris. Clear upper supernatant fluid (250 microliters) was used for the Sircol™ assay (Biocolor Ltd, Northern Ireland) according to manufacturer's instructions and OD readings were made at 550nm. Results were expressed as µg collagen per right lung.

MessengerRNA extraction, cDNA synthesis and real time quantitative PCR (Q-PCR)

Mouse mRNA extraction was performed using the NucleoSpin® RNA II kit (Macherey Nagel, Hoerdt, France) on non-lavaged frozen left mouse lung. The concentrations and quality of mRNA were determined by sprectrophotometry and agarose gel electrophoresis. Reverse transcription was performed with random hexamer primers, oligo(d)T and reverse transcriptase (MMLV-1, Promega, Charbonnières, France). Primers for polymerase chain reaction were designed with and the following genes were studied: 5-HT2A receptor, 5-HT2B receptor, transforming growth factor beta1 (TGF-beta1), connective growth factor (CTGF), procollagen 1 (alpha 2 chain of procollagen 1, COL1A2), procollagen 3 (alpha 1 chain of procollagen 3, COL3A1), plasminogen activator inhibitor 1 (PAI-1) (Table I). Hypoxanthine-phopho-ribosyl-transferase (HPRT) served as an endogenous mRNA control. Each amplification reaction was performed in duplicate with SybrGreen mix (Sigma, Saint Louis, USA) and specific primers (see online supplement Table 1). Signal detection and analysis of results was performed with ABI prism 7700 sequence detection software (Applied Biosystems, Courtaboeuf, France). The relative expression of the gene of interest was expressed as a ratio to the house keeping gene (HPRT) in relative number of copies calculated with a standard curve using 10^{-1}to10^{-4} dilutions. A negative control with water only was used for each primer.

Human lung samples

Human lung tissue samples obtained from 2 controls and 4 IPF patients were used for mRNA analysis and immunohistochemistry. Control lung was obtained at the time of surgery for a localized lung tumour from a non-involved segment. IPF lung tissue was obtained at the time of surgical lung biopsy or transplantation; IPF was diagnosed according to the ATS-ERS consensus criteria (14). The study was approved by local ethical committee. Informed consent was obtained for all patients. Samples were immediately frozen in liquid nitrogen and stored at -80°C until use. The histopathology of biopsies was evaluated on cryostat sections before their use for immunohistochemical studies.

For immunohistochemical staining, 4-6 micrometers thick cryostat sections fixed in acetone were incubated with 5-HT2A and 5-HT2B polyclonal antibodies (Santa Cruz Biotechnology) at room temperature for 1 hour and revealed using the Vectastain ABC-alkaline phosphatase kit system (Vector, Abcys, France) and the fast red substrate (Dako). Normal goat serum (Vector, Abcys, France) was used as a control and under these conditions no positive cells were identified.

Statistical analysis

A non parametric Mann Whitney test was used to compare 2 groups. Comparison of data between various treatments groups were performed with a non parametric Kruskal-Wallis test, followed by Mann Whitney *U*-test. A p value of less than 0.05 was considered as statistically significant. Differences between IPF and control fibroblasts were determined by the Mann-Whitney *U*-test. Data were expressed as mean +/-SEM. A p value of <0.05 was considered significant.

Results

Quantification of serotonin and its receptors 5-HT2A and 5-HT2B in murine lung

In order to determine whether the serotonin pathway was involved in the pathogenesis of bleomycin induced lung fibrosis, we measured serotonin concentration in lung homogenates and then evaluated the expression and the localisation of its two main receptors in the lung, 5-HT2A and 5-HT2B.

Serotonin was measured in lung homogenates from controls and bleomycin mice (day 3, 7 and 14). Serotonin concentrations increased over the time course of bleomycin induced fibrosis, with a maximum increase at day 7 (increased from 144 +/- 25 nM to 310 +/- 18 nM, p=0.008) and was still increased on day 14 (274 +/- 51 nM, p= 0.04) (Figure 1). In parallel to the increase of the serotonin concentration in lung homogenates, we observed an increased expression of its receptors 5-HT2A and 5-HT2B.

A very low level of 5-HT2A and 5-HT2B mRNA was detected in control lungs by real time quantitative PCR. After bleomycin intra-tracheal instillation, lung 5-HT2Areceptor mRNA content was increased 23-fold at day 3 (p=0.002) and such a high expression was maintained until day 14 (Figure 2A). Similarly, lung 5-HT2B receptor mRNA content was increased 16-fold at day 3 compared to controls (p=0.001) and high expression was maintained until day 14 (Figure 2B).

Binding experiments allowed us to quantify the relative availability of 5-HT2A and 5-HT2B receptors in the lung. In the normal lung, 5-HT2A specific binding was 6 fold higher than 5-HT2B specific binding. After bleomycin intra-tracheal instillation, 5-HT2A specific binding was unchanged whereas 5-HT2B specific binding increased 6 fold at day 3 (p=0.04) and was maintained until day 14 (Figure 3).

Immunohistochemical studies allowed us to localize the 5-HT2A and 5-HT2B receptors in lung tissue. In bleomycin lung, 5-HT2A and 5-HT2B receptors were expressed by bronchial

epithelial cells, alveolar macrophages, endothelial cells and peri-arterial smooth muscle cells (Figure 4A-E), In addition, the 5-HT2A receptor was expressed by mesothelial cells (Figure 4C). Over the time course of bleomycin induced lung fibrosis, the immunostaining of 5-HT2A and 5-HT2B receptors remained unchanged in resident cells, whereas the inflammatory cell infiltrate (polymorphonuclear neutrophils and lymphocytes) showed positive staining for 5-HT2B receptor (Figure 4F) but not for 5-HT2A. This parallels the effect of 5-HT2B on the inflammatory cells in BAL (see table 2 online supplement). In control lung, 5-HT2A and 5-HT2B receptors were mainly expressed by bronchial epithelial cells (Figure 4H and I), and at a lesser degree by alveolar macrophages and endothelial cells (not shown). Type II pneumocytes also expressed both receptors in control murine lungs (see insert on Figures 4H and 4I).

Together, these results demonstrate the expression of 5-HT2A and 5-HT2B receptors in bleomycin induced lung fibrosis.

Ketanserin and SB215505 reduced lung collagen content

In preliminary experiments, we checked that ketanserin and SB215505 at the doses of 2mg/Kg/day and 0.5mg/Kg/day respectively, decreased lung inositol 1,4,5 tri phosphate (IP3) concentration and that these concentrations were in the plateau of the dose-response curve (Figure 1 on line supplement). Then, we assessed the safety of the specific 5-HT2A and 5-HT2B receptors antagonists by giving intraperitoneal injections of ketanserin, SB215505 or the vehicle daily to naïve mice for 14 days. We observed a stable weight, normal lung histology and normal BAL cytology (data not shown). No mortality was associated with these treatments. In further experiments, we evaluated the protective effect of ketanserin and SB215505 in mice with bleomycin induced lung fibrosis. A group of animals received the vehicle only.

Intra-tracheal instillation of bleomycin produced a significant increase in lung collagen at 14 day when compared to saline treated mice (histological analysis on Masson trichrome and Picrosirius stains, Figure 5). Daily intra-peritoneal treatment with ketanserin or SB215505 significantly reduced collagen content (mean 86.4+/-6.8 micrograms/right lung with ketanserin, 97.2 +/- 6 micrograms/right lung with SB215505) compared to vehicle treated mice (116 micrograms/right lung +/- 6.4 micrograms/right lung, p=0.0006 for both antagonists) (Figure 6A). Ketanserin significantly reduced procollagen 1 mRNA (-46%, p=0.02) and procollagen 3 mRNA (-44%, p=0.046) content in lung homogenates compared to vehicle treated mice at day 14 (Figures 6B and 6C). Similarly, SB215505 reduced procollagen 1 (-31%, p=0.06) and procollagen 3 mRNA content (-39%, p= 0.047) compared to the vehicle group (Figures 6B and 6C).

These results demonstrate for the first time an anti fibrotic action of specific serotonin 5-HT2A and 5-HT2B receptor antagonists in lung fibrosis.

Serotonin antagonists have a minimal effect on alveolar inflammation

In order to determine whether the protective effect of serotonin antagonists was secondary to an anti-inflammatory effect, we evaluated BAL cytology on day 3, 7 and 14 after bleomycin. Serotonin antagonists did not significantly modify BAL cytology on day 3 and day 7. On day 14, SB215505 treated bleomycin mice displayed an increased percentage of macrophages (p=0.019), decreased percentages of neutrophils (p=0.02) and eosinophils (p=0.045) compared to vehicle treated mice, whereas ketanserin had no effect (see Table 2 online supplement).

Serotonin antagonists promote an anti-fibrotic environment in the lung

To evaluate the anti-fibrotic pathway of serotonin, we studied three important factors involved in lung fibrosis, TGF- beta1, CTGF and PAI-1 (15-17).

As evaluated by quantitative real time PCR, in vehicle treated mice, TGF- beta1 mRNA content was increased at day 14 after bleomycin when compared to controls (increased ratio to HPRT from 0,85+/- 0,12 to 1,1 +/- 0,008, p=0.03) (Figure 7A). Ketanserin and SB215505 decreased lung TGF-beta1 mRNA content in bleomycin mice (decreased ratio to HPRT from 1,1 +/- 0,12 for vehicle treated mice to 0,77 +/- 0, 085 for ketanserin treated mice (-29%) and 0,72 +/- 0,085 (-35%) for SB215505 treated mice, p=0.026 and p=0.014 respectively) (Figure 7A). BAL TGF-beta1 concentration was decreased in mice treated with ketanserin (p=0,01) whereas there was a non significant trend in mice treated with SB215505 (Figure 7B).

Part of the effect of TGF-beta1 is mediated by CTGF, which also has its own profibrotic effect (18).While CTGF mRNA content was increased in the fibrotic lung (ratio to HPRT 0,95 +/- 0,13 in control mice to 1,71 +/- 0,16 in bleomycin vehicle treated mice, +46%, p=0.009), CTGF mRNA was decreased in ketanserin and SB215505 treated mice (decreased ratio to HPRT from 1,71 +/- 0,16 for vehicle treated mice to 1,15 +/- 0,16 for ketanserin, -36% p=0.017 and to 1,16+/- 0,21 for SB215505, -29% p=0.033) (Figure 7C).

PAI-1 is a potent anti-proteinase with profibrotic properties in the lung (19). PAI-1 mRNA was strongly induced in the lung of bleomycin treated mice (ratio to HPRT increased from 0,49 +/- 0,4 to 19 +/- 5,6, p=0.0008, Figure 7D). Conversely, ketanserin and SB215505 profoundly inhibited PAI-1 increase (decreased ratio to HPRT from 19 +/- 5,6 to 2,37+/- 0,51 for ketanserin p=0.007, and to 3 +/- 0,8 for SB215505, p=0.008) compared to vehicle-treated mice (Figure 7D).

Altogether, these results demonstrate that serotonin antagonists induce an anti-fibrotic environment in the lung by reducing TGF-beta1, CTGF and PAI-1 expression.

Serotonin receptors 5-HT2A and 5-HT2B are expressed in human lung tissue and fibroblasts

We performed an immunohistochemical analysis of 5-HT2A and 5-HT2B receptors on sections from human control and IPF lung samples (Figure 4J and 4K). Both receptors were expressed in the normal and diseased lung. The distribution of the 5-HT2A receptor was similar in control and IPF lung. The 5-HT2A receptor was expressed by alveolar macrophages and bronchial epithelial cells, but was not detected on fibroblasts, particularly in the fibroblastic focus (Figure 4J). The 5-HT2B receptor was expressed by alveolar macrophages, bronchial epithelial cells and endothelial cells in control lung. In IPF lung, a similar distribution was observed, but we also demonstrated a strong expression of 5-HT2B receptor by fibroblasts in the fibroblastic focus, an area of proliferating fibroblasts located at the leading edge of active fibrosis (Figure 4K), contrasting with the negative staining of 5-HT2A in this area. Interestingly, the 5-HT2B receptor was expressed by fibroblasts in the surrounding fibrotic tissue.

Discussion

To our knowledge, this is the first study approaching the serotonin pathway in the pathophysiology of lung fibrosis. We have shown that lung serotonin content is increased in bleomycin-induced fibrosis, and that in parallel, the expression of the serotonin receptors 5-HT2A and 5-HT2B is increased in the fibrotic lung, as detected by quantitative PCR, binding experiments and immunohistochemical staining. Inhibition of 5-HT2A and 5-HT2B receptors by blockage with specific antagonists promoted an anti-fibrotic environment in bleomycin-induced lung fibrosis through the inhibition of key factors involved in lung fibrosis, TGF-beta1, CTGF and PAI-1. By contrast, inflammatory changes observed on BAL cytology were minimal, only following SB215505 administration. Finally, we have demonstrated the expression of 5-HT2A and 5-HT2B receptors in IPF, and particularly the specific expression of the 5-HT2B receptor by fibroblasts in the fibroblastic focus characteristic of UIP.

Serotonin is a peptide with well known properties in the lung (20). Serotonin is synthesized from tryptophan, and pooled in platelets, which store and release serotonin via its serotonin transporter (5-HTT). Very low levels of circulating free serotonin are detected in normal conditions. Serotonin is degraded by the monoamine oxydase A. In the lung, the main sources of serotonin include platelets, neuroendocrine cells, masts cell in certain inflammatory and fibrotic conditions (21), and endothelial cells as recently identified (2, 3). Serotonin release by these cells may explain the increase of serotonin contents following bleomycin lung fibrosis that we observed. Masts cells are increased in the fibrotic lung (22), and may release serotonin under certain stimuli (23). In addition, neuroendocrine cells are increased in human IPF lung samples (24) and platelets have been associated with lung fibrosis and may be considered as a profibrotic actor (25). Finally, endothelial cells are being recognised as cellular targets in pulmonary fibrosis (26).

We concentrated our study on 5-HT2A and 5-HT2B receptors in lung fibrosis since these receptors have been shown to play an important role in lung pathophysiology (27) and to have pro-fibrotic properties in respiratory (5, 6) and non respiratory tissues (8, 28). Our demonstration of 5-HT2A and 5-HT2B receptors expression in the lung vessels, including endothelial cells and peri-arterial smooth muscle cells, agrees with previous data (5, 6). We also show that type II pneumocytes, epithelial bronchial cells, pleural mesothelial cells and lung fibroblasts do express 5-HT2A and 5-HT2B receptors in normal and fibrotic lung. Thus the serotonin pathway appears to act on many different cell types, potentially involved in the pathogenesis of lung fibrosis. The expression of 5-HT2B by fibroblasts on human tissue sampled from IPF lungs, with a preferential location in the fibroblastic focus, thought to be the site of active fibrosis, puts into perspective our results in the mouse model of lung fibrosis.

We observed that lung expression of 5-HT2A and 5-HT2B mRNA was markedly increased after bleomycin. However, specific 5-HT2A binding did not change but 5-HT2B binding increased significantly, at day 3, day 7 and day 14. This suggests that the regulation of 5-HT2A and 5-HT2B receptors expression is different, and that post-transcriptional regulation plays a key role for both receptors *in vivo*.

In addition, immunohistochemical studies indicated that both resident cells and inflammatory cells expressed 5-HT2A and 5-HT2B serotonin receptors in the lung (Figure 4). Immunohistochemistry does not allow for a quantitative analysis of the level of expression per cell of the receptors. Since there was a strong inflammatory infiltrate in the initial phase of bleomycin-induced lung disease, we suspect that increased expression of 5-HT2A and 5-HT2B receptors was due to the combination of inflammatory cells infiltration and increased expression on a per cell basis.

Blockage of 5-HT2A and 5-HT2B receptors by specific antagonists (ketanserin and SB215505 respectively) support previous published data on the pro-fibrotic properties of

serotonin (7, 8). In the bleomycin mouse model of lung fibrosis used in this study, daily intraperitoneal administration of ketanserin and SB215505 significantly reduced collagen contents and procollagen 1 and 3 expression, which are known to be selectively increased in this model of lung fibrosis (29). The anti-fibrotic effect observed following treatment with ketanserin and SB215505 to selectively block 5-HT2A and 5-HT2B receptors appears to be associated with changes in the expression of potent key pro-fibrotic factors TGF-beta1, CTGF and PAI-1. These factors have been previously linked to serotonin, 5-HT2A and 5-HT2B receptors activation in other experimental settings. Indeed, serotonin upregulation of the TGF-beta1 pathway via its 5-HT2A receptor has been previously described in cultured mesangial cells (28), and also demonstrated in aortic valve interstitial cells cultured from aortic valve (10). Expression of CTGF is induced in renal mesangial cells by serotonin (5-HT) (30). Finally, PAI-1 displays a close relationship with the serotoninergic systems where 5-HT *in vitro* increases PAI-1 expression by rat aortic endothelial cells (31).

In this study, we have demonstrated the anti-fibrotic effect of specific 5-HT2A and 5-HT2B receptors antagonists *in vivo* using the animal model of bleomycin-induced lung fibrosis and the involvement of the TGF-beta1, CTGF and PAI-1 pathways. Our observations in human samples support the reasonable hypothesis that the serotonin pathway might be involved in the pathophysiology of human lung fibrosis. Treatment with specific antagonists of targeted receptors may offer a new therapeutic approach in humans, which need to be assessed in further studies.

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FIGURES LEGENDS

Figure 1: Serotonin concentration in lung homogenates (expressed as nM/right apical and cardiac lobes of right lung) at day 3, day 7 and day 14 post bleomycin instillation and in controls (naive mice).

Serotonin is increased from day 3 with a maximum at day 7 (p=0.008) and remains increased at day 14 (p=0.04) compared to controls (n=3-5 animals at each time).

Figure 2: 5-HT2A and 5-HT2B receptors mRNA expression in bleomycin-induced lung fibrosis.

Results are expressed as the ratio to the house keeping gene HPRT. Note the increased expression of both receptors in the bleomycin lung from day 3 compared to controls (naive mice), which is sustained significantly over time (n=3-5 animals at each time).

Figure 3: 5-HT2A and 5-HT2B receptors specific binding in bleomycin-induced lung fibrosis. Results are expressed as fM/mg of azygous and diaphragmatic lobes of right lung. No variation is observed between controls and bleomycin lung for 5-HT2A (A), whereas there is an increased specific binding for 5-HT2B receptor in bleomycin lung compared to controls (naive mice) at day 3 and day 14 (p= 0.004) (B) (n=3-5 animals at each time).

Figure 4: Immunohistochemical detection of 5-HT2A and 5-HT2B receptors in (A-H) mouse and (I-J) human lung.

In bleomycin lungs, 5-HT2A receptor is expressed by bronchial epithelial cells (A), macrophages (A insert, arrows) and mesothelial cells lining the pleura (C and insert). Smooth muscle and endothelial cells did weakly express 5-HT2A in our model (B). Scale bars=100µm.

5-HT2B receptor is expressed by bronchial epithelial cells (D) and alveolar macrophages (D insert, arrows), weakly by smooth muscle cells and focally by endothelial cells (E), and strongly by inflammatory cells within the lesions (macrophages, lymphocytes and polymorphonuclear cells) (F). Mesothelial cells remain negative for this receptor. Negative control using mouse IgG1 is shown for comparison in G. Scale bars=100µm

In control lungs, 5HT2A and 5HT2B receptors are mainly expressed by bronchial epithelial cells (H, I) and focally by pneumocytes along the alveolar wall (inserts 5H and 5I). Scale bars=50µm.

Immunohistochemical localisation of the 5-HT2A 5-HT2B receptors in cryosections of human controls and IPF lung using ABC-alkaline phosphatase and the fast red substrate. Staings showed that the 5-HT2A receptor is expressed by hyperplastic type II pneumocytes lining the alveolar space (as), but is not detected on fibroblasts, particularly in the fibroblastic focus (ff) (J). By contrast, 5-HT2B receptor is frankly expressed by fibroblasts in the fibroblastic focus (ff) and in the surrounding fibrotic tissue (arrows) (K). This receptor is also expressed by hyperplastic pneumocytes. Scale bars=100µm.

br: bronchiole; bv: blood vessel , ff: fibroblastic focus; as: alveolar space

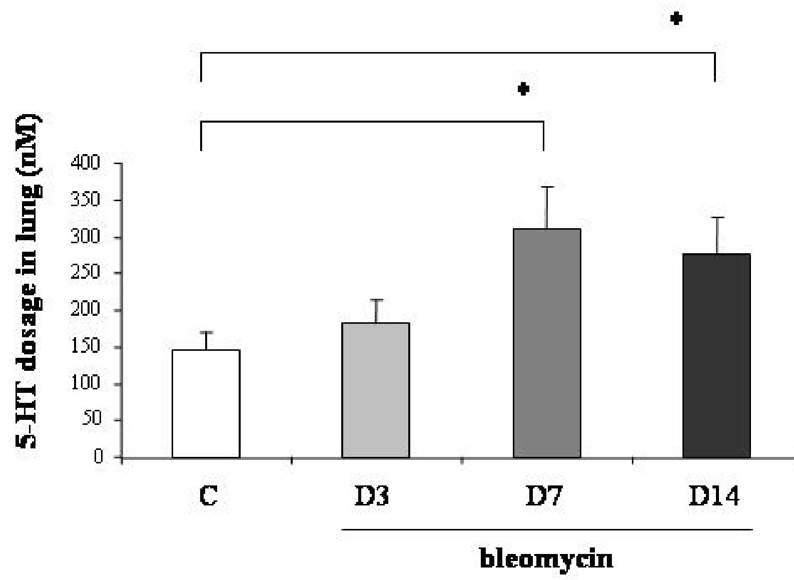
Figure 5: The morphological appearance on lung sections stained by hematoxylin-phloxin-safran (HPS, a, d, g, j), Masson's trichrome (MT, b, e, h, k) and Picrosirius (PS, c, f, i, l) shows hardly any collagen deposition in control mice, whereas 14 days post bleomycin instillation there is an increased collagen deposition evidenced by special stains in vehicle treated mice, which is clearly less marked in ketanserin- and SB215505 -treated mice. Scale bars=100µm.

Figure 6: (A) The total soluble collagen contents in the lung measured by the Sircol assay expressed as µg/right lung is reduced by treatment with ketanserin or SB215505 compared to vehicle treated mice (p=0.0006 for both antagonists, n=9-11 animals in each groups). (B) mRNA expression of procollagen I (COL1A2) and (C) mRNA expression of procollagen 3 (COL3A1) assessed by quantitative real time PCR (expressed as the ratio to the house keeping gene HPRT): ketanserin and SB21550 significantly reduce procollagen 1 mRNA (p=0.02 for ketanserin, p=0.06 for SB) and similarly procollagen 3 mRNA (p= 0.046 for ketanserin, p=0.047 for SB215505) at day 14 compared to vehicle treated mice (n=10-11 animals in each group).

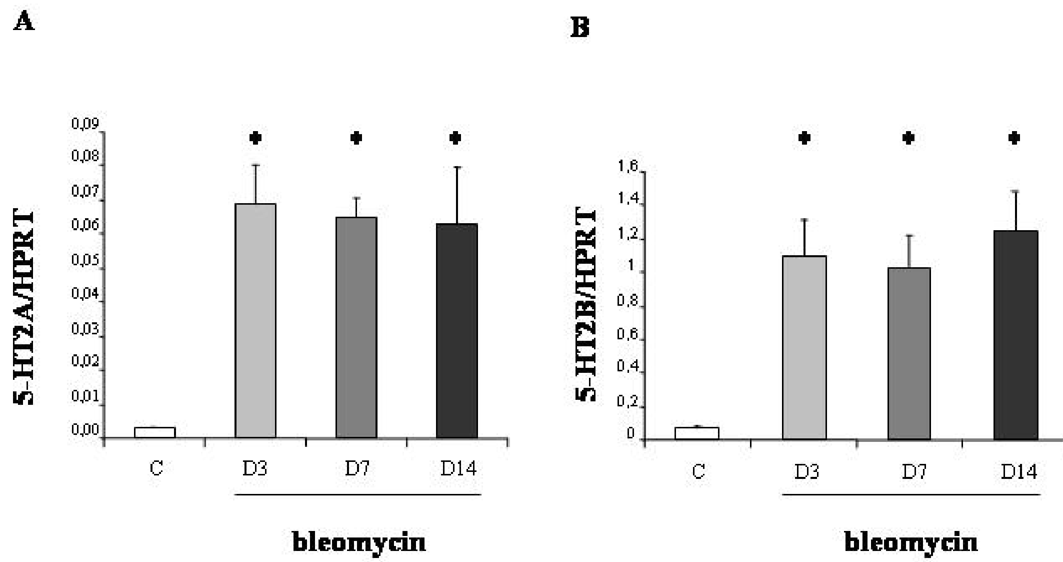
Figure 7: Inhibition of pro-fibrotic mediators by 5-HT2A and 5-HT2B receptors antagonists (A) TGF-beta1 mRNA in lung homogenates (expressed as the ratio to the house keeping gene HPRT) is reduced following treatment by ketanserin and SB215505 (p=0.026 and p=0.014 respectively) at day 14 post bleomycin instillation. (B) TGF-beta1 concentration in bronchoalveolar lavage fluid measured by ELISA (pg/ml) is reduced after treatment with ketanserin compared to vehicle treated mice (p=0.01). A trend was observed for SB215505 (p=0.33). C) CTGF mRNA content is increased in the fibrotic lung (p=0.009), CTGF mRNA is decreased in mice treated with ketanserin (p=0.017) and in mice treated with SB215505

(p=0.033) compared to vehicle treated mice. (D) PAI-1 mRNA (d) is markedly reduced following treatment by ketanserin and SB215505 compared to vehicle treated mice (p=0.007, and p=0.008, respectively) (n=9-11 animals in each group).

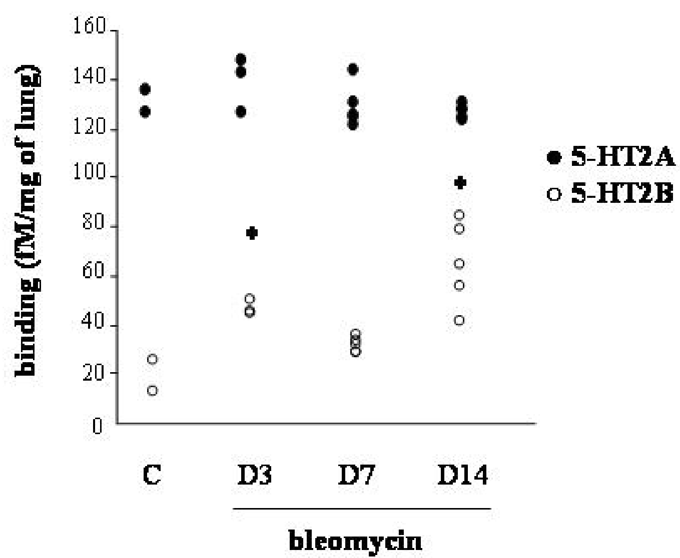
Fabre et al – Figure 1



Fabre et al – Figure 2



Fabre et al – Figure 3



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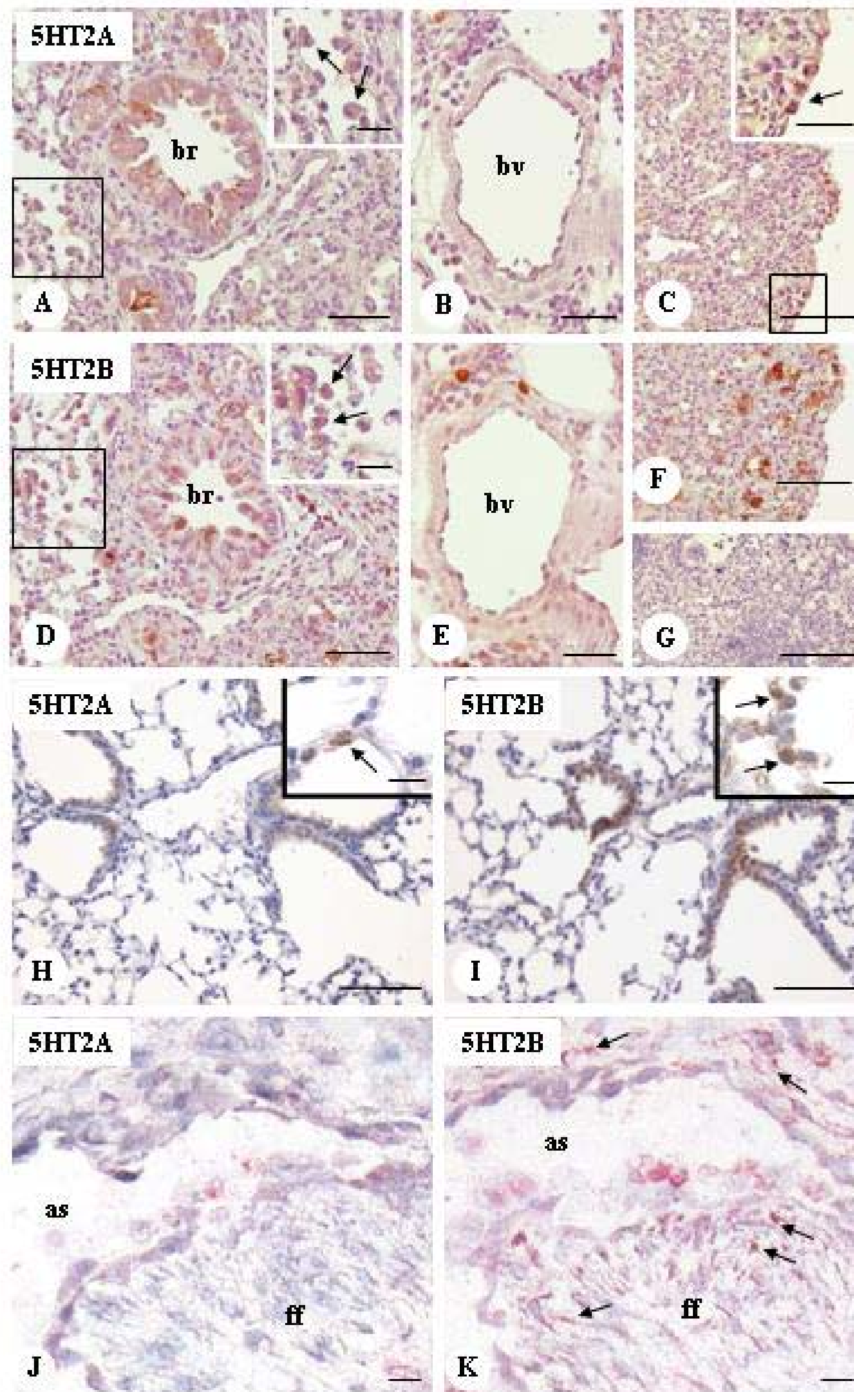

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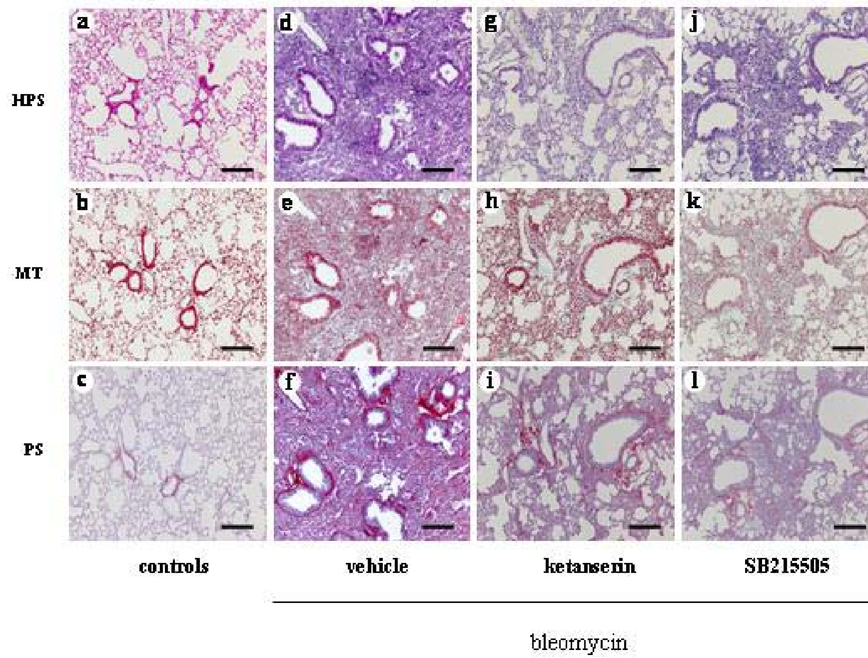
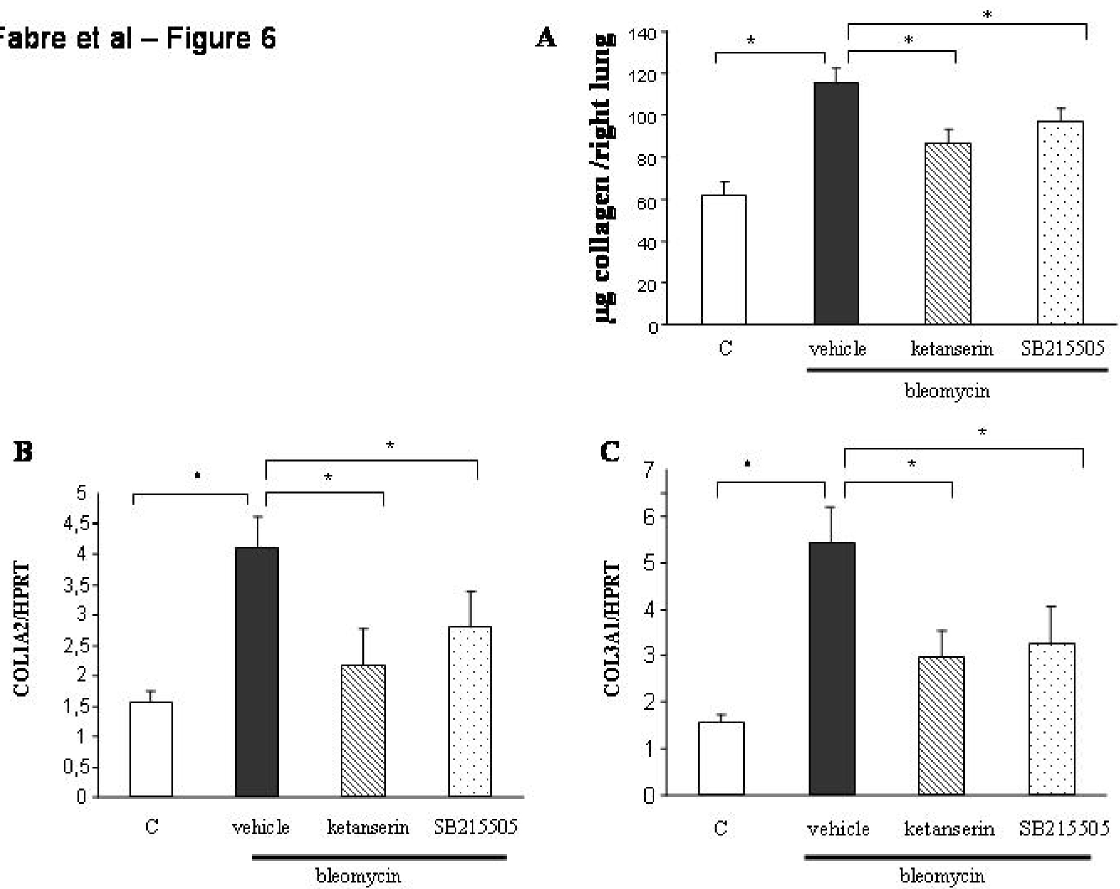
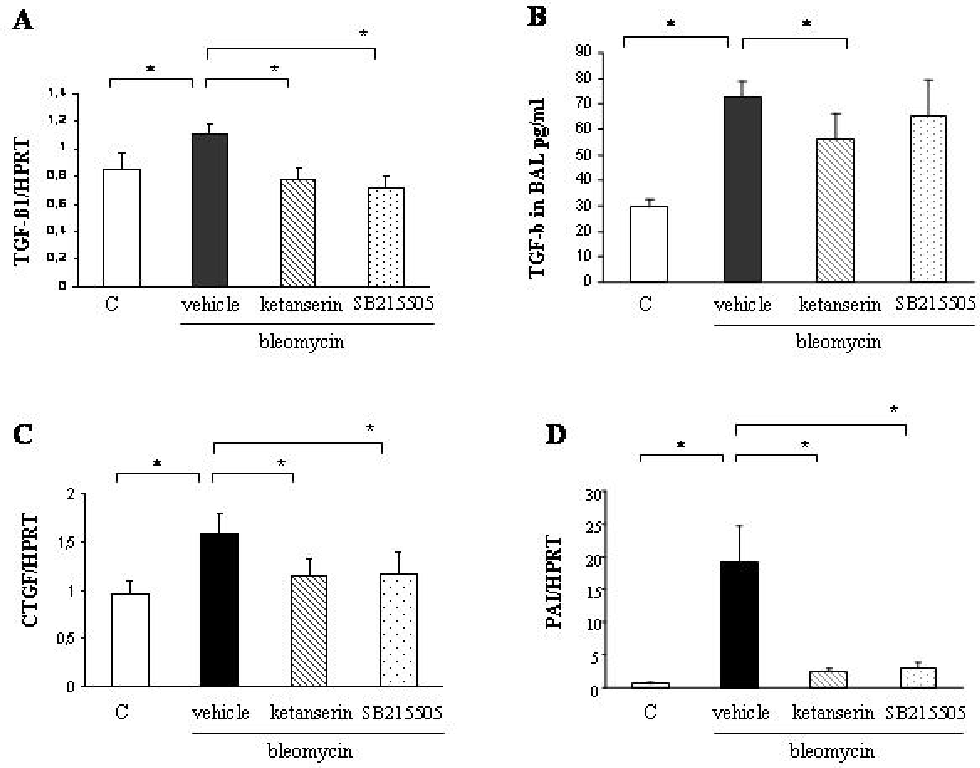


Fabre et al – Figure 6



Fabre et al – Figure 7



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