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Study code	937-16-V-0524

FINAL REPORT

Evaluation of skin sensitization hazard of APFHx by human Cell Line Activation Test (h-CLAT)

April, 2017

Chemicals Assessment and Research Center
Chemicals Evaluation and Research Institute, Japan

Study code: 937-16-V-0524

TITLE

Evaluation of skin sensitization hazard of APFHx by human Cell Line Activation Test (h-CLAT)

SPONSOR

DAIKIN INDUSTRIES, LTD.

1-1 Nishi Hitotsuya, Settsu-shi, Osaka 566-8585, Japan

TESTING FACILITY

Chemicals Assessment and Research Center

Chemicals Evaluation and Research Institute (CERI), Japan

1600 Shimotakano, Sugito-machi, Kitakatsushika-gun, Saitama 345-0043, Japan

PURPOSE OF STUDY

To evaluate the skin sensitization hazard of APFHx by human Cell Line Activation Test (h-CLAT).

METHOD OF STUDY

This study was conducted in accordance with the OECD TG442E.

PERIOD OF STUDY

Commencement of Study:	February 21, 2017
Dose finding assay:	February 21-22, 2017
CD86/CD54 expression measurement:	February 27 - March 2, 2017
Completion of Study:	April 4, 2017

NAMES, ASSIGNED SECTIONS AND JOB ASSIGNMENT OF STUDY DIRECTOR AND PERSONNEL

Study Director:	Yosuke Maeda Chemicals Assessment and Research Center
Study Staff:	Yosuke Maeda Chemicals Assessment and Research Center
	Toshiyuki Ohtake Chemicals Assessment and Research Center

SUMMARY

Skin sensitization hazard of APFHx was evaluated by human Cell Line Activation Test (h-CLAT), which was in accordance with the OECD TG442E. 1348.3 μg/mL of APFHx, derived from the dose finding assay, was employed as the highest dose for CD86/CD54 expression measurement. In two independent runs, the RFI of CD54 was over 200 while the RFI of CD86 was less than 150. As a result, APFHx was evaluated as positive by h-CLAT.

MATERIALS

1 Materials and Instrument

1.1 Test substance (Provided by the Sponsor)

1) Name

APFHx (C-1500N)

Synonym

APFHx

2) CAS

21615-47-4

3) Supplier

DAIKIN INDUSTRIES, LTD.

4) Lot No.

C150E62004

5) Purity

50 %

6) Appearance at normal temperature

Clear liquid

7) Storage Condition

The test substance was stored at normal temperature

8) Handling precautions

Gloves, a mask, a head cup and a lab coat were worn when handling

1.2 Positive control substance

1) Name

2, 4-Dinitrochlorobenzene (DNCB)

2) Supplier

Tokyo Chemical Industry Co., Ltd., Tokyo, Japan

3) Lot No.

JXPYL-KE

4) Storage Condition

The test substance was stored at normal temperature

5) Handling precautions

Gloves, a mask, a head cup and a lab coat were worn when handling

1.3 Solvent/vehicle

1.3.1

1) Name

Saline

2) Supplier

Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan

3) Lot No.

6F86N

4) Storage Condition

The solvent/vehicle was stored at 4°C

5) Handling precautions

Gloves, a mask, a head cup and a lab coat were worn when handling

1.3.2

1) Name

Dimethylsulfoxide (DMSO)

2) Supplier

Sigma-Aldrich Co., MO, USA

3) Lot No.

SHBG3462V

4) Storage Condition

The solvent/vehicle was stored at normal temperature

5) Handling precautions

Gloves, a mask, a head cup and a lab coat were worn when handling

1.4 Cells

THP-1 (ATCC, No. TIB-202)

1.5 Antibodies

Anti-CD86 antibody: #555657 (BD-PharMingen, CA, USA)

Anti-CD54 antibody: #F7143 (DAKO, Glostrup, Denmark)

FITC labelled-mouse IgG1: #X0927 (DAKO)

1.6 Instrument

Flow cytometer (Beckman Coulter, Cytomics FC500)

METHODS

1 Preparation of cells

1.1 Culture medium

RPMI-1640 (Thermo Fisher Scientific, CA, USA) supplemented following substances, was used to culture the cells during the assay. The culture medium was stored at 4°C.

Final Conc.	Substances	Suppliers
10%	Fetal Bovine Serum (FBS)	Thermo Fisher Scientific
0.05 mM	2-Mercaptoethanol	Wako Pure Chemical Industries, Ltd., Osaka, Japan
100 units/mL	Penicillin	Thermo Fisher Scientific
100 μg/mL	Streptomycin	Thermo Fisher Scientific

FBS was inactivated by heating (56°C, 30 min).

1.2 Maintenance of cells

The cells were maintained at 37°C in an incubator under an atmosphere of 5% CO_2 in air. Cells were routinely passaged every 2-3 days at the density of 0.16 to 0.2×10^6 cells/mL.

2 Dose finding assay

2.1 Day 1. Preparation of test chemicals and treatment

2.1.1 Solvent/vehicle selection

Saline was used as solvent/vehicle in this study.

2.1.2 Preparation of test solutions and exposure to cells

1) Stock and working solutions

Stock solutions (8 doses) were prepared by 1:2 serial dilutions from 100 mg/mL using medium. Working solutions were prepared by diluting each stock solution 50 times with culture medium just before use. Working solutions of 2500.0 μg/mL and 5000.0 μg/mL (final concentrations in the plate) were prepared directly from 100 mg/mL. (Final concentrations in the plate were 7.8 μg/mL, 15.6 μg/mL, 31.3 μg/mL, 62.5 μg/mL, 125.0 μg/mL, 250.0 μg/mL, 500.0 μg/mL, 1000.0 μg/mL, 2500.0 μg/mL, 5000.0 μg/mL).

2) Cell suspensions

Cell suspensions were prepared from culture dishes by centrifugation and then resuspended with fresh culture medium at the density of 2×10^6 cells/mL. 500 μL of cell suspensions were added to each well of a 24-well flat-bottom plate.

3) Exposure

500 μL of working solutions (10 doses) were added to each well, and the plate was shaken by hand before being placed in the incubator. Cells at a final density of 1.0×10^6 cells/mL were cultured for 24 ± 0.5 hours in 5 % CO_2 incubator.

2.2 Day 2. Staining, analysis, and calculation of CV75

2.2.1 Preparation

1) Staining buffer

0.1 % (w/v) Bovine Serum Albumin (BSA) solution was prepared in Phosphate Buffered Saline (PBS).

2) Propidium Iodide (PI) solution

12.5 μg/mL of PI solution was prepared by 1:100 dilutions from 1.25 mg/mL of stock solution using PBS.

2.2.2 Cell staining with PI

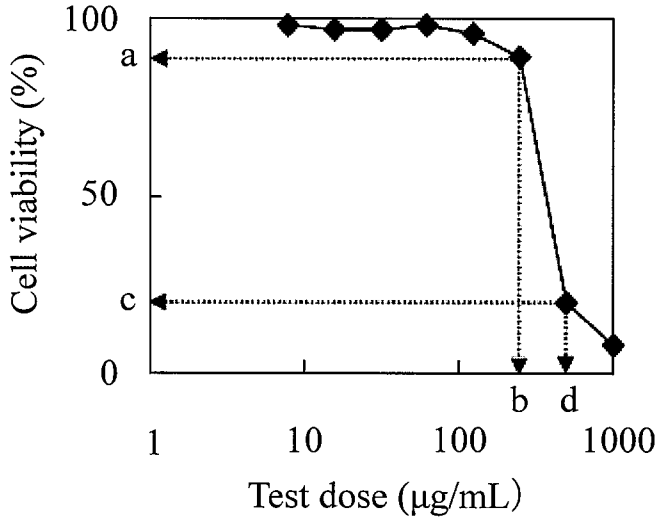
Cells treated with test substance were transferred into 2mL sample tubes and collected by centrifugation (300×g, 5 min, 4 °C). The supernatants were discarded and the remaining cells were resuspended with 600 μL of staining buffer. 200 μL of cell suspensions were transferred into 1.5mL sample tubes and washed twice with 600 μL of staining buffer and resuspended in 400 μL of staining buffer. 20 μL of PI solution (12.5 μg/mL) was added just before flow cytometry analysis (final concentration of PI was 0.625 μg/mL). All procedures were conducted on ice.

2.2.3 Flow cytometry analysis

The cell viability was measured by gating-out dead cells stained with PI. A total of 10,000 living cells was acquired from sample tubes.

2.2.4 Estimation of CV75 value

The CV75 value was derived from the dose response curve as shown in the figure (75% of cell viability is lying between "a" and "c"). CV75 was defined as the estimated concentration that was required to elicit 75% cell viability.



The CV75 value was calculated by log-linear interpolation utilizing the following equation:

$$\text{Log CV75} = \frac{(75-c)\times\text{Log}(b)-(75-a)\times\text{Log}(d)}{a-c}$$

2.2.5 Calculation of the test doses

As CV75 was estimated 1123.6 μg/mL, stock solutions (8 doses) were prepared by serial 1:1.2 dilutions from 67.4 mg/mL, which was the concentration corresponding to 50-fold of the 1.2 × CV75, as the highest dose.

3 Measuring CD86/CD54 expression

3.1 Day 1. Preparation of test chemicals and exposure

3.1.1 Preparation of test solutions and exposure to cells

1) Stock and working solutions

67.4 mg/mL of stock solution, 50 times the concentration corresponding to 1.2×CV75, was prepared as the highest dose and the other 7 stock solutions were prepared by serial 1:1.2 dilution using medium. Working solutions were prepared by diluting each stock solution 25 times with culture medium just before use. (Final concentrations in the plate were 376.3 μg/mL, 451.5 μg/mL, 541.8 μg/mL, 650.2 μg/mL, 780.2 μg/mL, 936.3 μg/mL, 1123.6 μg/mL, 1348.3 μg/mL).

2) Positive control

DNCB was used as the positive control in this assay. 2 mg/mL stock solution of DNCB was prepared with DMSO and further diluted 250-fold with culture medium to obtain 8 μg/mL of working solution (Final concentration in the plate was 4 μg/mL).

3) Solvent/vehicle controls

A culture medium and DMSO (Final concentration in the plate was 0.2%) samples were prepared as solvent/vehicle controls.

3.1.2 Cell suspensions

Cell suspensions were prepared from culture dishes by centrifugation and then resuspended with fresh culture medium at the density of 2×10^6 cells/mL. 500 μL of cell suspensions were added to each well of a 24-well flat-bottom plate.

3.1.3 Exposure

500 μL of working solutions (8 doses) were added to each well, and the plate was shaken by hand before being placed in the incubator. Cells at a final density of 1.0×10^6 cells/mL were cultured for 24±0.5 hours in 5 % CO_2 incubator.

3.1.4 Plate layout

The test doses were exposed to the cells as shown below;

	1	2	3	4	5	6
A	Medium	DMSO	DNCB 4 μg/mL			
B	APFHx 376.3 μg/mL	APFHx 451.5 μg/mL	APFHx 541.8 μg/mL	APFHx 650.2 μg/mL	APFHx 780.2 μg/mL	APFHx 936.3 μg/mL
C	APFHx 1123.6 μg/mL	APFHx 1348.3 μg/mL				
D						

The day of exposure;

1st run : February 27, 2017

2nd run : March 1, 2017

The day of staining and analysis;

1st run : February 28, 2017

2nd run : March 2, 2017

3.2 Day 2. Staining and analysis

3.2.1 Preparation

1) Blocking solution

The blocking solution was prepared by 1:100 dilution of 1% globulin solution in PBS with staining buffer just before use.

2) Antibody solution

Each antibody solution was prepared as listed below.

	Volume of antibody /sample	Volume of staining buffer /sample	Total volume of working solution /sample
Anti-CD86 antibody	6 μL	50 μL	56 μL
Anti-CD54 antibody	3 μL	50 μL	53 μL
FITC labelled-mouse IgG1	3 μL	50 μL	53 μL

3.2.2 Staining

Chemical-treated cells were transferred to 2mL sample tubes, collected by centrifugation (300×g, 5 min, 4 °C) and then washed twice with 1 mL of staining buffer. After the wash, cells were resuspended in 600 μL of blocking solution and incubated at 4°C for 15 min. After blocking, cells were divided into 3 aliquots of 180 μL in 1.5mL sample tubes. The three groups of cells were centrifuged and 50 μL of each antibody solution was added to each cell pellet. After gently mixing by hand, cells were incubated at 4°C for 30 min in the dark. After staining with antibodies, the cells were washed three times with 200 μL of staining buffer and resuspended in 400 μL of staining buffer. 20 μL of PI solution (12.5 μg/mL) was added just before flow cytometry analysis (final concentration of PI was 0.625 μg/mL). All procedures were conducted on ice.

3.2.3 Flow cytometry analysis

The FITC acquisition channel (FL-1) was set for the optimal detection of the FITC fluorescence signal, and the PI acquisition channel (FL-3) was set for the optimal detection of PI fluorescence signal. The cell viability was measured by gating-out dead cells stained with PI. A total of 10,000 living cells was acquired from sample tubes.

The cell viability was recorded from the isotype control cells which were stained with mouse IgG1 antibody.

3.2.4 Calculation of Relative Fluorescence Intensity (RFI)

Based on the geometric mean fluorescence intensity (MFI), the relative fluorescence intensity (RFI) of CD86 and CD54 were calculated according to the following equation;

$$\mathrm{RFI} = \frac{MFI\ of\ chemical\ treated\ cells - MFI\ of\ chemical\ treated\ isotype\ control\ cells}{MFI\ of\ solvent/vehicle\ treated\ control\ cells - MFI\ of\ solvent/vehicle\ treated\ isotype\ control\ cells} \times 100$$

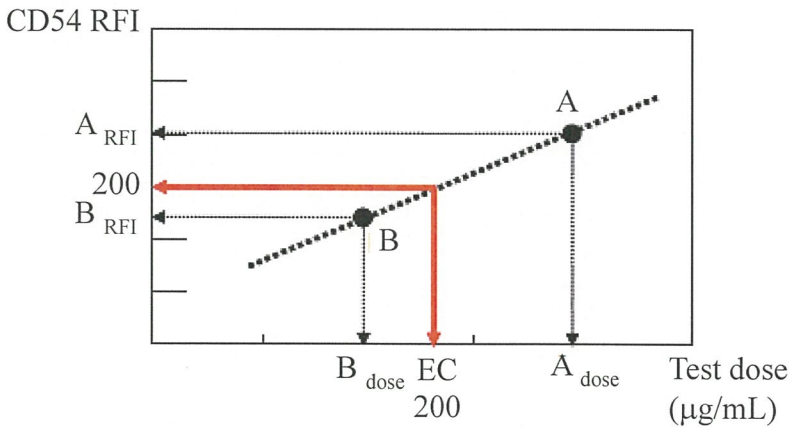
3.2.5 Evaluation

If the RFI of CD86 was equal or greater than 150 at any dose in at least 2 of 3 independent run data, or if the RFI of CD54 was equal or greater than 200 in at least 2 of 3 independent run data, the test chemical was evaluated as positive. Otherwise it was considered to be negative.

3.2.6 Calculation of EC200

Effective Concentration (EC200) values for CD54, the concentration at which the test chemical induced a RFI of 200, was estimated by linear regression as follows:

$$EC200 = B_{dose} + [(200 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{dose} - B_{dose})]$$



3.3 Acceptance criteria

All independent runs met the following acceptance criteria;

1) In the solvent/vehicle control (DMSO), the RFI values of both CD86 and CD54 did not exceed the positive criteria (CD86 < 150 and CD54 < 200) and cell viabilities were more than 90 %.
2) In the positive control (DNCB), RFI values of both CD86 and CD54 were over the positive criteria (CD86 > 150 and CD54 > 200) and cell viability showed more than 50 %.
3) For both medium and DMSO, the MFI ratios of both CD86 and CD54 to isotype control were greater than 105%.
4) For the test chemical, the cell viabilities showed more than 50% in at least four tested concentrations in each run.

RESULTS

1 Dose finding assay

The cell viabilities showed 85.5% and 3.1% when final concentrations in the plate were 1000.0 μg/mL and 2500.0 μg/mL, respectively (Table 1). Therefore, the dose of 1348.3 μg/mL was employed as the highest dose since the value of CV75 was calculated as 1123.6 μg/mL.

2 CD86 expression

APFHx was classified as negative since the maximum RFI was less than 150 (64.2 and 97.4) in two independent runs (Table 3-1 and Table 3-2).

All independent runs met the following acceptance criteria;

DNCB, used as the positive control, was classified as positive since the maximum RFI was over 150 (490.2 and 687.9) (Table 3-1 and Table 3-2). The solvent/vehicle control (DMSO) was estimated as negative since the maximum RFI was less than 150 (96.2 and 126.8). In addition, the MFI ratios of CD86 to isotype control were greater than 105% (Medium: 192.5% and 149.1%, DMSO: 198.1% and 162.6%) (Table 2-1, Table 2-2 and Table 4).

3 CD54 expression

APFHx was classified as positive since the maximum RFI was over 200 (232.9 and 231.5) in two independent runs (Table 3-1 and Table 3-2). The EC200 value could not be calculated as the RFI value of the lowest test dose was over 200.

All independent runs met the following acceptance criteria;

DNCB, used as the positive control, was classified as positive since the maximum RFI was over 200 (568.0 and 555.8) (Table 3-1 and Table 3-2). The solvent/vehicle control (DMSO) was estimated as negative since the maximum RFI was less than 200 (147.1 and 116.9). In addition, the MFI ratios of CD86 to isotype control were greater than 105% (Medium: 113.5% and 119.2%, DMSO: 122.0% and 122.5%) (Table 2-1, Table 2-2 and Table 4).

4 Cell viability

The cell viabilities of APFHx in all tested concentrations and DNCB were greater than 50% in each run. Additionally, a culture medium and DMSO controls showed higher than 90% of cell viability (Table 3-1 and Table 3-2).

DISCUSSION

Skin sensitization hazard of APFHx was evaluated by human Cell Line Activation Test (h-CLAT), which was in accordance with the OECD TG442E.

Saline was used as solvent/vehicle in this study. The cell viabilities showed 85.5% and 3.1% when final concentrations in the plate were 1000.0 μg/mL and 2500.0 μg/mL, respectively. Therefore, the dose of 1348.3 μg/mL (1.2×CV75) was employed as the highest dose since the value of CV75 was calculated as 1123.6 μg/mL. In two independent runs, the RFI of CD54 was over 200 (the maximum values were 232.9 and 231.5) while the RFI of CD86 was less than 150 (the maximum values were 64.2 and 97.4).

As a result, APFHx was evaluated as positive by h-CLAT. The EC200 value could not be calculated as the RFI value of the lowest test dose was over 200.

All independent runs met the following acceptance criteria;

DNCB, used as the positive control, met the positive criteria and showed higher than 50% of cell viability. In the solvent/vehicle control, the RFI values of both CD86 and CD54 did not exceed the positive criteria. In addition, the MFI ratios of both CD86 and CD54 to isotype control were greater than 105% and cell viabilities were more than 90%. For the test chemical, the cell viabilities showed more than 50% in at least four tested concentrations in each run.

【Addendum 1】

Table 1 Dose finding assay

Final conc. (μg/mL)	Cell viability (%)
7.8	99.4
15.6	99.4
31.3	99.4
62.5	99.4
125.0	99.2
250.0	99.1
500.0	96.4
1000.0	85.5
2500.0	3.1
5000.0	0.7

Table 2-1 The MFI of APFHx in the 1st run

Test substance	Final conc. (μg/mL)	MFI		
		CD86	CD54	IgG1
Medium	-	0.995	0.587	0.517
APFHx	376.3	0.779	0.674	0.511
	451.5	0.834	0.672	0.527
	541.8	0.834	0.664	0.533
	650.2	0.826	0.653	0.535
	780.2	0.817	0.649	0.544
	936.3	0.829	0.629	0.542
	1123.6	0.816	0.62	0.54
	1348.3	0.787	0.609	0.541
DMSO	-	0.929	0.572	0.469
DNCB	4.0	2.75	1.08	0.495

Table 2-2 The MFI of APFHx in the 2nd run

Test substance	Final conc. (μg/mL)	MFI		
		CD86	CD54	IgG1
Medium	-	0.692	0.553	0.464
APFHx	376.3	0.645	0.675	0.469
	451.5	0.683	0.654	0.468
	541.8	0.701	0.636	0.479
	650.2	0.674	0.632	0.485
	780.2	0.694	0.639	0.484
	936.3	0.679	0.608	0.486
	1123.6	0.685	0.595	0.521
	1348.3	0.681	0.57	0.514
DMSO	-	0.751	0.566	0.462
DNCB	4.0	2.46	1.05	0.472

Table 3-1 The RFI and cell viability of APFHx in the 1st run

Test substance	Final conc. (μg/mL)	RFI		Cell viability (%)
		CD86	CD54	
Medium	-	100.0	100.0	99.6
APFHx	376.3	56.1	232.9	99.2
	451.5	64.2	207.1	99.0
	541.8	63.0	187.1	98.6
	650.2	60.9	168.6	98.0
	780.2	57.1	150.0	98.4
	936.3	60.0	124.3	96.6
	1123.6	57.7	114.3	93.9
	1348.3	51.5	97.1	84.8
DMSO	-	96.2	147.1	99.7
DNCB	4.0	490.2	568.0	91.3

The RFI of DNCB was calculated based on the MFI of DMSO

Table 3-2 The RFI and cell viability of APFHx in the 2nd run

Test substance	Final conc. (μg/mL)	RFI		Cell viability (%)
		CD86	CD54	
Medium	-	100.0	100.0	99.7
APFHx	376.3	77.2	231.5	99.3
	451.5	94.3	209.0	98.8
	541.8	97.4	176.4	98.1
	650.2	82.9	165.2	97.3
	780.2	92.1	174.2	97.3
	936.3	84.6	137.1	96.4
	1123.6	71.9	83.1	93.7
	1348.3	73.2	62.9	83.9
DMSO	-	126.8	116.9	99.8
DNCB	4.0	687.9	555.8	94.1

The RFI of DNCB was calculated based on the MFI of DMSO

Table 4 The MFI ratios of both CD86 and CD54 to isotype control in solvent/vehicle controls

1st run	Isotype control	CD86		CD54	
	MFI	MFI	%*	MFI	%*
DMSO	0.469	0.929	198.1	0.572	122.0
Medium	0.517	0.995	192.5	0.587	113.5
2nd run	Isotype control	CD86		CD54	
	MFI	MFI	%*	MFI	%*
DMSO	0.462	0.751	162.6	0.566	122.5
Medium	0.464	0.692	149.1	0.553	119.2

* Ratio of CD86 or CD54 to isotype control

REFERENCES

Organization for Economic Corporation and Development (OECD, 2016). *In Vitro* Skin Sensitisation: human Cell Line Activation Test (h-CLAT), TG-442E (Adopted: 29 July 2016).

Nukada Y, Ashikaga T, Miyazawa M, Hirota M, Sakaguchi H, Sasa H, Nishiyama N. (2012). Prediction of skin sensitization potency of chemicals by human Cell Line Activation Test (h-CLAT) and an attempt at classifying skin sensitization potency. Toxicology in Vitro, 26. 1150–1160.

Takenouchi O, Miyazawa M, Saito K, Ashikaga T, Sakaguchi H. (2013) Predictive performance of the human Cell Line Activation Test (h-CLAT) for lipophilic chemicals with high octanol-water partition coefficients. Journal of Toxicological Sciences, 38. 599-609.

Authorized signature of this final report

Study Director:

前田洋祐　　April 4, 2017

Yosuke Maeda　　Date

Chemicals Assessment and Research Center

Chemicals Evaluation and Research Institute, Japan