Ameliorative effect of mepenzolate bromide against pulmonary fibrosis

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Abbreviations: BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; CPFE, combined pulmonary fibrosis and emphysema; CS, cigarette smoke; DAPI, 4,6-diamidino-2-phenylindole; DTPA, diethylenetriamine-\(N, N', N''\)-pentaacetic acid; EGTA, ethylene glycol tetraacetic acid; EMT,
epithelial-mesenchymal transition; FVC, forced vital capacity; GST, glutathione S-transferase; H & E, hematoxylin and eosin; IPF, idiopathic pulmonary fibrosis; PBS, phosphate buffered saline; ROS, reactive oxygen species; SMA, smooth muscle actin; SOD, superoxide dismutase; SpO₂, percutaneous arterial oxygen saturation; TCA, trichloroacetic acid; TGF, transforming growth factor.

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is thought to involve lung injury caused by reactive oxygen species (ROS), which in turn is followed by abnormal fibrosis. A transforming growth factor (TGF)-β1-induced increase in myofibroblast number plays an important role in this abnormal fibrosis. We recently found that mepenzolate bromide (mepenzolate), which has been used clinically to treat gastrointestinal disorders, has ROS-reducing property. In the present study, we examined the effect of mepenzolate on bleomycin-induced pulmonary fibrosis and lung dysfunction in mice. The severity of pulmonary fibrosis was assessed by histopathologic evaluation and determination of hydroxyproline levels. Lung mechanics (elastance) and respiratory function (forced vital capacity, FVC) were assessed using a computer-controlled ventilator. Respiratory function was also evaluated by monitoring percutaneous arterial oxygen saturation (SpO₂). Intratracheal administration of mepenzolate prior to bleomycin treatment reduced the extent of pulmonary fibrosis and changes in lung mechanics and led to a significant recovery of both FVC and SpO₂ compared with control. Furthermore, mepenzolate produced a therapeutic effect even when it was
administered after the development of fibrosis. Administration of mepenzolate also prevented bleomycin-induced pulmonary cell death, inflammatory responses and increased myofibroblast number. Mepenzolate also decreased NADPH oxidase activity and active TGF-β1 level or increased glutathione S-transferase (GST) activity in the presence of bleomycin treatment. These results show that the intratracheal administration of mepenzolate reduced bleomycin-induced pulmonary fibrosis and lung dysfunction in mice. These effects may be due to this drug’s inhibitory effect on NADPH oxidase and TGF-β1 activities and its stimulatory effect on GST.
Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating chronic lung condition with poor prognosis; the reported mean length of survival from the time of diagnosis is 2.8-4.2 years. IPF progresses insidiously and slowly, and acute exacerbations of the condition are highly lethal (Kim et al., 2006; Raghu et al., 2011). Current agents for the treatment of IPF, such as steroids and immunosuppressors, have not been found to improve prognosis (Luppi et al., 2004; Walter et al., 2006; Raghu et al., 2011). Pirfenidone, a novel antifibrotic drug, was reported in some (but not all) clinical studies to slow the rate of FVC decrease in IPF patients. While this drug is licensed in Japan and Europe as a treatment for IPF (Azuma et al., 2005; Maher, 2010; Taniguchi et al., 2010), the FDA declined to approve its use due to inconclusive evidence of its clinical efficacy, and because of severe side effects such as photosensitivity in dermatitis, as well as nausea and anorexia (Maher, 2010; Noble et al., 2011). Thus, the development of new types of drugs to treat IPF is warranted.

Although the aetiology of IPF is not yet fully understood, recent studies have suggested that it is triggered by reactive oxygen species (ROS)-induced lung injury and
promoted by abnormal wound repair and remodelling, resulting in abnormal fibrosis (collagen deposition) (Maher et al., 2007; du Bois, 2010). The body contains a number of endogenous anti-oxidant proteins such as superoxide dismutase (SOD) and glutathione S-transferase (GST) that offer protection against ROS-induced tissue damage. In addition to ROS, transforming growth factor (TGF)-β1 also seems to play an important role in the pathogenesis of IPF (Sheppard, 2006; Kinnula, 2008). Lung myofibroblasts, consisting of a cell type that is intermediate between fibroblasts and smooth muscle cells, produce considerable amounts of extracellular matrix components, such as collagen, which may give rise to abnormal fibrosis (Hinz et al., 2007). Myofibroblasts are produced by both the activation of fibroblasts and by the transformation of epithelial cells through the process of epithelial-mesenchymal transition (EMT) (Hinz et al., 2007). TGF-β1 appears to increase the number of lung myofibroblasts by activating fibroblasts and inducing EMT of epithelial cells (Willis and Borok, 2007; Strieter and Mehrad, 2009). It has also been reported that ROS can activate TGF-β1 (Barcellos-Hoff and Dix, 1996; Bellocq et al., 1999).
The co-existing of emphysema and fibrosis in the same patient is known as combined pulmonary fibrosis and emphysema (CPFE) syndrome. The prognosis for CPFE syndrome is especially poor and an optimal treatment protocol for patients with this syndrome has not been established (Jankowich and Rounds, 2012). This is because pulmonary fibrosis and emphysema are characterized by distinct clinical, radiological, pathological and functional characteristics. For example, pulmonary fibrosis and emphysema respectively increase and decrease lung elastance (Papiris et al., 2013). On the other hand, since ROS-induced pulmonary damage also plays an important role in the pathogenesis of pulmonary emphysema (Nadeem et al., 2005; Mak, 2008), drugs that could decrease the pulmonary level of ROS may also be effective for treating both pulmonary fibrosis and emphysema. Supporting this notion, we recently reported that inhalation of lecithinized SOD ameliorates both bleomycin-induced pulmonary fibrosis and elastase- or cigarette smoke (CS)-induced pulmonary emphysema by decreasing the pulmonary level of ROS (Tanaka et al., 2010a; Tanaka et al., 2011; Tanaka et al., 2012a; Tanaka et al., 2012b).
We also reported that mepenzolate bromide (mepenzolate), an orally administered muscarinic receptor antagonist used to suppress the gastrointestinal hypermotility associated with irritable bowel syndrome (Long and Keasling, 1954; Buckley et al., 1957; Chen, 1959), could prevent elastase- or CS-induced pulmonary emphysema in mice by decreasing the pulmonary level of ROS (Tanaka et al., 2013). As for the mechanism governing the ROS-reducing activity of mepenzolate, we found that this activity is independent of the muscarinic receptor, because other muscarinic receptor antagonists such as ipratropium bromide (ipratropium) and tiotropium bromide (tiotropium) could not exert ameliorative effects against elastase-induced pulmonary disorders; scopolamine and pirenzepine also have no discernible effects against elastase-induced pulmonary disorders, even though, as for mepenzolate, these drugs are orally administered drugs used to treat gastrointestinal disorders and their clinical doses are similar to that of mepenzolate; the dose of mepenzolate required to affect elastase-induced pulmonary disorders was much higher than that required to show the bronchodilation activity (Tanaka et al., 2013). On the other hand, we suggested that the ROS-reducing activity of mepenzolate is mediated by both the inhibition of NADPH
oxidase activity and the stimulation of GST activity (Tanaka et al., 2013). Based on these findings, we proposed that mepenzolate could serve as a candidate drug for the treatment of patients with pulmonary emphysema.

In the present study, we have examined the effect of mepenzolate on bleomycin-induced pulmonary fibrosis in mice. The results obtained show that the intratracheal administration of mepenzolate suppresses bleomycin-induced pulmonary fibrosis and lung dysfunction in a manner that is probably mediated by this drug’s inhibitory effect on NADPH oxidase and TGF-β1 activities and by its stimulatory effect on GST.
Materials and Methods

Chemicals and animals. Chloramine T, 4-(dimethylamino)-benzaldehyde (DMBA), potassium dichromate, phosphotungstic acid, mepenzolate, phosphomolybdic acid, Orange G, ipratropium and acid fuchsin were obtained from Sigma (St. Louis, MO). Apocynin was from Santa Cruz Biotechnology (Santa Cruz, CA). Tiotropium was from ChemReagents (Sugarland, TX). Bleomycin was purchased from Nippon Kayaku (Tokyo, Japan), Novo-Heparin (5000 units) was from Mochida Pharmaceutical Co. (Tokyo, Japan), chloral hydrate was from Nacalai Tesque (Kyoto, Japan), and Diff-Quik was from the Sysmex Corporation (Kobe, Japan). The ELISA kit for quantifying TGF-β1 was from R&D Systems (Minneapolis, MN), while the assay kit for GST was from PromoKine (Heidelberg, Germany). Antibody against α-smooth muscle actin (α-SMA) was purchased from Abcam (Cambridge, Cambridgeshire), and Alexa Fluor 594 goat anti-rabbit immunoglobulin G was from Invitrogen (Carlsbad, CA). L-hydroxyproline, sodium acetate, trichloroacetic acid (TCA), azophloxin, aniline blue and formalin neutral buffer solution were obtained from WAKO Pure Chemicals (Tokyo, Japan). Mounting medium for immunohistochemical analysis
(VECTASHIELD) was purchased from Vector Laboratories (Burlingame, CA), while Mayer’s haematoxylin, 1% eosin alcohol solution, mounting medium for histological examination (malinol) and Weigert’s iron haematoxylin were from MUTO Pure Chemicals (Tokyo, Japan). Diethylenetriamine-$N, N', N'', N'''$-pentaacetic acid (DTPA) and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Dojindo (Kumamoto, Japan). Xylidine ponceau was from WALDECK GmbH & Co. KG, DIVISION CHROMA (Muenster, Germany). Isoflurane was from Pfizer (New York, NY). ICR mice (5-6 weeks old, male) were purchased from Charles River (Yokohama, Japan). The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and were approved by the Animal Care Committee of Keio University.

_Treatment of mice with bleomycin and mepenzolate._ Mice maintained under anaesthesia with isoflurane were intratracheally administered bleomycin (5 mg/kg or 3 mg/kg, once-only) in PBS or mepenzolate (various doses) in PBS via a
The first administration of mepenzolate was performed 1 h before the bleomycin administration (except for experiments shown in Fig. 2).

**Preparation of BALF, cell count and measurement of enzyme activities and TGF-β1.** Bronchoalveolar lavage fluid (BALF) was collected by cannulating the trachea and lavaging the lung with 1 ml of sterile PBS containing 50 units/ml heparin (2 times). About 1.8 ml of BALF was routinely recovered from each animal. The total cell number was counted using a haemocytometer. Cells were stained with Diff-Quik reagents after centrifugation with Cytospin® 4, and the ratios of macrophages and neutrophils to total cells were determined.

NADPH oxidase activity was measured by using lucigenin as a substrate (chemiluminescence) (Griendling et al., 1994). Samples were incubated with 0.1 mM NADPH in 50 mM phosphate buffer containing 1 mM EGTA, 150 mM sucrose and 0.5 mM lucigenin, and lucigenin chemiluminescence was recorded for 15 min in a microplate reader (MicroLumat Plus LB96V, Berthold Technologies).

The GST activity and the amount of active TGF-β1 were measured by employing each assay kit according to the manufacturer’s protocol.
Histological and immunohistochemical analyses. Lung tissue samples were fixed in 10% formalin neutral buffer solution for 24 h, after which they were embedded in paraffin before being cut into 4 μm-thick sections.

For histological examination, sections were stained first with Mayer’s haematoxylin and then with 1% eosin alcohol solution (H & E staining). Samples were mounted with malinol and inspected with the aid of an Olympus BX51 microscope.

For staining of collagen (Masson’s trichrome staining), sections were treated sequentially with solution A (5% w/v potassium dichromate and 5% w/v TCA), Weigert’s iron haematoxylin, solution B (1.25% w/v phosphotungstic acid and 1.25% w/v phosphomolybdic acid), 0.75% w/v Orange G solution, solution C (0.12% w/v xylidine ponceau, 0.04% w/v acid fuchsin and 0.02% w/v azophloxin), 2.5% w/v phosphotungstic acid, and finally with aniline blue solution. Samples were mounted with malinol and inspected with the aid of an Olympus BX51 microscope.

For immunohistochemical analysis, sections were blocked with 2.5% goat serum for 10 min, then incubated for 12 h with antibodies against α-SMA (1:100
dilution) in the presence of 2.5% bovine serum albumin, followed by incubation with Alexa Fluor 594 goat anti-rabbit immunoglobulin G (1:500 dilution) and DAPI (5 μg/ml) for 2 h. Samples were mounted with VECTASHIELD and inspected with the aid of a fluorescence microscope (Olympus BX51).

For TdT-mediated biotinylated UTP nick end labelling (TUNEL) assay, sections were incubated first with proteinase K for 15 min at 37 ºC, then with TdT and biotin 14-ATP for 1 h at 37 ºC, and finally for 2 h with Alexa Fluor 488 conjugated with streptavidin (1:500 dilution). Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus IX73).

**Measurement of SpO$_2$.** Measurement of percutaneous arterial oxygen saturation (SpO$_2$) was performed with the MouseOx system (STARR Life Sciences Corp., Allison Park, PA), as described previously (Tanaka et al., 2012a). MouseOx sensor attached to the thigh of mouse under anaesthesia with chloral hydrate (500 mg/kg). All data were analysed using MouseOx software (STARR Life Sciences Corp., Allison Park, PA).
Measurement of lung mechanics and FVC. Measurement of lung mechanics was performed with a computer-controlled small-animal ventilator (FlexiVent; SCIREQ, Montreal, Canada), as described previously (Tanaka et al., 2012a). Mice were anaesthetised with chloral hydrate (500 mg/kg), a tracheotomy was performed, and an 8 mm-long section of metallic tube (outer and inner diameters of 1.27 mm and 0.84 mm, respectively) was inserted into the trachea. Mice were mechanically ventilated at a rate of 150 breaths/min, using a tidal volume of 8.7 ml/kg and a positive end-expiratory pressure of 2-3 cmH₂O.

Total respiratory system elastance and tissue elastance were measured by the snapshot and forced oscillation techniques, respectively. All data were analysed using FlexiVent software (version 5.3; SCIREQ, Montreal, Canada).

Determination of FVC was performed with the same computer-controlled small-animal ventilator connected to a negative pressure reservoir (SCIREQ, Montreal, Canada), as described previously (Tanaka et al., 2012a). Mice were anaesthetised, then tracheotomized and ventilated as described above. The lungs were inflated to 30 cmH₂O over one second and held at this pressure. After 0.2 sec, the pinch valve was
(connected to the ventilator) was closed, and after 0.3 sec, the shutter valve (connected to the negative pressure reservoir) was opened, exposing the lung to the negative pressure, which was held for 1.5 sec to ensure complete expiration. FVC was determined using FlexiVent software (version 5.3).

**Hydroxyproline determination.** Hydroxyproline content was determined as described previously (Woessner, 1961). Briefly, the lung was removed and homogenized in 0.5 ml of 5% TCA. After centrifugation, pellets were hydrolysed in 0.5 ml of 10 N HCl for 16 h at 110 °C. Each sample was incubated for 20 min at room temperature after the addition of 0.5 ml of 1.4% w/v chloramine T solution and then incubated at 65 °C for 10 min after the addition of 0.5 ml of Ehrlich’s reagent (1M DMBA, 70% v/v isopropanol and 30% v/v perchloric acid). Absorbance was measured at 550 nm, and the amount of hydroxyproline was determined.

**Statistical analysis.** All values are expressed as the mean ± S.E.M. One-way ANOVA followed by the Tukey test or the Student’s t-test for unpaired results were used to evaluate differences between three or more groups or between two groups, respectively. Differences were considered to be significant for values of \( P<0.05 \).
Results

Effects of mepenzolate on bleomycin-induced pulmonary fibrosis.

Pulmonary fibrosis was induced by administering mice a single (on day 0) intratracheal dose of bleomycin. To begin with, we examined the preventive effect of mepenzolate on pulmonary fibrosis; mepenzolate was administered intratracheally once daily for 11 days (from day 0 to day 10) and pulmonary fibrosis was assessed on day 21 by histopathological analysis and measurement of pulmonary hydroxyproline levels (an indicator of collagen levels). Histopathological analysis of pulmonary tissue following H & E staining revealed that severe pulmonary damage was induced by the bleomycin and that this damage was suppressed by the intratracheal administration of mepenzolate (Fig. 1A). Masson’s trichrome staining of collagen revealed that bleomycin-induced increase in collagen deposition was clearly suppressed by the mepenzolate treatment (Fig. 1A, B). We also found that the bleomycin-induced elevation of pulmonary hydroxyproline was significantly suppressed by mepenzolate in a dose-dependent manner (Fig. 1C). The administration of mepenzolate did not affect pulmonary
hydroxyproline levels in mice that had not been subjected to bleomycin treatment (Fig. 1C).

Changes in lung mechanics associated with pulmonary fibrosis are characterized by an increase in elastance (Tanaka et al., 2010b). Total respiratory system elastance (elastance of the total lung, including the bronchi, bronchioles and alveoli) and tissue elastance (elastance of the alveoli) increased following bleomycin treatment. These effects were suppressed by the intratracheal administration of mepenzolate, again in a dose-dependent manner (Fig. 1D).

We next examined the effect of mepenzolate on respiratory function. As shown in Fig. 1E, FVC was clearly decreased in bleomycin-treated mice, and this decrease could be significantly reversed with concomitant mepenzolate treatment. We also evaluated respiratory function by monitoring SpO₂, which showed a decrease in bleomycin-treated mice and subsequent return towards control values following treatment with mepenzolate (Fig. 1F). In summary, the results in Fig. 1 show that the intratracheal administration of mepenzolate prevents bleomycin-induced pulmonary
damage and fibrosis, and reduces alterations in lung mechanics and respiratory dysfunction.

We next tested the efficacy of mepenzolate when the treatment protocol was initiated after the development of fibrosis (i.e., 10 days after the administration of bleomycin), with pulmonary fibrosis, lung mechanics and respiratory function parameters assessed on day 21. We first suggested that pulmonary fibrosis and alterations of lung mechanics and respiratory function were occurred on day 10 under these conditions and the extent of these alterations on day 10 was less apparent than that on day 21, although the differences were not statistically significant (supplemental Fig. S1). We subsequently found that treatment with mepenzolate (from day 10 to day 19) decreased the extent of pulmonary damage, pulmonary fibrosis and lung elastance changes on day 21 (Fig. 2A-C). It should however be noted that although mepenzolate showed a tendency to restore FVC in the presence of bleomycin treatment, the recovery was not statistically significant (Fig. 2D). We showed that the bronchodilatory effect of mepenzolate does not directly affect the monitoring of lung mechanics and respiratory function, based on observations that intratracheal administration of
mepenzolate from day 10 to day 19 did not affect the lung mechanics and respiratory function monitored on day 21 in control (without treatment with bleomycin) mice (same washout period as that in Fig. 2C and D) in supplemental Fig. S2. Results in Fig. 2 indicate that mepenzolate could be an effective agent for the treatment of pre-existing pulmonary fibrosis.

Effects of mepenzolate on bleomycin-induced pulmonary cell death, inflammatory responses and increase in myofibroblast number. As described above, pulmonary fibrosis involves various phenomena, such as pulmonary cell death, inflammation and an increase in myofibroblast number. To this end, we next examined the manner in which mepenzolate affects these bleomycin-induced physiological changes.

The level of pulmonary cell death was monitored by TUNEL assay. While treatment with bleomycin increased the number of TUNEL-positive cells, this increase was suppressed by the simultaneous administration of mepenzolate (Fig. 3).

Next, we monitored bleomycin-induced pulmonary inflammatory responses by determining the number of leucocytes in BALF. As shown in Fig. 4A, the total
number of leucocytes increased following bleomycin treatment, an effect which was partially suppressed by the concomitant treatment of animals with mepenzolate. Similar results were observed in relation to the numbers of macrophages and neutrophils (Fig. 4A).

We also used immunohistochemical analysis with antibodies against α-SMA, a marker for myofibroblasts (Hinz et al., 2007), to examine the effect of mepenzolate on the pulmonary level of myofibroblasts. As shown in Fig. 4B, C, bleomycin administration increased the number of α-SMA-positive cells, whereas simultaneous treatment with mepenzolate decreased this level, thus indicating that this drug suppresses the bleomycin-induced increase in lung myofibroblast number.

As described above, we reported that in elastase-induced pulmonary emphysema model of mice, anti-inflammatory and ROS-reducing activities of mepenzolate are independent of its muscarinic receptor-mediated bronchodilatory activity (Tanaka et al., 2013). To test the contribution of the bronchodilatory activity of mepenzolate to its ameliorative effect against bleomycin-induced pulmonary fibrosis, we examined the effect of other muscarinic antagonists (bronchodilators), such as
ipratropium and tiotropium on bleomycin-induced pulmonary fibrosis and alteration of lung mechanics and respiratory functions. Since we previously reported that bronchodilatory activity was indistinguishable between mepenzolate and ipratropium (Tanaka et al., 2013), we here used the ipratropium dose (500 µg/kg) that is equivalent to that of mepenzolate required to suppress bleomycin-induced pulmonary fibrosis (Fig. 1) and the tiotropium dose (56 µg/kg), considering the clinical doses of ipratropium and tiotropium. As shown in Fig. 5, intratracheal administration of each of these muscarinic antagonists could not suppress bleomycin-induced pulmonary fibrosis and alteration of lung mechanics and respiratory functions, suggesting that the ameliorative effect of mepenzolate against bleomycin-induced pulmonary fibrosis is independent of its muscarinic receptor-mediated bronchodilatory activity.

Effects of mepenzolate on NADPH oxidase, GST and TGF-β1. As described above, we recently reported that mepenzolate decreases the pulmonary level of ROS by suppressing the NADPH oxidase activation and by stimulating GST activity in elastase- or CS-administered mice (Tanaka et al., 2013). With the results of those studies in mind, we next examined here the effect of mepenzolate on NADPH oxidase
and GST activities in bleomycin-administered mice. As shown in Fig. 6A, treatment with bleomycin increased pulmonary NADPH activity in a manner that could be partially suppressed by the simultaneous administration of mepenzolate. Administration of mepenzolate increased the pulmonary GST activity in the presence of bleomycin treatment (Fig. 6B). Although treatment with bleomycin showed a tendency to decrease the GST activity, the decrease was not statistically significant (Fig. 6B).

To test the contribution of inhibitory effect of mepenzolate on NADPH oxidase activity to its ameliorative effect against bleomycin-induced pulmonary damage, we examined the effect of apocynin (an inhibitor of NADPH oxidase) and/or mepenzolate on bleomycin-induced pulmonary cell death, inflammatory responses and increase in myofibroblast number. As shown in Fig. 7, intratracheal administration of apocynin suppressed bleomycin-induced pulmonary cell death, inflammatory responses and increase in myofibroblast number, however, this administration of apocynin did not affect these indexes in the presence of treatment with mepenzolate, suggesting that the
The ameliorative effect of mepenzolate against bleomycin-induced pulmonary damage is mediated by its inhibitory effect on NADPH oxidase.

Finally, we monitored the pulmonary level of active TGF-β1, which is also an important endogenous factor implicated in promoting pulmonary fibrosis (see above). Treatment with bleomycin increased the pulmonary level of active TGF-β1 as described previously (Takemasa et al., 2012), and this increase was suppressed by the simultaneous administration of mepenzolate (Fig. 6C). The results in Figs. 6 and 7 thus suggest that the ameliorative effect of mepenzolate on bleomycin-induced pulmonary fibrosis is mediated by its inhibitory effect on NADPH oxidase and TGF-β1 activities, and by its stimulatory effect on GST activity.
Discussion

As IPF is a disease that affects lung mechanics and respiratory function, it is important to examine the effect that a candidate drug has not only on pulmonary fibrosis, but also on these other parameters. We have shown here that the concomitant administration of mepenzolate can reduce bleomycin-induced pulmonary fibrosis, increased lung elastance and respiratory dysfunction seen with the bleomycin treatment. The extent of amelioration by mepenzolate was similar to that afforded by pirfenidone (a drug used clinically to treat IPF patients) or PC-SOD (a drug being developed to treat IPF), which were investigated in the same animal model under similar conditions (Tanaka et al., 2010a; Tanaka et al., 2012a). Furthermore, in terms of clinical relevance, it is important to examine not only the preventive value of candidate compounds but also their therapeutic efficacy; to this extent we found here that mepenzolate is effective in combating pre-existing pulmonary fibrosis. Taken together, these results suggest that mepenzolate could be beneficial for the treatment of IPF patients.
To understand the mechanism governing the ameliorative effect of mepenzolate on pulmonary fibrosis, we also examined its effects on bleomycin-induced pulmonary cell death, inflammatory responses and increase in myofibroblast number, and found that mepenzolate could suppress all of these phenomena. To understand the mechanism at the molecular level, we focused on NADPH oxidase, GST and TGF-β1, given that ROS play an important role in causing the pulmonary cell damage associated with IPF, and that TGF-β1 can increase the number of lung myofibroblasts (Kinnula and Myllarniemi, 2008; Strieter and Mehrad, 2009). Furthermore, we recently reported that mepenzolate not only inhibited NADPH oxidase activity but also stimulated GST activity in elastase- or CS-administered mice (Tanaka et al., 2013). We found here that mepenzolate suppressed both the activation of NADPH oxidase and the increase in the active form of TGF-β1 brought about by bleomycin treatment. Furthermore, administration of mepenzolate restored the pulmonary GST activity in the presence of bleomycin treatment. The result suggests that these effects are involved in its ameliorative activity on bleomycin-induced pulmonary fibrosis. Since it was reported that ROS induce the activation of TGF-β1 (Barcellos-Hoff and Dix, 1996;
Bellocq et al., 1999), the inhibitory effect of mepenzolate on the pulmonary level of active TGF-β1 can likely be explained by its ROS-reducing activity. On the other hand, although some previous reports suggested that tiotropium shows therapeutic effects on lipopolysaccharide-induced pulmonary inflammatory responses and remodelling in vivo and suppresses acetylcholine-induced proliferation of fibroblasts and myofibroblasts in vitro (Pieper et al., 2007; Pera et al., 2011), we here showed that tiotropium is inert for bleomycin-induced pulmonary fibrosis.

As described in the Introduction, pulmonary fibrosis and emphysema are characterised by distinct clinical, radiological, pathological and functional characteristics. For example, imaging and pathological examinations highlight the fact that pulmonary fibrosis and emphysema are manifested in different ways in terms of the pulmonary regions involved and the parenchymal modifications that take place (Jankowich and Rounds, 2012). Furthermore, it was believed that pulmonary fibrosis or emphysema involve excess or insufficient wound repair, respectively (Chilosi et al., 2013). For these reasons, the clinical treatment of CPFE syndrome is rendered very difficult. Since ROS are involved in the pathogenesis of both pulmonary fibrosis and
emphysema, ROS-decreasing drugs may be effective for treating both conditions. To this end, we found here that, as for the animal model of pulmonary emphysema, mepenzolate is effective in treating the animal model of pulmonary fibrosis.

Chronic obstructive pulmonary disease (COPD) is characterized by airflow limitation and abnormal inflammatory responses, for which a combination of anti-inflammatory drugs (such as steroids) and bronchodilators forms the standard treatment regimen (Rabe et al., 2007). On the other hand, mepenzolate not only exhibits anti-inflammatory activity but also bronchodilatory activity due to its muscarinic receptor antagonizing action (Tanaka et al., 2013). It is reasonable to postulate, therefore, that this drug may be beneficial for treating COPD without the concomitant use of other medications. A feasible approach might be to initially develop mepenzolate for the treatment of COPD, followed by the clinical testing of its effects on CPFE syndrome.

The number of drugs reaching the marketplace each year is decreasing, mainly as a consequence of unexpected adverse effects of potential drugs being revealed at advanced clinical trial stages. For this reason, we proposed a new strategy for drug
discovery and development (drug re-positioning) (Mizushima, 2011). In this strategy, compounds with therapeutically beneficial activity are screened from a library of approved medicines with a view to developing them for new indications. We previously applied this strategy to the development of drugs to treat COPD patients by testing potential drugs on animal models of COPD (elastase- or CS-induced pulmonary emphysema), with mepenzolate identified as a potential candidate (Tanaka et al., 2013) and we here found that this drug is effective for bleomycin-induced pulmonary fibrosis in mice, which is a useful model for IPF (Moore et al., 2013). Thus, we proposed that mepenzolate may be good candidate drug for IPF, because its safety has already confirmed clinically.
Authorship Contributions.

**Participated in research design:** Kurotsu, Tanaka, Mizushima.

**Conducted experiments:** Kurotsu, Tanaka, Niino, Asano, Sugizaki.

**Performed data analysis:** Kurotsu, Tanaka.

**Wrote or contributed to the writing of the manuscript:** Kurotsu, Tanaka, Azuma, Suzuki, Mizushima.
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lecithinized superoxide dismutase on bleomycin-induced pulmonary fibrosis.


Footnotes

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Figure legends.

Fig. 1. Effect of mepenzolate on bleomycin-induced pulmonary fibrosis and lung dysfunction.

Mice were treated with bleomycin (BLM, 5 mg/kg) or vehicle once only on day 0. Mepenzolate (Mep) at 500 µg/kg (A, B) or the indicated dose (C-F) was administered intratracheally once daily for 11 days (from day 0 to day 10). Sections of pulmonary tissue were prepared on day 21 and subjected to histopathological examination (H & E staining (upper images) and Masson’s trichrome staining (lower images); scale bar, 100 µm) (A) and the percentage of area stained with Masson’s trichrome was determined using Image J software (B). Pulmonary hydroxyproline levels were determined on day 21 (C). Measurement of total respiratory system elastance (D), tissue elastance (D), FVC (E) and SpO2 (F) was carried out on day 21 as described in the Materials and Methods. Values represent the mean ± S.E.M. *P<0.05.

Fig. 2. Effect of mepenzolate on pre-developed pulmonary fibrosis.
Mice were treated with bleomycin (BLM, 3 mg/kg) or vehicle on day 0 only. Mepenzolate (Mep, 500 µg/kg) was administered intratracheally once daily for 10 days (from day 10 to day 19). Sections of pulmonary tissue were prepared on day 21 and subjected to histopathological examination (H & E staining (upper images) and Masson’s trichrome staining (lower images); scale bar, 100 µm) (A) and the percentage of area stained with Masson’s trichrome was determined (B). Measurement of total respiratory system elastance (C), tissue elastance (C) and FVC (D) was carried out on day 21. Values represent the mean ±S.E.M. *P<0.05; n.s., not significant.

**Fig. 3.** Effect of mepenzolate on bleomycin-induced pulmonary cell death.

Mepenzolate (Mep, 500 µg/kg) was administered intratracheally once only. Mice were treated with bleomycin (BLM, 5 mg/kg) or vehicle 1 h after the mepenzolate administration. Sections of pulmonary tissue were prepared 24 h after the BLM administration and subjected to TUNEL assay and DAPI staining (A). TUNEL-positive cells were counted (B). Values represent mean ±S.E.M. *P<0.05.
Fig. 4. Effect of mepenzolate on bleomycin-induced pulmonary inflammatory responses and increase in myofibroblast number.

Mice were treated with bleomycin (BLM, 5 mg/kg) or vehicle once only on day 0. Mepenzolate (Mep) at the indicated dose (A) or 500 µg/kg (B, C) was administered intratracheally once daily for 10 days (from day 0 to day 9). Total cell number, and individual numbers of macrophages and neutrophils in BALF were determined on day 10 as described in the Materials and Methods (A). Sections of pulmonary tissue were prepared on day 10 and subjected to immunohistochemical analysis with an antibody against α-SMA (B; scale bar, 100 µm). The percentage of area stained with the antibody was determined using Image J software (C). Values represent the mean ± S.E.M. **P<0.01; *P<0.05.

Fig. 5. Effect of other muscarinic antagonists on bleomycin-induced pulmonary fibrosis and lung dysfunction.

Mice were treated with bleomycin (BLM, 5 mg/kg) or vehicle once only on day 0. Ipratropium (Ipr, 500 µg/kg) or tiotropium (Tio, 56 µg/kg) was administered
intratracheally once daily for 11 days (from day 0 to day 10). Sections of pulmonary tissue were prepared on day 21 and subjected to histopathological examination (H & E staining (upper images) and Masson’s trichrome staining (lower images); scale bar, 100 μm) (A). Pulmonary hydroxyproline levels were determined on day 21 (B). Measurement of total respiratory system elastance (C), tissue elastance (C) and FVC (D) was carried out on day 21. Values represent the mean ± S.E.M. *P<0.05; n.s., not significant.

Fig. 6. Effect of mepenzolate on pulmonary activities of NADPH oxidase, GST and TGF-β1.

Mice were treated with bleomycin (BLM, 5 mg/kg) or vehicle once only on day 0. Mepenzolate (Mep, 500 μg/kg) was administered intratracheally once daily for 3 days (from day 0 to day 2) (A), once only on day 0 (B) or once daily for 10 days (from day 0 to day 9) (C). Cells in BALF were prepared on day 3 and NADPH oxidase activity was determined (A). On day 1 (B) or day 10 (C), lung homogenates were prepared
and pulmonary levels of GST activity (B) or the active form of TGF-β1 (C) were determined. Values represent the mean ± S.E.M. *P<0.05; n.s., not significant.

Fig. 7. Effect of mepenzolate and/or apocynin on bleomycin-induced pulmonary cell death, inflammatory responses and increase in myofibroblast number.

Mice were treated with bleomycin (BLM, 5 mg/kg) or vehicle once only on day 0 (A-E). Mepenzolate (Mep, 500 µg/kg) and/or apocynin (Apo, 1 mg/kg) was intratracheally administered once only 1 h before the BLM administration (A, B) or once daily for 11 days (from day 0 to day 10) (C-E). Sections of pulmonary tissue were prepared 24 h after the BLM administration and subjected to TUNEL assay and DAPI staining (A). TUNEL-positive cells were counted (B). Total cell number in BALF was determined on day 10 (C). Sections of pulmonary tissue were prepared on day 10 and subjected to immunohistochemical analysis with an antibody against α-SMA (D; scale bar, 100 µm). The percentage of area stained with the antibody was determined using Image J software (E). Values represent the mean ± S.E.M. *P<0.05; n.s., not significant.
**Fig. 1**

A

** vehicle **

** vehicle **

** BLM **

** + Mep **

H & E

Masson's trichrome

B

![Bar chart showing collagen area (%)](chart1)

C

![Bar chart showing hydroxyproline (µg/lefth lung)](chart2)

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Fig. 1

D

Total respiratory system elastance (cmH₂O/ml)

Mep (µg/kg) 0 500 0 125 250 500
BLM (mg/kg) 0 5

Tissue elastance (cmH₂O/ml)

Mep (µg/kg) 0 500 0 125 250 500
BLM (mg/kg) 0 5

E

FVC (ml)

Mep (µg/kg) 0 500 0 125 250 500
BLM (mg/kg) 0 5

SpO₂ (%)

Mep (µg/kg) 0 500 0 125 250 500
BLM (mg/kg) 0 5
**Fig. 2**

A. H&E and Masson's trichrome staining of lung tissue from BLM and BLM + Mep treated groups.

B. Bar graph showing collagen area (%).

C. Bar graph showing total respiratory system elastance (cmH$_2$O/ml).

D. Bar graph showing FVC (ml).
Fig. 3

A

vehicle  BLM  + Mep

TUNEL

DAPI

B

TUNEL positive cells (cells/HPF)

vehicle  BLM  + Mep

*  *  

n=8  n=8  n=8
Fig. 4

A

**

* n=4

n=3

n.d.

n=4

n=4

n=3

Mep (µg/kg) 0 0 125 250 500

BLM (mg/kg) 0 0 5

Total cells (×10^5 cells)

Macrophages (×10^5 cells)

Neutrophils (×10^5 cells)
Fig. 4

B

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<th>+ Mep</th>
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<tr>
<td>DAPI</td>
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C

Area of α-SMA (%/HPF)

- **vehicle**
- **BLM**
- **+ Mep**

* n=4
Fig. 5

A

Vehicle | BLM | + Ipr | + Tio

H&E

Masson’s trichrome

B

Hydroxyproline (µg/left lung)

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<th>+ Ipr</th>
<th>+ Tio</th>
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* n.s.
Fig. 6

A

![Graph showing NADPH oxidase activity (%) for vehicle, BLM, and + Mep with n=7 and n=6 for each group.]

B

![Graph showing GST activity (%) for vehicle, BLM, and + Mep with n=6 for each group.]

C

![Graph showing Active TGF-β1 (pg/lung) for vehicle, BLM, and + Mep with n=16, n=25, n=24 for each group.]

* indicates statistical significance.
Fig. 7

D

\[
\begin{array}{cccc}
\text{vehicle} & \text{BLM} & + \text{Apo} & + \text{Mep} \\
\alpha\text{-SMA} & & & \\
\text{DAPI} & & & \\
\end{array}
\]

\[\text{n=5} \quad \text{n=5} \quad \text{n=5} \quad \text{n=5}\]

E

\[
\begin{array}{cccc}
\text{Apo} & - & - & + & + \\
\text{Mep} & - & - & + & + \\
\text{BLM (mg/kg)} & 0 & 5 \\
\end{array}
\]

\[
\text{Area of } \alpha\text{-SMA (%/HPF)}
\]

\[
\begin{array}{c}
\text{n=5} \\
\text{n=5} \\
\text{n=5} \\
\text{n=5} \\
\end{array}
\]

\[
\begin{array}{c}
* \\
* \\
\text{n.s.} \\
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