Ameliorative Effect of Mepenzolate Bromide against Pulmonary Fibrosis

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ABSTRACT

Idiopathic pulmonary fibrosis 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 properties. 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 histopathological 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 and 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 forced vital capacity (FVC) decrease in patients with IPF. Although this drug is licensed in Japan and Europe as a treatment of IPF (Azuma et al., 2005; Maher, 2010; Taniguchi et al., 2010), the Food and Drug Administration declined to approve its use because of inconclusive evidence of its clinical efficacy and because of severe side effects, such as photosensitivity in dermatitis and nausea and anorexia (Maher, 2010; Noble et al., 2011). Thus, the development of new types of drugs to treat IPF is warranted.

Although the etiology of IPF is not yet fully understood, recent studies have suggested that it is triggered by reactive oxygen species (ROS)–induced lung injury and is promoted by abnormal wound repair and remodeling, resulting in abnormal fibrosis (collagen deposition) (Maher et al., 2007; du Bois, 2010). The body contains a number of endogenous antioxidant proteins such as superoxide dismutase (SOD) and glutathione S-transferase (GST) that offer protection against the oxidizing environment. Of these, GST offers protection against ROS by converting them to water-soluble conjugates that can be excreted in the urine. Activated TGF-β1 was the first cytokine recognized as a mediator of fibrosis. A recent study showed that a TGF-β1–induced increase in myofibroblast number plays an important role in IPF (Lampugnani & Mantovani, 1994). This study demonstrates that mepenzolate decreases 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.
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 the transformation of epithelial cells through the process of epithelial-mesenchymal transition (Hinz et al., 2007). TGF-β1 appears to increase the number of lung myofibroblasts by activating fibroblasts and inducing epithelial-mesenchymal transition 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 coexistence 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, radiologic, pathologic, and functional characteristics. For example, pulmonary fibrosis and emphysema increase and decrease lung elastance, respectively (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 SODameliorates 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, 2011, 2012a,b).

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 cause 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 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-Aldrich (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), chlorylate was from Nacalai Tesque (Kyoto, Japan), and Diff-Quik was from the Sysmex Corporation (Kobe, Japan). The enzyme-linked immunosorbent assay (ELISA) kit for quantifying TGF-β1 was from R&D Systems (Minneapolis, MN), and the assay kit for GST was from Promokine (Heidelberg, Germany). Antibody against α-smooth muscle actin (α-SMA) was purchased from Abeam (Cambridge, Cambridge-shire, UK), 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 (VECTASHEILD) was purchased from Vector Laboratories (Burlingame, CA), and Mayer's hematoxylin, 1% eosin alcohol solution, mounting medium for histologic examination (malinol), and Weigert's iron hematoxylin were from Muto Pure Chemicals (Tokyo, Japan). Diethylenetriamine-N,N,N',N''-pentaaetic acid (DTPA) and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Dojindo (Kumamoto, Japan). Xyline Ponceau was from Waldeck GmbH & Co. KG, Division Chroma (Muenster, Germany). Isoflurane was from Pfizer (New York, NY). ICR mice (5–6-week-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 anesthesia with isoflurane were intratracheally administered bleomycin (5 or 3 mg/kg; once only) in phosphate-buffered saline (PBS) or mepenzolate (various doses) in PBS via a micropipette (P200; Gilson, Inc., Middleton, WI). The first administration of mepenzolate was performed 1 hour before the bleomycin administration (with the exception of experiments shown in Fig. 2).

Preparation of Bronchoalveolar Lavage Fluid, 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 (two times). Approximately 1.8 ml of BALF was routinely recovered from each animal. The total cell number was counted using a hemocytometer. Cells were stained with Diff-Quik reagents after centrifugation with Cytospin 4 (Thermo Fisher Scientific, Inc., Waltham, MA), 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 the ratios of macrophages and neutrophils to total cells were determined.
TGF-β1 were measured by using each assay kit according to the manufacturer’s protocol.

**Histologic and Immunohistochemical Analyses.** Lung tissue samples were fixed in 10% formalin neutral buffer solution for 24 hours, after which they were embedded in paraffin before being cut into 4-μm-thick sections. For histologic examination, sections were stained first with Mayer’s hematoxylin 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 hematoxylin, 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 minutes and then incubated for 12 hours 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 hours. Samples were mounted with VECTASHIELD and inspected with the aid of a fluorescence microscope (Olympus BX51).

For TdT-mediated biotinylated UTP terminal deoxynucleotidyl transferase-mediated digoxigenin-labeled nick-end labeling (TUNEL) assay, sections were incubated first with proteinase K for 15 minutes at 37°C and then with TdT and biotin 14-ATP for 1 hour at 37°C, and finally for 2 hours with Alexa Fluor 488 conjugated with streptavidin (1:500 dilution). Samples were mounted with VECTASHIELD and inspected using a fluorescence microscope (Olympus IX73; Olympus Corporation, Tokyo, Japan).

**Measurement of Percutaneous Arterial Oxygen Saturation.** Measurement of percutaneous arterial oxygen saturation (SpO₂) was performed with the MouseOx system (Starr Life Sciences Corp., Allison Park, PA), as described previously (Tanaka et al., 2012a). The MouseOx sensor attached to the thigh of a mouse under anesthesia with chloral hydrate (500 mg/kg). All data were analyzed using MouseOx software (Starr Life Sciences Corp.).

**Measurement of Lung Mechanics and FVC.** Measurement of lung mechanics was performed with a computer-controlled small animal ventilator (FlexiVent; SCIREQ, Montreal, QC, Canada), as described previously (Tanaka et al., 2012a). Mice were anesthetized 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 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 cm H₂O.

Total respiratory system elastance and tissue elastance were measured by the snap shot and forced oscillation techniques, respectively. All data were analyzed using FlexiVent software (version 5.3; SCIREQ).

Determination of FVC was performed with the same computer-controlled small-animal ventilator connected to a negative pressure reservoir (SCIREQ), as described previously (Tanaka et al., 2012a). Mice were anesthetized and then tracheotomized and ventilated as described earlier. The lungs were inflated to 30 cm H₂O over 1 second and held at this pressure. After 0.2 second, the pinch valve (connected to the ventilator) was closed, and after 0.3 second, the shutter valve (connected to the negative pressure reservoir) was opened, exposing the lung to the negative pressure, which was held for 1.5 seconds to ensure complete expiration. FVC was determined using FlexiVent software (version 5.3).

**Hydroxyproline Determination.** Hydroxyproline content was determined as described previously (Woessner, 1961). In brief, the lung was removed and homogenized in 0.5 ml of 5% TCA. After centrifugation, pellets were hydrolyzed in 0.5 ml of 10 N HCl for 16 hours at 110°C. Each sample was incubated for 20 minutes 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 minutes after the addition of 0.5 ml of Ehrlich’s reagent (1 M DMBA, 70% w/v isopropanol, and 30% w/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 analysis of variance followed by the Tukey test or 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 if P values < 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. 1, A and 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 toward 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 occurred on day 10 under these conditions, and the extent of these alterations on day 10 was less apparent than on day 21, although the differences were not statistically significant (Supplemental Fig. 1). 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. 2, A–C). However, it should 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 in Supplemental Fig. 2 that intra-tracheal administration of mepenzolate from day 10 to day 19 did not affect the lung mechanics and respiratory function monitored on day 21 in control mice (without treatment with bleomycin; same washout period as that in Fig. 2, C and D). 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 earlier, 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 physiologic changes. The level of pulmonary cell death was monitored by TUNEL assay. Although treatment with bleomycin increased the number of TUNEL-positive cells, this increase was suppressed by the simultaneous administration of mepenzolate (Fig. 3).

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Next, we monitored bleomycin-induced pulmonary inflammatory responses by determining the number of leukocytes in BALF. As shown in Fig. 4A, the total number of leukocytes 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. 4, B and 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 earlier, we reported that, in an 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), here we used the ipratropium dose (500 µg/kg) equivalent to that of mepenzolate that is 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

**Fig. 2.** Effect of mepenzolate on predeveloped 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 (top images) and Masson’s trichrome staining (bottom 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.
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 earlier, 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 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 pulmonary GST activity in the presence of bleomycin treatment (Fig. 6B). Although treatment with bleomycin showed a tendency to decrease GST activity, the decrease was not statistically significant (Fig. 6B).

To test the contribution of the 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 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 earlier discussion). 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 bleomycin treatment. The extent of amelioration by mepenzolate was similar to that afforded by pirfenidone (a drug used clinically to treat patients with IPF) or lecithinized human Cu/Zn-superoxide dismutase (a drug being developed to treat IPF), which were investigated in the same animal model under similar conditions (Tanaka et al., 2010a, 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 that mepenzolate is effective in combating pre-existing pulmonary fibrosis. Taken together, these results suggest that mepenzolate could be beneficial for the treatment of patients with IPF.
To understand the mechanism governing the ameliorative effect of mepenzolate on pulmonary fibrosis, we also examined its effects on bleomycin-induced pulmonary cell death and inflammatory responses, as well as 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). Here,

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 and 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 under Materials and Methods (A). Sections of pulmonary tissue were prepared on day 10 and subjected to immunohistochemical analysis with an antibody against α-SMA (scale bar, 100 μm) (B). The percentage of area stained with the antibody was determined using ImageJ software (C). Values represent the mean ± S.E.M. **P < 0.01; *P < 0.05. HPF, high-power field; n.d., not determined.
we found 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 pulmonary GST activity in the presence of bleomycin treatment. The result suggests that these effects are involved in the ameliorative activity of mepenzolate 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 remodeling in vivo and suppresses acetylcholine-induced proliferation of fibroblasts and myofibroblasts in vitro (Pieper et al., 2007; Pera et al., 2011), here we show that tiotropium is inert for bleomycin-induced pulmonary fibrosis.

As described under Introduction, pulmonary fibrosis and emphysema are characterized by distinct clinical, radiologic, pathologic, and functional characteristics. For example, imaging and pathologic 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 involves excess or insufficient wound repair, respectively (Chilosi et al., 2013). For these reasons, the clinical treatment of CPFE syndrome is rendered very difficult. Because 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 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 exhibits not only anti-inflammatory activity but also bronchodilatory activity due to its muscarinic receptor–antagonizing action (Tanaka et al., 2013). Therefore, it is reasonable to postulate 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 repositioning) (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 patients with COPD 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 in this study, we 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 a good candidate drug for IPF, because its safety has already been confirmed clinically.

**References**


**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 hour before the BLM administration (A and B) or once daily for 11 days (from day 0 to day 10) (C–E). Sections of pulmonary tissue were prepared 24 hours 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 (scale bar, 100 µm) (D). The percentage of area stained with the antibody was determined using ImageJ software (E). Values represent the mean ± S.E.M. *P < 0.05; n.s., not significant. HPF, high-power field.
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Supplemental Fig. S1

Supplemental Fig. S1. The extent of bleomycin-induced pulmonary fibrosis and lung dysfunction on day 10 and 21.

Mice were treated with bleomycin (BLM, 3 mg/kg) or vehicle on day 0 only. Sections of pulmonary tissue were prepared on day 10 and 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 10 and 21 (B). Measurement of total respiratory system elastance (C), tissue elastance (C) and FVC (D) was carried out on day 10 and 21. Values represent the mean ± S.E.M. *P<0.05; n.s., not significant.
Supplemental Fig. S2. Effect of mepenzolate on lung function in control mice. Mepenzolate (Mep, 500 µg/kg) was administered intratracheally once daily for 10 days (from day 10 to day 19). Measurement of total respiratory system elastance (A), tissue elastance (A) and FVC (B) was carried out on day 21. Values represent the mean ± S.E.M. n.s., not significant.