FINAL REPORT

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

Study Number: CJ16050

Study Initiation Date: 28 November 2016

Study Completion Date: 16 February 2017

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

STATEMENT OF STUDY DIRECTOR

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

I declare that the following report constitutes a true and faithful account of the procedures adopted and the results obtained in the performance of the study.

Study Director: _____ _____
Taro SENDO, DVM, PhD Date

Drug Safety Research,
Kaku Pharmaceutical Co., Ltd.

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1. SUMMARY

1.1 INTRODUCTION

The purpose of this study was to assess the effects of Compound A on respiratory function when once administered orally to rats.

1.2 STUDY DESIGN

Compound A (Lot number: ABC123) was administered orally, by gavage, to male Sprague-Dawley rats (6 animals/group) at single doses of 100 mg/kg (10 mL/kg). A control group received an equivalent volume of vehicle (0.5% methylcellulose solution, 10 mL/kg).

1.3 MEASUREMENTS

The following parameters were evaluated: respiratory function (respiratory rate, tidal volume, and minute volume). The respiratory function was measured before and 1, 2, 4, and 8 hours after administration using the whole body plethysmography.

1.4 RESULTS

At 100 mg/kg, there were significant increases in respiratory rate and minute volume at 1 to 8 hours after administration.
At 1000 mg/kg, there were significant decreases in respiratory rate, tidal volume, and minute volume at 2 to 4, 1 to 8, and 2 to 8 hours after administration, respectively.

1.5 CONCLUSION

Compound A was administered orally, by gavage, as a single dose of 100 or 1000 mg/kg to male Sprague-Dawley rats to evaluate the effects on respiratory function. Accelerative respiratory effects were induced at 100 mg/kg and suppressive respiratory effects were induced at 1000 mg/kg.

2. INTRODUCTION

The purpose of this study was 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).

3. STUDY PERIOD

Study Initiation Date:	28 November 2016
Animal Receipt:	29 November, 2016
Study Initiation Date:	6 December, 2016
Dosing Date:	7, 8 and 9 December, 2016
Study Completion Date:	16 February 2017

4. MATERIALS AND METHODS

4.1 TEST ARTICLE

Chemical name:	Compound A
Lot number:	ABC123
Source:	Kaku Pharmaceuticals Co., Ltd., Japan

4.2 CONTROL ARTICLE

General name:	0.5w/v% methylcellulose 400 solution
Lot number:	ECE7022
Source:	Wako Pure Chemical Industries, Ltd., Japan
Specification:	For biochemistry use

4.3 PREPARATION OF DOSING FORMULATIONS

Vehicle:	0.5w/v% methylcellulose 400 solution
Preparation method:	Compound A were suspended in vehicle at concentrations of 10 and 100 mg/mL.
Preparation frequency:	3 times (prior to use)

4.4 ANIMALS

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 was performed in compliance with Laboratory Animal Policy at Kaku

Pharmaceutical Co., Ltd.

Species/Strain: Rat/Sprague Dawley (Crl:CD(SD))

Sex/Age at supply: Male/7 weeks old

Source: Charles River Laboratories Japan, Inc., Japan

Receipt date: 29 Nov 2016

Number of animals supplied: 20 males

Quarantine and acclimatization periods: Period: 29 Nov 2016 to 05 Dec 2016
The following were examined: daily clinical signs (all animals), body weights and food consumption on the first and last days of the period (selected 5 animals).

Abnormalities during quarantine or acclimatization period: None

Number of animals used: 18 males

Age at initiation of dosing: 8 weeks old

Body weight at dosing: 239.2 to 273.7 g

Animal assignment method: Animal number was assigned in order of temporary animal number for animals without abnormalities during the quarantine and acclimatization periods. Animals that were not assigned to groups were excluded from the study on 09 Dec 2016 and their records were filed separately.
Each animal was assigned to test chambers as 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

* Defined as Day 1

Animal identification method: Quarantine and acclimatization periods: An abbreviated temporary animal number was marked on the tail with ink. Temporary animal number was indicated on a cage card.
Experimental period (after grouping): An abbreviated animal number was marked on the tail with ink. Animal number was indicated on a cage card.

4.5 ANIMAL HUSBANDRY

Diet: Solid chow CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan), approximately 21 g/day

Water: Sterilized tap water from an automatic water dispenser, ad libitum

Temperature: Set at 20 to 26°C

Humidity: Set at 35 to 75%

Housing: Single housing in steel cages, in clean air room with 12-hour light cycles

4.6 STUDY DESIGN

Dosing dates: 07, 08, or 09 Dec 2016
The study design is shown in the following Table:

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

Animals in the control group were given vehicle.

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

4.7 DRUG ADMINISTRATION

Route: Oral by gavage

Frequency: Once

Dosing period: 1 day

Dosing volume: 10 mL/kg body weight. The dosing volume for each rat was calculated based on the body weight measured on the dosing day.

4.8 MEASUREMENTS AND OBSERVATIONS

4.8.1 Clinical Signs

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

4.8.2 Respiratory Function

The respiratory function was 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 were put into a chamber (1 animal per chamber) for about 30 minutes on the day before administration. On the dosing day, the rats were placed in chambers about 30 minutes before each measurement time. The data for 3 minutes, which were stable with less influence by moving of rats, were selected out of 5 or 6 minutes before and after each measurement time. The average value of the data for 3 minutes was used for evaluation. For all animals in Group No. 1 (compound A 100 mg/kg group) and Animal No. 02M05, the data sometimes could not be obtained for 3 minutes due to unstable conditions and the average values were calculated with the available data for multiples of 30-second periods (ie, the duration of the data was 30 seconds, 1, 1.5, 2, or 2.5 minutes) in those animals; especially for Animal No. 01M02, no data could be obtained at 2 hours after administration.
As the respiratory function, respiratory rate (breaths/min) and tidal volume (mL/breath) were measured before and 1, 2, 4, and 8 hours after administration, and minute volume (mL/min) was calculated by multiplying tidal volume by respiratory rate.
After completion of the measurement on each dosing day, all used animals were euthanized by CO_2 gas asphyxia.

4.9 STATISTICAL ANALYSIS

The test results for respiratory rate, tidal volume and minute volume are expressed as mean ± S.D. The parametric Dunnett's test was 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. The analysis was performed using the software package GraphPad Prism.

4.10 UNFORESEEABLE EVENTS AND DEVIATIONS FROM THE PROTOCOL

There were no significant deviations in animal husbandry including temperature and humidity in the animal room during the quarantine, acclimatization and experimental periods. There were no deviations from the SOPs that compromised the validity or integrity of the study.

4.11 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.

5. RESULTS AND DISCUSSION

5.1 CLINICAL SIGNS

(Table 1 and Appendices 1-1 to 1-3)

Decreased activity and scrotum relaxation were observed in all animals at 1000 mg/kg. No abnormalities were observed at 100 mg/kg.

5.2 RESPIRATORY FUNCTION

(Tables 2 to 4 and Appendices 2 to 4)

At 100 mg/kg, there were significant increases in respiratory rate and minute volume at 1 to 8 hours after administration.
At 1000 mg/kg, there were significant decreases in respiratory rate, tidal volume, and minute volume at 2 to 4, 1 to 8, and 2 to 8 hours after administration, respectively.

6. CONCLUSION

Compound A was administered orally, by gavage, as a single dose of 100 or 1000 mg/kg to male Sprague-Dawley rats to evaluate the effects on respiratory function. Accelerative respiratory effects were induced at 100 mg/kg and suppressive respiratory effects were induced at 1000 mg/kg.

Table 1 Clinical signs

Study No.: CJ16050				Study: Respiratory Function					
Species: Rat		Strain: Crl:CD(SD)		Route: p.o.				Sex: Male	
Treatment		Days							
			-1	1		2		3	
	Finding			pre	post	pre	post	pre	post
Control		-	6	6	2	4	2	2	2
		Total	6	6	2	4	2	2	2
Compound A		-	6	6	2	4	2	2	2
100 mg/kg		Total	6	6	2	4	2	2	2
Compound A		-	6	6	0	4	0	2	0
1000 mg/kg	Decreased activity	+	0	0	2	0	2	0	2
	Scrotum relaxation	+	0	0	2	0	2	0	2
		Total	6	6	2	4	2	2	2

-: No abnormality, +: Positive
Note: On the treatment day, clinical signs were observed pre- and post-administration.

Table 2 Respiratory rate

Study No.: CJ16050				Study: Respiratory Function		
Species: Rat		Strain: Crl:CD(SD)		Route: p.o.		Sex: Male
Treatment		Time after administration (hours)				
		pre	1	2	4	8
Control	N	6	6	6	6	6
	Mean	91.7	94.8	112.5	118.1	106.7
	SD	18.0	21.3	14.8	22.1	11.9
Compound A	N	6	6	5	6	6
100 mg/kg	Mean	89.5	186.2 *	164.9 *	184.8 *	171.8 *
	SD	12.3	10.0	19.3	27.4	29.9
Compound A	N	6	6	6	6	6
1000 mg/kg	Mean	89.3	102.1	85.2 *	79.2 *	82.2
	SD	15.6	12.9	13.2	7.7	6.0

*: $P<0.05$, comparison with the control group (parametric Dunnett's test) (breaths/min)

Table 3 Tidal volume

Study No.: CJ16050				Study: Respiratory Function		
Species: Rat		Strain: Crl:CD(SD)		Route: p.o.		Sex: Male
Treatment		Time after administration (hours)				
		pre	1	2	4	8
Control	N	6	6	6	6	6
	Mean	1.379	1.454	1.568	1.632	1.520
	SD	0.141	0.097	0.174	0.102	0.128
Compound A	N	6	6	5	6	6
100 mg/kg	Mean	1.557	1.494	1.504	1.547	1.477
	SD	0.162	0.261	0.113	0.177	0.255
Compound A	N	6	6	6	6	6
1000 mg/kg	Mean	1.423	1.050 *	1.040 *	0.998 *	0.978 *
	SD	0.318	0.181	0.196	0.251	0.272

*: $P<0.05$, comparison with the control group (parametric Dunnett's test) (mL/breath)

Table 4 Minute volume

Study No.: CJ16050				Study: Respiratory Function		
Species: Rat		Strain: Crl:CD(SD)		Route: p.o.		Sex: Male
Treatment		Time after administration (hours)				
		pre	1	2	4	8
Control	N	6	6	6	6	6
	Mean	126.7	136.8	176.6	191.3	162.4
	SD	24.9	21.6	32.2	34.8	24.2
Compound A	N	6	6	5	6	6
100 mg/kg	Mean	134.5	269.6 *	243.8 *	288.4 *	257.3 *
	SD	9.6	40.7	36.6	65.8	60.0
Compound A	N	6	6	6	6	6
1000 mg/kg	Mean	121.4	106.0	88.6 *	78.9 *	81.6 *
	SD	18.0	17.8	17.3	22.4	24.4

*: $P<0.05$, comparison with the control group (parametric Dunnett's test) (mL/min)

Appendix 1-1 Clinical signs

Study No.: CJ16050			Study: Respiratory Function							
Species: Rat		Strain: Crl:CD(SD)	Route: p.o.						Sex: Male	
Treatment	Control	Days								
			-1	1		2		3		
Animal No.	Finding			pre	post	pre	post	pre	post	
00M01			-	-	-	NA		NA		
00M02			-	-	-	NA		NA		
00M03			-	-	NA	-	-	NA		
00M04			-	-	NA	-	-	NA		
00M05			-	-	NA	-	NA	-	-	
00M06			-	-	NA	-	NA	-	-	

-: No abnormality, NA: Not applicable

Note: On the treatment day, clinical signs were observed pre- and post-administration.

Appendix 1-2 Clinical signs

Study No.: CJ16050			Study: Respiratory Function							
Species: Rat		Strain: Crl:CD(SD)	Route: p.o.						Sex: Male	
Treatment	Compound A	100 mg/kg Days								
			-1	1		2		3		
Animal No.	Finding			pre	post	pre	post	pre	post	
01M01			-	-	-	NA		NA		
01M02			-	-	-	NA		NA		
01M03			-	-	NA	-	-	NA		
01M04			-	-	NA	-	-	NA		
01M05			-	-	NA	-	NA	-	-	
01M06			-	-	NA	-	NA	-	-	

-: No abnormality, NA: Not applicable

Note: On the treatment day, clinical signs were observed pre- and post-administration.

Appendix 1-3 Clinical signs

Study No.: CJ16050			Study: Respiratory Function			
Species: Rat		Strain: Crl:CD(SD)	Route: p.o.			Sex: Male
Treatment	Compound A	1000 mg/kg Days				
			-1	1	2	3
Animal No.	Finding			pre post	pre post	pre post
02M01	Decreased activity		-	- +	NA	NA
	Scrotum relaxation			- +		
02M02	Decreased activity		-	- +	NA	NA
	Scrotum relaxation			- +		
02M03	Decreased activity		-	- NA	- +	NA
	Scrotum relaxation				- +	
02M04	Decreased activity		-	- NA	- +	NA
	Scrotum relaxation				- +	
02M05	Decreased activity		-	- NA	- NA	- +
	Scrotum relaxation					- +
02M06	Decreased activity		-	- NA	- NA	- +
	Scrotum relaxation					- +

-: No abnormality, +: Positive, NA: Not applicable

Note: On the treatment day, clinical signs were observed pre- and post-administration.

Appendix 2 Respiratory rate

Study No.: CJ16050		Code:			Study: Safety Pharmacology		
Species: Rat		Strain: Crl:CD(SD)			Route: p.o.		Sex: Male
Treatment	Animal No.	Time after administration (hours)					
		pre	1	2	4	8	
Control	00M01	99.5	137.4	130.7	129.6	110.9	
	00M02	77.6	87.6	123.6	113.9	88.3	
	00M03	71.8	79.7	122.4	92.6	107.0	
	00M04	88.0	88.8	94.9	101.2	101.4	
	00M05	90.7	84.0	102.9	155.0	124.5	
	00M06	122.4	91.3	100.6	116.0	107.9	
	Mean	91.7	94.8	112.5	118.1	106.7	
	SD	18.0	21.3	14.8	22.1	11.9	
Compound A 100 mg/kg	01M01	90.3	191.3	159.1	133.3	182.3	
	01M02	106.5	201.8	-	198.4	214.5	
	01M03	94.6	185.3	178.1	187.9	169.4	
	01M04	71.6	186.1	134.1	207.9	125.8	
	01M05	79.8	180.5	181.9	203.1	183.4	
	01M06	94.3	172.0	171.2	178.1	155.4	
	Mean	89.5	186.2 *	164.9 *	184.8 *	171.8 *	
	SD	12.3	10.0	19.3	27.4	29.9	
Compound A 1000 mg/kg	02M01	84.4	97.4	82.6	83.8	83.4	
	02M02	104.5	119.3	111.5	91.3	89.6	
	02M03	77.9	117.5	83.8	80.5	87.5	
	02M04	71.5	92.9	79.7	70.8	79.6	
	02M05	85.8	94.4	76.3	72.4	73.2	
	02M06	111.6	90.8	77.5	76.3	79.7	
	Mean	89.3	102.1	85.2 *	79.2 *	82.2	
	SD	15.6	12.9	13.2	7.7	6.0	

*: $P < 0.05$, comparison with the control group (parametric Dunnett's test)

-: no data due to unstable condition

(breaths/min)

Appendix 3 Tidal volume

Study No.: CJ16050		Code:			Study: Safety Pharmacology		
Species: Rat		Strain: Crl:CD(SD)			Route: p.o.		Sex: Male
Treatment	Animal No.	Time after administration (hours)					
		pre	1	2	4	8	
Control	00M01	1.453	1.312	1.560	1.640	1.598	
	00M02	1.545	1.593	1.817	1.697	1.539	
	00M03	1.444	1.522	1.340	1.639	1.361	
	00M04	1.161	1.412	1.412	1.444	1.388	
	00M05	1.262	1.424	1.603	1.629	1.532	
	00M06	1.408	1.460	1.677	1.745	1.701	
	Mean	1.379	1.454	1.568	1.632	1.520	
	SD	0.141	0.097	0.174	0.102	0.128	
Compound A 100 mg/kg	01M01	1.575	1.370	1.323	1.323	1.161	
	01M02	1.372	1.241	-	1.517	1.380	
	01M03	1.452	1.468	1.577	1.361	1.359	
	01M04	1.674	1.619	1.465	1.665	1.535	
	01M05	1.809	1.953	1.572	1.756	1.923	
	01M06	1.461	1.310	1.585	1.660	1.501	
	Mean	1.557	1.494	1.504	1.547	1.477	
	SD	0.162	0.261	0.113	0.177	0.255	
Compound A 1000 mg/kg	02M01	1.693	1.257	1.313	1.431	1.460	
	02M02	0.988	1.065	0.940	0.856	0.864	
	02M03	1.820	0.835	0.877	0.868	1.069	
	02M04	1.552	0.847	0.816	0.854	0.918	
	02M05	1.195	1.063	1.085	0.801	0.649	
	02M06	1.290	1.234	1.208	1.177	0.907	
	Mean	1.423	1.050 *	1.040 *	0.998 *	0.978 *	
	SD	0.318	0.181	0.196	0.251	0.272	

*: $P < 0.05$, comparison with the control group (parametric Dunnett's test)

-: no data due to unstable condition

(mL/breath)

Appendix 4 Minute volume

Study No.: CJ16050 Code: Study: Safety Pharmacology
Species: Rat Strain: Crl:CD(SD) Route: p.o. Sex: Male

Treatment	Animal No.	Time after administration (hours) pre	1	2	4	8
Control	00M01	147.3	178.9	205.0	211.4	178.8
	00M02	118.6	137.0	224.6	191.0	134.6
	00M03	107.4	124.2	161.0	155.5	147.0
	00M04	102.1	125.3	136.2	148.0	140.8
	00M05	118.5	120.7	165.0	239.9	189.9
	00M06	166.3	134.8	167.8	202.1	183.3
	Mean	126.7	136.8	176.6	191.3	162.4
	SD	24.9	21.6	32.2	34.8	24.2
Compound A 100 mg/kg	01M01	125.7	260.2	212.5	175.6	217.0
	01M02	143.5	238.7	-	302.1	313.4
	01M03	136.3	264.2	272.3	255.9	234.9
	01M04	120.5	295.6	196.1	355.8	194.4
	01M05	144.3	335.8	270.0	343.4	348.4
	01M06	136.4	223.0	268.2	297.8	235.9
	Mean	134.5	269.6 *	243.8 *	288.4 *	257.3 *
	SD	9.6	40.7	36.6	65.8	60.0
Compound A 1000 mg/kg	02M01	143.2	122.4	109.4	117.9	121.5
	02M02	103.3	127.2	104.8	77.6	78.9
	02M03	131.8	96.8	73.2	70.1	93.1
	02M04	109.7	79.0	66.0	59.9	73.9
	02M05	103.4	100.1	84.0	58.2	48.2
	02M06	136.9	110.4	94.1	89.6	73.9
	Mean	121.4	106.0	88.6 *	78.9 *	81.6 *
	SD	18.0	17.8	17.3	22.4	24.4

*: $P < 0.05$, comparison with the control group (parametric Dunnett's test)
-: no data due to unstable condition

(mL/min)