Effect of 4[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]benzoic acid (Am80) on alveolar regeneration in adiponectin deficient-mice showing a chronic obstructive pulmonary disease-like pathophysiology

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2. Running Title Page.

Alveolar regeneration in COPD-like pathophysiology of mice

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Abbreviations

Am80, 4[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]benzoic acid; APL, acute promyelocytic leukemia; ATRA, all-trans-retinoic acid; COPD, chronic obstructive pulmonary disease; CRABP, cellular retinoic acid binding protein; CT, computed tomography; CYP, cytochrome P450 enzymes; DMSO, dimethyl sulfoxide; HU, Hounsfield unit; LAA, low attenuation area; Lm, mean linear intercept; RAR, nuclear retinoic acid receptor.

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3. Abstract.

Chronic obstructive pulmonary disease (COPD) is an intractable pulmonary disease that causes widespread and irreversible alveolar collapse. Although COPD occurs worldwide, only symptomatic therapy is currently available. The objective of the present study was the development of therapeutic agents to eradicate COPD. Therefore, we focused on 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl) carbamoyl] benzoic acid (Am80), which is a derivative of all-trans retinoic acid. We evaluated the effects of Am80 on alveolar repair in a novel COPD model of adiponectin-deficient mice. This mouse model has more symptoms similar to human COPD than the classic elastase-induced emphysema mouse model. Lung volume, CT values, low attenuation area (LAA) ratios, and bone and fat mass were measured by computed tomography. However, the administration of Am80 did not affect these results. To examine the degree of destruction in the alveoli, the mean linear intercept of the alveolar walls was calculated, and assessment of this value confirmed that there was a significant difference between the control (46.3 ± 2.3 μm) and 0.5 mg/kg Am80-treated group (34.4 ± 1.7 μm). All mice survived because of treatment that lasted for more than 6 months, and we did not observe any abnormalities in autopsies performed at 80 weeks of age. These results suggested that Am80 was effective as a novel therapeutic compound for the treatment of COPD.
5. Introduction

Introduction

Chronic obstructive pulmonary disease (COPD) leads to chronic airflow obstruction because of different causes such as smoking (Pauwels et al., 2001). COPD had been classified as emphysema or chronic bronchitis until the Global Initiative for Chronic Obstructive Lung Disease established the concept of the disease in 2001. According to the World Health Organization, COPD is expected to become the third largest cause of death in the world by 2030 (World Health Organization, COPD. Fact sheet No. 315, October 2013). Currently, no agent is available that can achieve a radical cure of the alveolar destruction caused by this disease (Feldman, 2013). As first-line drugs for symptomatic therapy, long-acting muscarinic antagonists, inhaled steroids or long-acting β2-agonists have been used (Karel, 2016; Rodrigo and Neffen, 2017). However, the rate of satisfaction with these agents for COPD is approximately only 40% (Massaro and Massaro, 1997), which indicates patient dissatisfaction with such treatments. A drug incorporating a new strategy of treatment for COPD is needed.

We have already shown that 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl) carbamoyl] benzoic acid (Am80) (Chemical formula: C_{22}H_{25}NO_{3}, CAS Number: 94497-51-5) can induce the differentiation of human alveolar epithelial progenitor cells into type I and II alveolar epithelial cells (Sakai et al., 2014). We also reported that Am80 showed clear improvement effects
on COPD in a mouse model of elastase-induced emphysema (Sakai et al., 2014). This is a pulmonary emphysema model that can be easily produced and has been widely used (Antunes and Rocco, 2011). However, this model is problematic in that it lacks long-term inflammation, and thus, the reduction of emphysema is also transient. Therefore, we need to focus on a model that better reflects the disease state of human COPD. It is thought that adiponectin-deficient mice are more suitable as a disease model of COPD than the elastase-induced emphysema model in regard to these points. However, the effect of Am80 on alveolar regeneration and systemic symptoms has not been evaluated. Adiponectin is a protein secreted from adipocytes (Ouchi et al., 1999). Because of its antiinflammatory effects and improvements in insulin resistance, it has been suggested to contribute to metabolic syndrome (Welty et al., 2016). Because a decrease in adiponectin also occurs in patients with COPD, adiponectin is strongly suggested to be associated with the pathogenesis of COPD (Couillin et al., 2009), which is still largely unknown. It is thought that the enhancement of apoptosis in endothelial cells caused by the decreased expression of vascular endothelial growth factor-2 and platelet endothelial cell adhesion molecule-1 is responsible for the development of COPD-like symptoms in adiponectin-deficient mice (Nakanishi et al., 2011).

In the present study, we used adiponectin-deficient mice as a new disease model of COPD and examined the effects of Am80 on alveolar regeneration.
6. Materials and Methods

6-1. Reagents and animals

Am80 was a gift from Dr. Koichi Shudo of the Itsu Institute (Tokyo, Japan). Physiological saline was purchased from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan), and ethanol and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Isoflurane used for anesthesia in the mice were purchased either from Intervet KK (Osaka, Japan) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

The adiponectin-deficient mouse (apM1 Homo) was originally created in the Osaka University School of Medicine. The mice were transferred and have been maintained at CLEA Japan, Inc. (Tokyo, Japan). The male and female mice were provided by CLEA Japan Inc., and they were bred in our laboratory. All animal care and use procedures were approved by the Tokyo University of Science Ethics Committee (Approval Numbers Y12058, Y13008, 1542). Male mice were used in the experiments. After evaluating the development of emphysema in the adiponectin-deficient mice, we carried out pulmonary administration with the method described below in a control group (6 mice) and an Am80-treated group (5 mice).

The method of pulmonary administration was reported in our earlier papers (Horiguchi et al., 2015; Horiguchi et al., 2016). Pulmonary administration was carried out with a gastric tube for oral administration (No. KN-348, for mouse; Natsume Seisakusho Co., Ltd., Tokyo, Japan). The gastric
tube was equivalent in diameter to the mouse airway. Using the Mouse Intubation Platform-Model MIP (Penn-Century, Inc., Wyndmoor, PA, USA) as a mouse retainer for pulmonary administration, the front teeth of the mouse were retained in the retaining position approximately at a 90° angle to facilitate tracheal access of the tube. The airway was confirmed with the Small Animal Laryngoscope-Model LS-2 (Penn-Century, Inc.) as a tracheal endoscope for the mouse, the tube was inserted to the airway, and the drug solution was administered in synchronization with the air intake of the mouse.

6-2. Evaluation by computed tomography (CT)

Lung volume, CT values, and low attenuation area (LAA) ratios in mice were measured using an animal experimental X-ray CT apparatus (Latheta LCT-200, Hitachi Aloka Medical, Ltd., Tokyo, Japan). The machine was automatically calibrated by computer software. Mice were anesthetized by the continuous inhalation of isoflurane. For imaging, the field of view was set at 48 mm, and slice thickness was set at 192 μm. The lung volume, average CT value, and their standard deviations were calculated with the following settings. The range of CT values (Hounsfield unit; HU) was set to −1000 to −200 HU for detecting the region of interest. The radius of noise removal was set at 3 pixels. The LAA was calculated with the range of CT values previously described (−871 to −610 HU) (Kobayashi et al., 2013). We used the default setting of the machine to calculate bone and fat.
mass. Slice thickness was 192 μm in the abdomen and 96 μm in the femoral region. The scan range of the abdomen was set from the bottom of the ribs to the root of both thighbones.

In addition to the CT image analysis, lung CT images were used to create 3D models by Amira, a system for 3D visualization and analysis (Visualization Sciences Group, Burlington, MA, USA). LAA sites in the emphysema area were visualized in red.

6-3. Evaluation of alveolar wall distance using histochemistry

After the mice were euthanized by an overdose of isoflurane, lung tissue was removed and fixed with 4% paraformaldehyde/phosphate buffered saline. The fixed lung tissue was embedded with TissueTech OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan). Frozen sections with a thickness of 10 μm were prepared using a CM3050S cryostat (Leica Microsystems GmbH, Wetzlar, Germany) and then stained with hematoxylin and eosin. The stained sections were examined at any five points with both 4× and 20× magnification using an HS All-in-one Fluorescence Microscope (Keyence Corp., Osaka, Japan). To examine the degree of destruction in the alveoli, the mean linear intercept (Lm) of the alveolar walls was calculated on the image taken at 20× magnification. We used ImageJ (National Institutes of Health, MD, USA) for this analysis. Lm was calculated by applying the equation to five different images. The five different images were chosen from each of three sections of lung (upper, middle, and lower parts).
Mean linear intercept of alveolar walls (μm) = Number of intersections of the grid lines \times \text{lattice width (50 μm)} / \text{the number of alveoli in the field}

6-4. Evaluation of alveolar repair by long-term administration of Am80 in adiponectin-deficient mice

We used male adiponectin-deficient mice with a C57BL/6J genetic background. We monitored the time course of the degree of emphysema from 5 to 30 ± 1 weeks of age. We justified the use of the adiponectin-deficient mouse model by comparing it to the degree of emphysema in the elastase-induced emphysema model.

Am80 (0.5 mg/kg) was dissolved in 5% DMSO/saline solution. We gave 50 μl of Am80 solution via pulmonary administration twice a week to the mice. These mice were at 50 ± 2 weeks of age and had advanced emphysematous lesions. Control mice received 50 μl of 5% DMSO/saline solution. At 78–82 weeks of age, all mice were euthanized by an overdose of isoflurane. Lung tissue was removed and fixed with 4% paraformaldehyde/phosphate buffered saline.

6-5. Statistics

For each parameter measured, the values from individual samples were averaged, and the S.E. was
calculated. Data were compared using the unpaired \( t \)-test when only two groups were compared. The Dunnet method was used to evaluate differences between three or more groups. A 5% probability was considered significant.
7. Results

7-1. Time course for the development of emphysema in adiponectin-deficient mice

The elastase-induced emphysema model is a typical COPD disease model that has been used to evaluate the effect of alveolar repair. However, the pathogenesis of emphysema in this model is different from that of human COPD. Moreover, it does not exhibit any systemic symptoms. In contrast, the adiponectin-deficient mouse shows emphysematous symptoms with age. This mouse also exhibits systemic symptoms, such as increased inflammatory cytokines and decreased bone mass (Nakanishi et al., 2011). Because of these observations, we decided to use this mutant mouse to evaluate the development of emphysema. We evaluated CT values every 5 weeks from 5 to 30 weeks of age.

According to previous reports, in the elastase-induced emphysema mouse model, the administration of elastase (4.05 EU) twice a week significantly increased the CT value: from $-408 \pm 7$ HU 2 weeks before injection to $-437 \pm 8$ HU 2 days after injection, $P < 0.05$. On the basis of this result, we set the CT value of $-450$ HU as being representative of the onset of emphysema. At 5 weeks of age, the CT value of the adiponectin-deficient mice was $-420 \pm 4$ HU, which did not reach the set threshold for emphysema. The CT value in mice of 20 weeks of age or older dropped to less than $-450$ HU. Thus, the adiponectin-deficient mice exhibited moderate progression of emphysema along with age (Figure 2A). Lung volume was also increased with age (Figure 2B). As with the CT
value, a gradual change in LAA% was observed, which indicated moderate progression of emphysema with age (Figure 2C, D). Bone mass of the chest gradually increased up to 20 weeks of age because of growth and became stable without any significant fluctuations (Figure 2E). Body weight increased from 19 ± 1 g at 5 weeks of age to 31 ± 1 g at 20 weeks of age. After 20 weeks of age, there was no significant increase in body weight. The LAA% data are representative of two individual experiments, and the other data are representative of one individual experiment.

7-2 Effect of long-term administration of Am80 on alveolar repair

From a dose-determination study for Am80, we found that 0.5 mg/kg is the highest dose that can be administered without mortality and obvious adverse events. We chose as the model animal the adiponectin-deficient mouse because it exhibits moderate progression of emphysema and systemic symptoms, such as reduction of bone mass with age. Therefore, we evaluated the effects of the long-term administration of 0.5 mg/kg of Am80 on alveolar repair by calculating CT or Lm values in these mice.

We measured the lung volume and CT value in both Am80 and control animals at 80 weeks of age. The lung volume was 0.748 ± 0.023 cm³ in the control group and 0.705 ± 0.018 cm³ in the Am80-treated group. The CT value was −496 ± 6 HU in the control group and −484 ± 6 HU in the Am80-treated group. For LAA%, the values were 9.90 ± 1.8% in the control group and 7.12 ±
0.92% in the Am80 group (Figure 3A). Cortical bone, bone surface, total bone, and planar bone densities were not significantly different in the chest, abdomen, and femur between treatments (Figure 3B).

Total fat amounts in the abdomen, visceral fat mass, and subcutaneous fat mass were also not significantly different between the treatments (Figure 3C). The percentage of fat in the abdomen was 16.8 ± 4.2% in the control and 15.2 ± 1.4% in the Am80-treated group, whereas that in the femur was 20.5 ± 3.6% in the control group and 16.8 ± 1.4% in the Am80-treated group.

At the beginning of the treatment at 52 weeks of age, the body weight was 37 ± 2 g in the control group and 36 ± 1 g in the Am80-treated group. At the end of the treatment at 80 weeks of age, it was 36 ± 2 g in the control group and 34 ± 0.4 g in the Am80-treated group. Although the body weight was observed to slightly decrease before 80 weeks of age, we did not observe any individual whose body weight decreased to approximately 25 g, as reported previously (Nakanishi et al., 2011).

The length of Lm was 46.3 ± 2.3 µm in the control group and 34.4 ± 1.7 µm in the Am80-treated group. Because this difference was statistically significant, we concluded that Am80 has a significant effect on alveolar regeneration (Figure 4). It should be noted that all mice survived because of the treatment that lasted for more than 6 months, and we did not observe any abnormalities in the autopsy performed at 80 weeks of age. The LAA% data are representative of two individual experiments, and the Lm data are representative of three individual experiments. Other data are
representative of one individual experiment.
8. Discussion

Although COPD occurs worldwide, only symptomatic therapy is currently available. The development of a therapeutic drug to eradicate COPD was the objective of the present study.

All-trans-retinoic acid (ATRA) is known to have an effect on alveolar repair in COPD (Massaro and Massaro, 1997). Am80 is a derivative of ATRA and yet is more stable than ATRA. In the present study, we evaluated the effects of Am80 on alveolar repair in adiponectin-deficient mice, and we give our conclusions below.

First, we administered Am80 to adiponectin-deficient mice, which is a novel COPD model. This mouse model has more symptoms similar to human COPD than the classic elastase-induced emphysema mouse model. It exhibits COPD-related systemic symptoms, such as emphysema, bone loss, and fat mass loss (Nakanishi et al., 2011). The progression of emphysema symptoms was observed between 5 and 30 weeks of age (Figure 2A–D). This result is similar to that of previous studies. Although a decrease in body weight and bone mass with age has been reported, we could not reproduce this finding (Figure 2E). Although the total bone density at 30 weeks of age was $455 \pm 9 \text{ mg/cm}^3$, that at 80 weeks of age was $445 \pm 15 \text{ mg/cm}^3$ in the control group and $438 \pm 5 \text{ mg/cm}^3$ in the Am80-treated group. The difference between 30 and 80 weeks of age was not statistically significant (Figure 3B). The total bone density in the femur was $546 \pm 13 \text{ mg/cm}^3$ in the control group and $546 \pm 10 \text{ mg/cm}^3$ in the Am80-treated group, which is essentially the same as the data in
previous reports. Our results suggest that the adiponectin-deficient mouse is a useful COPD model because of the high reproducibility of spontaneous emphysema and bone loss. We did not observe any significant effect on systemic symptoms, such as bone or fat mass loss, in the Am80-treated group. However, we found significant improvement in alveolar regeneration as indicated by the Lm value (Figure 4). In previous reports, correlations between micro-CT and histopathological findings were observed, but not with complete consistency (Kawakami et al., 2008; Saito and Murase, 2012). We thought that 0.5 mg/kg Am80 showed mild potency for the repair of collapsed alveoli and that this effect was observed only in the histological data.

We conducted pulmonary administration of elastase in mice in a previous study. In fact, CT imaging performed after 1 week of elastase administration indicated that the drug solution was delivered to the ends of the lungs (Horiguchi et al., 2015). Using this method of administration, we confirmed a significant improvement in Lm by Am80 treatment. The results of the measurement of tissue levels of Am80 showed a level in lung tissue of 10.9 ng/mg and that in blood of 0.447 μg/mL, indicating a higher level of Am80 in lung than in blood. In the present study, we found that Am80 improves alveolar regeneration in adiponectin-deficient mice, which is a new COPD mouse model. Taken together from the above results, our findings indicate that Am80 may be a new therapeutic agent for the radical cure of COPD.

Am80 was kindly provided by Dr. Koichi Shudo (Japan Pharmaceutical Information Center).
10. Authorship Contributions.

Participated in research design: Hitomi Sakai, Michiko Horiguchi, Tomomi Akita, Chikamasa Yamashita

Conducted experiments: Hitomi Sakai, Mai Hirokawa, Yuki Oiso, Harumi Kumagai

Wrote or contributed to the writing of the manuscript: Michiko Horiguchi, Tomomi Akita, Chihiro Ozawa, Yoshito Takeda, Isao Tachibana, Norikazu Maeda, Chikamasa Yamashita
11. References.


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12. Footnotes.

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13. Figure Legends.

**Fig. 1.** Chemical structure of Am80.

**Fig. 2.** Micro-CT assessment revealed that adiponectin-deficient mice showed progressive COPD-like phenotype development. (A) The average CT values, (B) average lung volumes (C) and average LAA%. (D) CT image and three-dimensional image by integrating CT images at 30 weeks of age (LAA% is 1.91%). The LAA (−871 to −610 HU) is colored in red, and the whole lung field (−1000 to −200 HU) is colored in blue. (E) Overall, cortical, spongy and planar bone densities of the chest. Data represent the mean ± S.E. (n = 5–15).

**Fig. 3.** CT assessment of the repair of lung emphysema in adiponectin-deficient mice at 80 weeks of age. (A) The average CT values, lung volumes and LAA%. (B) The overall, cortical, spongy and planar bone densities of the chest, abdomen and femur. (C) The volume of muscle and total, visceral and subcutaneous fat of the abdomen and femur in control (5% DMSO-treated, n = 6) and 0.5 mg/kg Am80-treated mice (n = 5). Data represent the mean ± S.E.
Fig. 4. Lung emphysema repaired with Am80 in adiponectin-deficient mice at 80 weeks of age by intercept assessment. Lung sections and the mean distance between alveolar walls (Lm) from control (5% DMSO-treated, n = 6) and 0.5 mg/kg Am80-treated mice (n = 5) are shown. Sections were stained with hematoxylin and eosin. Scale bar = 50 μm. Data represent the mean ± S.E., *p < 0.05.
15. Figures.

Figure 1
Figure 2

A

Average of CT value (HU)

Weeks of age

5 10 15 20 25 30

-550

-500

-450

-400


B

Lung volume (cm³)

Weeks of age

5 10 15 20 25 30

0.4

0.5

0.6

0.7

0.8


C

% of low-attenuation area (LAA)%

Weeks of age

5 10 15 20 25 30

0

5

10


D

Dorsal

Ventral


E

Bone density (mg/cm²)

Weeks of age

5 10 15 20 25 30

0

100

200

300

400

500

600

- All
- Cortical bone
- Spongy bone
- Planar bone
Figure 3

A

B

C
Figure 4

[Diagram showing control and Am80 0.5 mg/kg comparisons with mean linear intercept (μm) on the y-axis and condition on the x-axis.]