YM-40461 Improves Airway Clearance in Guinea Pigs with Induced Subacute Bronchitis

Aishi Kimoto,* Yasuno Hirano, Takaya Iwai, Munetoshi Saitou, Kenichi Tomioka, Keiji Miyata, and Toshimitsu Yamada
Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyakigaoka, Tsukuba, Ibaraki 305-0841, Japan. Received April 4, 2000; accepted September 29, 2000

The effects of the surfactant secretagogue YM-40461 on the mucociliary transport (MCT) velocity were examined in guinea pigs with induced bronchitis. Guinea pigs were exposed to SO2 gas (900 ppm, 3 h/d) for 5 d. MCT velocity was measured by the movement of a 3% gelatin solution containing Evans blue dye placed on the tracheal mucosal surface. Repeated doses of YM-40461 improved the MCT in guinea pigs with bronchitis within 5 d after the completion of SO2 exposure, with an ED50 value of 3.1 mg/kg p.o. At a dose of 10 mg/kg p.o., YM-40461 restored MCT to the control level (98.0% recovery). Ambroxol, bromhexine and salbutamol also improved MCT, but were far less effective than YM-40461. Airway fluid collected from bronchitic animals revealed increased disaturated phosphatidylcholine (DSPC, a major component of surfactants)-to-protein ratio and decreased surface tension produced by YM-40461 treatment (10 mg/kg). These results suggest that YM-40461 ameliorates MCT dysfunction in animals with SO2 gas-induced bronchitis by increasing the DSPC-to-protein ratio in the airway.

Key word surfactant; mucociliary transport; bronchitis

Chronic obstructive pulmonary diseases (COPD) such as chronic bronchitis, lung emphysema and severe asthma, reduce airway clearance.1) Airway clearance is determined by such factors as ciliary function and the physiologic properties of airway secretions.2) Indeed, suppression of ciliary activity, biochemical changes and rheological abnormalities of mucus have been demonstrated in COPD patients.3) Dysfunction in mucociliary transport (MCT) causes difficulties in sputum expectoration as well as antigen and toxic substances removal. This in turn leads to signs and symptoms of chest discomfort including coughing, wheezing, shortness of breath and expiratory dyspnea.

Pulmonary surfactant, a thin layer of tensioactive material lining the alveoli and terminal bronchioles, lowers the surface tension of alveoli and stabilizes the air-liquid interface in the lungs. In addition, surfactant has many important physiologic roles in the airway including acceleration of MCT and protection of mucous membranes from toxic substances, antigens and bacteria.4) Thus, alteration in mucociliary clearance caused by a dysfunction of the surfactant, e.g. decreasing of amount and tensio-active property, may be an important factor in COPD pathogenesis.

Presently, there are few effective clinical therapies for COPD. A potent pulmonary surfactant secretagogue, YM-26818 was recently synthesized and powerfully ameliorates lung compliance with increasing surfactant in guinea pig which was induced surfactant depression by lung lavage.3) YM-40461, which is not hygroscopic, is the free base form of YM-26818. In the present study, the effect of YM-40461 on the MCT function and physical properties of the airway fluid were examined using a subacute bronchitis model.

MATERIALS AND METHODS

Animals Adult male Hartley guinea pigs (Charles River Co.; Yokohama, Japan) weighing 350–450 g were used in this study. All experiments were performed in compliance with the regulation of the Animal Ethical Committee of Yamanouchi Pharmaceutical.

Induction of Chronic Bronchitis in Guinea Pigs Bronchitis was induced by exposing the guinea pigs to SO2 gas as follows. Each guinea pig was put into a plastic chamber (W 290 mm×L 180 mm×H 375 mm) and exposed to 1000 ppm SO2 gas in air 3 h/d for 5 d. The gas concentration was constantly monitored by a gas concentration detector (Gastec Co.; Tokyo, Japan). All drugs used in this study were suspended in 0.5% methylcellulose solution and orally administered twice a day for 5 days starting the day after the last exposure to SO2. The reason that the drugs were administered twice a day was based on the results of pharmacokinetics study of YM-40461 and ambroxol in guinea pigs (Data not shown). In cases of bromhexine and salbutamol were followed the setting of YM-40461 and ambroxol. Each drugs were administered during 9–10 a.m. and 6–7 p.m. in each days. The concentration of each drugs were used in these experiments were as follows; YM-40461 1, 3 and 10 mg/kg; salbutamol 2, 6 and 20 mg/kg; ambroxol 100, 300 and 600 mg/kg; bromhexine 100 and 300 mg/kg. Measurements of the MCT velocity and the collection of airway fluid were performed 2 h after the last drug dosing.

Measurement of MCT Velocity The MCT velocity was assessed by a newly established method.6) Guinea pigs were anesthetized by an intraperitoneal (i.p.) injection of urethane at a dose of 1.2 g/kg, 10 min before measurement of MCT velocity. The animals were fixed in a dorsal position on a fixation board inclined 10° from horizontal, with the head up. The trachea was exposed, and blood vessels and connective tissues over the trachea were separated carefully over 4 cm from approximately 1 cm below the larynx. The exposed trachea was punctured with a sterilized needle, and 2 μl of a 30% gelatin saline solution containing 0.5% Evans blue dye was introduced into the trachea using a micro-syringe. After 2 min, the trachea was opened and the distance the dye moved from the injection point was measured with calipers to determine the MCT velocity. The recovery (%) of MCT function produced by the test drugs was calculated using the

* To whom correspondence should be addressed. e-mail: kimoto@yamanouchi.co.jp

© 2000 Pharmaceutical Society of Japan
following formula:

\[
\text{Recovery (\%)} = \frac{\text{MCT velocity (SO}\_2\text{-drug treated)} - \text{MCT velocity (SO}\_2\text{-nontreated)}}{\text{MCT velocity (non-exposed)} - \text{MCT velocity (SO}\_2\text{-nontreated)}} \times 100
\]

Collection of Airway Fluid from Bronchitic Animals
The procedures for collecting the airway fluid were based on the method of Kase et al.\(^7\) Guinea pigs were anesthetized by an i.p. injection of urethane at a dose of 1.2 g/kg, 10 min after the ninth oral dosing. The guinea pigs were restrained in the supine position with their heads downwards on a 25° inclined board. The trachea was exposed and a specially devised Y-shaped polyethylene cannula was intubated into the trachea. One of its openings was connected to an air outlet of an Engelhorn-improved humidifier so that the animals could spontaneously breathe water-saturated air (RH approximately 100%) maintained at 39±1°C. Airway fluids which flowed out of the respiratory tract by means of postural drainage were collected for 8 h in a tube using the other opening of the cannula. Samples from 4 guinea pigs were used to measure surface tension with a Face Automatic Surface Tension-meter (Model CBVP-A3; Kyowa Kaimenkagaku Co., Tokyo).

Determination of Airway Fluid Contents
Collected airway fluid samples were centrifuged at 300×g at 4°C for 10 min and the supernate were used for determination of airway fluid contents. The total protein content in the supernate was determined by the method of Lowry et al.\(^8\) A 100 μl aliquot of the supernate of the airway fluids was used to determine the disaturated phosphatidylcholine (DSPC) content in the airway fluid as follows. Total phosphatidylcholine was prepared by extraction with chloroform–methanol 2:1 (v/v) and washing according to the procedure of Folch et al.\(^9\) An aliquot of this resulting solution containing 1 mg of lipid was evaporated and the residue was redissolved in 0.3 ml of carbon tetrachloride containing 3.1 mg of osmium tetroxide (Wako Pure Chemicals, Tokyo). After standing 20 min to allow completion of the reaction, the solution was evaporated and the residue was redissolved in chloroform–methanol 20:1 (v/v). This material was applied to a column formed by placing 0.8 g of aluminum oxide (neutral alumina, Biorad Laboratories, Richmond, CA, U.S.A.) on a plug of glass wool in the neck of a disposable 9-in Pasteur pipette. An aliquot of DSPC was measured with the Nescoat PL kit-K (Nippon Shoji, Osaka, Japan).\(^10\)

Statistical Analysis
All statistical analysis was performed using the SAS statistical software package (SAS Institute, Cary, NA, U.S.A.). Results are expressed as the mean±S.E.M. of the indicated number of experiments. The ED\(_{50}\) value is the dose of YM-40461 producing a 50% recovery of MCT function. Either a Student’s t test (when there were only two groups) or a Dunnnett’s test (based on a one-way ANOVA, when there were more than two groups) was used to compare control-group means with treatment-group means. A value of p≤0.05 was considered significant.

Drugs
YM-40461, 1-(2-dimethylaminoethyl)-1-(3,4,5-trimethoxyphenyl)urea was prepared by Yamanouchi Pharmaceutical Co. (Tsukuba, Japan).

Ambroxol HCl, bromhexine HCl and salbutamol hemisulfate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RESULTS

Effect of Repeated SO\_2 Exposure on MCT Function in Guinea Pigs
In guinea pigs not exposed to SO\_2, MCT velocity was 6.3±0.3 mm/min. SO\_2 gas-exposure significantly lowered MCT velocity to 3.4±0.5 mm/min. MCT dysfunction in the SO\_2-exposed group lasted at least 5 days after the last SO\_2 exposure. MCT velocity at that time was 3.9±0.3 mm/min (Fig. 1).

Effect of Test Compounds on MCT Dysfunction in SO\_2-Induced Chronic Bronchitis Model
Oral treatment with YM-40461 at doses of 1, 3, 10 mg/kg induced a dose-dependent recovery of MCT function, with an ED\(_{50}\) value of 3.1 mg/kg 2 h after the last dosing. Maximum recovery was observed in animals that received 10 mg/kg of YM-40461 (98.2±20.8% recovery, Fig. 2). Ambroxol, a representative mucocactive drug, induced a 49.7±12.9% recovery of MCT function at 300 mg/kg p.o., but no dose-dependency was observed between the 300 and 600 mg/kg doses (Fig. 2). Bromhexine induced significant recovery of MCT function, the degrees of recovery being 24.7±9.3% and 23.8±1.6% at the doses of 100 and 300 mg/kg p.o., respectively, but again showed no dose-dependency. Salbutamol improved MCT function at doses of 6 mg/kg p.o. or more, but showed little dose-dependency (6 mg/kg: 41.2±10.7%; 20 mg/kg: 42.0±9.1% recovery; Fig. 2).

Effect of YM-40461 on Surface Tension and DSPC-to-Protein Ratio
Surface tension values were 50.1±0.7 and 55.8±0.8 dyn/cm in the non-exposed and SO\_2-exposed guinea pig group, respectively (p<0.05). YM-40461 at a dose of 10 mg/kg p.o. significantly reduced the surface tension increased by the SO\_2 exposure, with a surface tension value of 52.8±0.6 dyn/cm (Fig. 3).

There was no significant difference in DSPC-to-protein ratio in supernate of airway fluid between the non-exposed (0.10±0.03 μg DSPC/μg protein) and SO\_2-exposed (0.07±0.009 μg DSPC/μg protein) guinea pigs. YM-40461 at a
Fig. 2. Effects of YM-40461, Ambroxol, Bromhexine and Salbutamol on MCT Dysfunction in Guinea Pigs with SO₂-Induced Chronic Bronchitis

YM-40461 (●, 1, 3 or 10 mg/kg; ambroxol (●) 100, 300 or 600 mg/kg; bromhexine (▲) 100 or 300 mg/kg; salbutamol (■) 2, 6 or 20 mg/kg; or the vehicle alone (0.5% methylcellulose saline solution) were orally administered twice a day for 5 days beginning the day following the last SO₂-exposure. On the fifth day, MCT velocity was measured. 1 (salbutamol), 2 (YM-40461) or 3 (ambroxol, bromhexione) after the last dosing. The percent recovery of MCT velocity was obtained from the formula described in the Materials and Methods section. Values are expressed as mean±S.E.M. In the SO₂-exposed animals, the significance of the difference between the vehicle-treated and drug-treated groups was calculated using Dunnett’s multiple range test (*p<0.05, **p<0.01).

Fig. 3. Effect of YM-40461 on the Surface Tension of Airway Fluid

The open column indicates the surface tension in the airway fluid from non-exposed guinea pigs. The closed column indicates the surface tension in the airway fluid from bronchitis guinea pigs. The shaded column indicates the surface tension in airway fluid from the bronchitis guinea pigs treated with 10 mg/kg. p.o. doses of YM-40461. Values are mean±S.E.M. The significance of the differences between each pair of groups was calculated using the unpaired t-test (**p<0.001).

dose of 10 mg/kg p.o. significantly increased DSPC-to-protein ratio (0.257±0.07 µg DSPC/µg protein) compared with the SO₂ exposed group (p<0.05, Fig. 4).

DISCUSSION

SO₂ exposure in animals causes injury to the airway epithelium which mimics changes observed in COPD. COPD patients with a tidal expiratory flow limitation have MCT impairment in the airway and obstructive mucus hypersecretion. In the present study, a lasting alteration of MCT function and an increase in the protein contents of the airway fluid in SO₂ gas-exposed guinea pigs were observed. Therefore, this bronchitis model, induced by repeated SO₂ exposure, reflects the clinical symptoms of COPD patients and is useful for evaluation of compounds used to treat COPD.

Chest discomfort and airway obstruction observed in COPD patients may be mainly caused by impaired clearance of excess mucus caused by MCT dysfunction. The main factors influencing MCT function are ciliary motility and the physical properties of the airway fluid. Lusuardi et al. reported a marked decrease in total phospholipids in COPD patients compared with healthy subjects.

Pulmonary surfactant exists in thin layer lining the alveoli and terminal bronchioles. And surface active materials like pulmonary surfactant is also produced by both nonciliated bronchiolar cells and bronchial mucous glands. In many airway diseases, surfactant degradation is reflected by efficiency of mucociliary clearance and lymphatic drainage. YM-40461, a novel agent that promotes secretion of pulmonary surfactant, was discovered in the chemical modification study of ambroxol and our compounds library. As demonstrated in the present work, repeated administration of YM-40461 dose-dependently improves MCT function strongly. YM-40461 also lowers surface tension in airway fluid collected from animals with chronic bronchitis. There is an optimal rheological range of viscosity and elasticity of airway fluid well adapted to MCT function. The surface tension of airway fluid is regulated by surfactant materials, including surfactant. However other factors such as its ability to adhere to the airway wall and its transportability by MCT also determine the proper physiologic function of airway fluid. Airway fluid from YM-40461-treated animals exhibited significantly lower surface tension than that from the control animals. The alteration of the surface tension of the airway fluid by YM-40461 was accompanied by increased DSPC-to-protein ratio compared with the control group. Considering that YM-40461 has no activities on radical scavenging, LTs production and normal MCT function in our preliminary studies, these results indicate that changes in the physical properties of airway fluid, e.g. adhesiveness or viscoelasticity, produced by increased secretion of surfactant may aid in the recovery of MCT velocity.

Although there was no significant difference in the DSPC-to-protein ratio of the airway fluid between the non-exposed and SO₂-exposed guinea pig lungs, it is difficult to explain the relation between the surface tension and the DSPC-to-protein ratio as simply due to an increase in a protein that might inhibit surfactant activity. In this bronchitis model, physiological alteration of surfactant component may in fact

Fig. 4. Effect of YM-40461 on the DSPC-to-Protein Ratio of Airway Fluid

The open column indicates the surfactant activity in the airway fluid from non-exposed guinea pigs. The closed column indicates the surfactant activity in the airway fluid from bronchitis guinea pigs. The shaded column indicates the surfactant activity in the airway fluid from the bronchitis guinea pigs treated with a 10 mg/kg, p.o. dose of YM-40461. Values are mean±S.E.M. The significance of the differences between each pair of group was calculated using the unpaired t-test (*p<0.05).
affect surfactant activity. The hydrophobic components of surfactant, namely, phospholipid and surfactant proteins SP-B and SP-C, may contribute to the alteration of surfactant activity. Although these components were not quantified in the present study, it may be that YM-40461 ameliorates the alteration of the quantity of surfactant components.

In the present study, ambroxol, which has been reported to increase surfactant and mucus secretion, improved MCT at a low dose, but at a high dose failed to improve MCT. The failure of ambroxol may be due to hypersecretion of mucus which causes obstruction of the trachea, one of the non-selective side effects of ambroxol on airway secretion. Bromhexine, the mother compound of ambroxol, improved the MCT function at a dose of 300 mg/kg significantly. But the efficacy and potency of bromhexine are far less than that of YM-40461. Salbutamol had a weak effect on MCT at doses high enough to exhibit a bronchodilation effect.

Although these mucoregulating agents and bronchodilators are used for clinical treatment of COPD, they do not show as marked an effect as YM-40461 on alleviating MCT dysfunction in SO_2-induced bronchitis.

Taken together, previous and present findings suggested that YM-40461 improves MCT chiefly by increased DSPC-to-protein ratio, and that there is a close relation between the improvement of MCT function and the increased secretion of surfactant. Therefore, like YM-40461, compounds which increase surfactant and improve MCT function may relieve COPD patients from their chest discomfort and airway obstruction.

REFERENCES


Vol. 23, No. 12

64 (1992).


