Study Protocol

Study Title: Effects of Compound A on Respiratory Function in Rats

Study Number: CJ16050

Testing Facility: Drug Safety Research,
Kaku Pharmaceutical Co., Ltd.
1-1-1, Dokoka-cho, Chiyoda-ku, Tokyo, Japan

SIGNATURE OF STUDY PERSSONEL

Study Title: **Effects of Compound A on Respiratory Function in Rats**
Study Number: **CJ16050**

Study Director:

Taro SENDO, DVM, PhD	Date

Test Facility Management:

Ittetsu UNKAN, PhD	Date

1. STUDY PURPOSE

The purpose of this study is to assess the effects of Compound A on respiratory function when once administered orally to rats using the relocated system (Respiratory DSI Whole Body Plethysmography).

2. ANIMAL WELFARE

Before the beginning of study, Laboratory Animal Care and Use Committee at Kaku Pharmaceuticals Co., Ltd. had approved this study plan. This study is performed in compliance with Laboratory Animal Policy at Kaku Pharmaceutical Co., Ltd.

3. STUDY PERIOD

Animal Receipt:	29 November, 2016
Study Initiation Date:	6 December, 2016
Dosing Date:	7, 8 or 9 December, 2016
Study Completion Date:	February, 2017

4. PERSONNEL INVOLVED IN THE STUDY

Study Director	:	Taro Sendo
Test Article Supervisor	:	Jiro Kamei

5. MATERIALS AND METHODS

5.1 TEST ARTICLE

5.1.1 Chemical Name

Compound A

5.1.2 Lot Number

ABC123

5.1.3 Source

Kaku Pharmaceuticals Co., Ltd., Japan

5.1.4 Purity

100.3%

5.1.5 Stability

Not confirmed

5.1.6 Storage Condition

The test article is stored in a light resistant tight container at room temperature.

5.2 CONTROL ARTICLE

5.2.1 General Name

0.5w/v% methylcellulose 400 solution

5.2.2 Source

Wako Pure Chemical Industries, Ltd., Japan

5.2.3 Specification

For biochemistry use

5.3 PREPARATION OF DOSING FORMULATIONS

5.3.1 Preparation Method

Compound A is suspended in vehicle by a magnetic stirring and sonication.

5.3.2 Preparation Frequency

Prior to use at the dosing day

5.3.3 Stability and Homogeneity

Unknown

5.3.4 Confirmation of the Concentration

Not to be conducted

5.3.5 Storage

Not applicable due to the preparation at the time of use

5.4 ANIMALS

5.4.1 Species/Strain

Sprague-Dawley Rat (Crl:CD(SD))

5.4.2 Rationale for Selection

Male rats are widely used as a test system for evaluating the effect on respiratory function, and SD rats are used as experimental animals in various fields and have experience.

5.4.3 Number of Animals Supplied

20, male

5.4.4 Sex, Age and the Range of Body Weight

Sex	Upon shipment		At the time of start dosing
	Age (weeks of age)	Body Weight (g)	Age (weeks of age)
Male	7	215～245	8

5.4.5 Source

Hino Breeding Center, Charles River Laboratories Japan, Inc., Japan

5.4.6 Animal Selection and Allocation

Animal number is assigned in order of temporary animal number for animals without abnormalities during the quarantine and acclimatization periods (Date: December 6, 2016). Animals not assigned at the time of allocation are reserve animals and excluded from the study after the final administration.

5.4.7 Animal Identification Method

Before allocation each animal is identified with the abbreviation of the temporary animal number marked on the tail (coloring method with oily ink), and the temporary animal number is displayed in the cage.
After allocation each animal is identified with the abbreviation of the animal number marked on the tail (coloring method with oily ink), and the animal number is displayed in the cage.
The reserve animals are identified with the abbreviation of the temporary animal number, and

the temporary animal number is displayed in the cage until the exclusion from the study.

5.5 ANIMAL HUSBANDRY

Temperature (acceptable range):	20 to 26°C
Humidity(acceptable range):	35 to 75%
Lighting:	12-hour/day (7:00 to 19:00)
Ventilation:	Ventilate with clean air (about 10 times / hour)
Cage type:	Stainless steel cages (W160 × D340 × H200 mm)
Rack type:	Stainless steel racks
Diet:	CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan). Each rat is fed 6 pieces (approximately 3.5 g per piece) once daily. On the day of administration, rats are fed after the measurement of respiratory function one hour after administration. For all lots to be used, the certificate of analysis is obtained, and it is confirmed that there is no influence on the study.
Water:	Sterilized tap water from an automatic water dispenser, ad libitum. Inspections that comply with the water quality standards of the Waterworks Law are conducted twice a year at external analytical facilities and it is confirmed that there is no influence on the study.

5.6 QUARANTINE AND ACCLIMATION

Period:	November 29, 2016 to December 5, 2016
Room No.:	1113
Number of animals per cage:	One animal per cage
Quarantine:	General observation, weight and food intake are measured, and health condition of the animals is evaluated.

5.7 ENVIRONMENT AT EXPERIMENT

Period:	December 6 to 9, 2016
Animal room No.:	1113
Laboratory No.:	1111
Number of animals per cage:	One animal per cage

5.8 DOSING

5.8.1 Dosing Route and Rationale for Selection

Dosing route:	Oral by gavage
Rationale:	The effect by oral administration has been confirmed.

5.8.2 Dosing Period, Dosing Frequency and Rationale for Selection

Dosing period:	Single The animal with the last letter of the animal number are 01 and 02: December 7, 2016 The animal with the last letter of the animal number are 03 and 04: December 8, 2016 The animal with the last letter of the animal number are 05 and 06: December 9, 2016
Withdrawal period:	Not applicable
Frequency:	Once
Rationale:	The effect by oral administration has been confirmed and the dosing period is single based on Guidelines for Safety Pharmacology Studies (Notification No. 902, Jun. 21, 2001, Ministry of Health, Labour and Welfare, Japan).

5.8.3 Method of Dosing and Rationale for Selection

Method of dosing:	Each animal is given orally using a sterile plastic syringe and a gavage tube.
Rationale:	A predetermined volume can be reliably administered.

5.9 STUDY DESIGN

5.9.1 Organization of Study Group

The study design is shown in the following Table:

Group No.	Dose Group (mg/kg)		Concentration (mg/mL)	Volume (mL/kg)	Number of Animals (Animal Numbers)
00	Control	0	0	10	6 (00M01-00M06)
01	Compound A	100	10	10	6 (01M01-01M06)
02	Compound A	1000	100	10	6 (02M01-02M06)

Animals in the control group were given vehicle.

5.9.2 Dose Justification

The effects of Compound A for respiratory functions are unknown. The dose levels of 1000 and 100 mg/kg were therefore selected as high and low dose levels, respectively.

5.9.3 Dosing Volume

The dosing volume (mL) for each individual rat is calculated by rounding up the second digit after the decimal point based on the body weight measured on the dosing day.

5.10 MEASUREMENTS AND OBSERVATIONS

The first day of dosing (07 Dec 2016) is designated as Day 1. Data on excluded animals should not be reflected in the final report.

5.10.1 Clinical Signs

Clinical signs are checked for all animals at least once a day in the experimental period. On the day of dosing, clinical signs are checked before and after administration.

5.10.2 Respiratory Function

The respiratory function is measured using the method of whole body unrestrained plethysmograph (Respiratory DSI Whole Body Plethysmography, DSI, USA). To ensure acclimation to the chamber for the measurement, all surviving animals are put into a chamber (1 animal per chamber) for about 30 minutes on the day before administration. On the day of dosing, the rats are placed in chambers about 30 minutes before each measurement time. The data for 3 minutes, which are stable with less influence by moving of rats, are selected out of 5 or 6 minutes before and after each measurement time. The average value of the data for 3 minutes is used for evaluation. If continuous measurement data for 3 minutes cannot be obtained, continuous data of 30 seconds or more is extracted within the measurement time range and added from the longest time in order for 3 minutes (in the case of more than 3 minutes as a result of the summation, the portion closer to the start time for each piece of data is added in units of 30 seconds to make it 3 minutes). Regarding the measurement before administration, the range of the measurement time is not specifically defined. As the respiratory function, respiratory rate (breaths/min) and tidal volume (mL/breath) are measured before and 1, 2, 4, and 8 hours after administration, and minute volume (mL/min) is calculated by multiplying tidal volume by respiratory rate.
After completion of the measurement on each dosing day, all used animals are euthanized by CO_2 gas asphyxia.

The animal number, date of dosing / measurement and the measurement chamber are assigned as shown in the following table.

Chamber No. and Animal No.

Chamber No.	Dates of dosing and measurement		
	07 Dec 2016	08 Dec 2016	09 Dec 2016
A	00M01		01M05
B	00M02		01M06
C	01M01	02M03	
D	01M02	02M04	
E	02M01	01M03	00M05
F	02M02	01M04	00M06
G		00M03	02M05
H		00M04	02M06

5.11 STATISTICAL ANALYSIS

The test results for respiratory rate, tidal volume and minute volume are expressed as mean ± S.D. The parametric Dunnett's test is used to examine the significance of differences between the control and treatment groups at each measurement point, with findings of $P<0.05$ (two-sided) considered significant.

6. ARCHIVES

All records and the final report are stored in the archives of Drug Safety Research, Kaku Pharmaceutical Co., Ltd., 1-1-1, Dokoka-cho, Chiyoda-ku, Tokyo, Japan.

7. DATA COLLECTION METHODS

Data will be collected using worksheets, experiment notes or measurement instrument.