

Eskes et al.:

The HaCaT/THP-1 Cocultured Activation Test (COCAT) for Skin Sensitization: A Study of Intra-Laboratory Reproducibility and Predictivity

Supplementary Data

Tab. S1: Reproducibility of cytotoxicity and upregulation of CD86 and CD54 in the valid runs in COCAT

Shown is the lowest cytotoxic dilution for the valid runs of the tested chemicals and the lowest dilution inducing CD54 and CD86 above the prediction model thresholds (grey indicates differences between runs of ≥ 2 dilution). Serial 1:2 dilution starting at 4 mM (Dilution 1) based on molecular weight provided for the blind study.

Test chemical	Lowest concentration interfering with cell viability based on PI staining #			Lowest dilution with CD86 Δ MF > 10.8			Lowest dilution with CD54 Δ MF > 300		
	1 st valid run	2 nd valid run	3 rd valid run	1 st valid run	2 nd valid run	3 rd valid run	1 st valid run	2 nd valid run	3 rd valid run
Diphenylcyclopropenone	Dilution 7	Dilution 7	Dilution 7	Dilution 9	Dilution 9	Dilution 9	Dilution 10	Dilution 10	Dilution 10
<i>p</i> -Phenylenediamine	Dilution 2	Dilution 3	Dilution 4	Dilution 3	Dilution 5	Dilution 6	Dilution 7	Dilution 8	Dilution 6
Formaldehyde	Dilution 5	Dilution 5	Dilution 5	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6
Methyldibromo glutaronitrile	Dilution 6	Dilution 6	Dilution 7	Dilution 8	Dilution 8	Dilution 9	Dilution 9	Dilution 8	Dilution 9
Isoeugenol	> Dilution 4*	> Dilution 4*	> Dilution 4*	Dilution 4	Dilution 4	Dilution 4	Dilution 5	Dilution 4	Dilution 4
2-Mercaptobenzothiazole	> Dilution 5*	> Dilution 5*	> Dilution 5*	-	-	-	Dilution 6	Dilution 5	Dilution 6
Resorcinol	> Dilution 1*	> Dilution 1*	Dilution 1	Dilution 2	Dilution 2	-	Dilution 4	Dilution 4	Dilution 5
Coumarin	> Dilution 1*	> Dilution 1*	> Dilution 1*	Dilution 5	Dilution 2	Dilution 2	-	-	-
Linalool	> Dilution 3*	> Dilution 3*	> Dilution 3*	-	-	-	Dilution 4	-	Dilution 3
<i>p</i> -Aminobenzoic acid	> Dilution 1*	> Dilution 1*	> Dilution 1*	-	-	-	Dilution 1	-	-
Benzalkonium chloride	Dilution 9	Dilution 9	Dilution 9	-	-	-	-	Dilution 11	-
Glycerol	> Dilution 1*	> Dilution 1*	> Dilution 1*	-	-	-	-	-	-
Hydrocortisone	> Dilution 4*	> Dilution 4*	> Dilution 4*	-	-	-	-	-	-
Propylparaben	> Dilution 5*	> Dilution 5*	> Dilution 5*	-	-	-	-	-	-
Salicylic acid	> Dilution 1*	> Dilution 1*	> Dilution 1*	-	-	-	Dilution 2	Dilution 2	Dilution 3

*maximum tested or maximum soluble concentration; '-' indicates that none of the tested concentrations exceeded the respective threshold; #corresponding to a compound induced cytotoxicity of maximal 35%; PI, propidium iodide

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COCAT Standard Operating Procedure



DB-ALM Protocol

Template for Data Content

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Part A. Protocol Introduction

Protocol Name: Cocultured Activation Test (COCAT)

Abstract: The COCAT is an *in vitro* method which quantifies the expression of the cell surface molecules CD86 and CD54 as a measure of the activation of the cell line THP-1, used as surrogate for dendritic cells (DC), after exposure with test chemicals in direct coculture with the keratinocyte cell line HaCaT. The activation of DC is considered as one key event in the adverse outcome pathway (AOP) for skin sensitization (OECD, 2014). The activation of DC by sensitizers comprises the upregulation of CD86 and CD54, and other surface molecules (reviewed by Hubo et al., 2013). These molecules interact with their counterparts on T cells and generate a costimulatory signal (signal 2) that synergizes with the T cell receptor-mediated signal (signal 1) to promote an adaptive immune response. Thus, results obtained from the COCAT are considered relevant for the assessment of skin sensitization potential and potency of chemicals.

Résumé

The purpose of the assay is to contribute to the evaluation of the skin sensitization potential and potency of chemicals. COCAT provides information on the potential and potency of a chemical to upregulate molecules on the cell surface that are associated with the activation of dendritic cells (DC). The activation of DC is considered as the third key event in the adverse outcome pathway (AOP) for skin sensitization (OECD, 2012).

Experimental Description

Biological Endpoint and Endpoint Measurement:

UPREGULATION OF THE EXPRESSION OF CELL SURFACE PHENOTYPIC BIOMARKERS (CD86 and CD54): quantified on the DC-related cell line THP-1 by flow cytometric analysis following 24 h exposure to test chemicals in direct coculture with the keratinocyte cell line HaCaT.

Endpoint Value:

Difference in expression (Δ MFI) of CD86 and CD54 cell surface molecules compared to solvent control.

EC Δ 10.8: Estimated concentration needed for an upregulation of CD86 with MFI of 10.8 above solvent control.

EC Δ 300: Estimated concentration needed for an upregulation of CD54 with MFI of 300 above solvent control.

Experimental System:

Direct coculture of THP-1 cells with HaCaT cells: THP-1 cells, a monocytic cell line (purchased at an appropriate cell bank such as ATCC), specifically upregulate CD86 and CD54 after exposure to chemical sensitizers (Yoshida, 2003), HaCaT cells are an immortalized keratinocytes cell line (purchased at an appropriate cell bank such as CLS Cell Lines Service). Keratinocytes support DC activation by the release of danger molecules among other factors (Galbiati et al., 2014, Matjeka et al., 2012).

Discussion

Given the complexity of the mechanism underlying skin sensitization, it is likely that combinations of test methods within *Integrated Approaches to Testing and Assessment* (IATA) are needed to be able to substitute the regulatory animal tests that have been used to satisfy regulatory requirements for this endpoint. Since the COCAT addresses a key biological event involved in the induction of skin sensitization, it provides useful information for the assessment of skin sensitization potential of chemicals.

Status

In Development:

COCAT is currently in development and has not yet been formally validated.

Known Laboratory Use:

University Trier

Participation in Evaluation Study:

COCAT was optimized in a study supported by the Federal Office of Public Health (FOPH, Switzerland).

Participation in Validation Study:

The COCAT has not yet undergone a validation study.

Regulatory Accepted:

-

Proprietary and/or Confidentiality Issues

NA

Health and Safety Issues

General precautions:

General safety instructions should be followed and appropriate protective safety equipment worn at all times. Unknown and coded chemicals should be considered potential sensitizing agents or toxins and must be handled with extreme care.

MSDS Information:

Propidium iodide (CAS 25535-16-4) is a known carcinogen. MSDS available at www.sigmaaldrich.com.

Abbreviations and Definitions

AOP: adverse outcome pathway

APC: allophycocyanin

ATCC: American Type Culture Collection

CLS: Cell Lines Service

ΔMFI: difference in mean fluorescence intensity between cells exposed to test chemical versus solvent

DC: dendritic cells

DMSO: dimethylsulfoxide

DNCB: 2,4-dinitrochlorobenzene

ECΔ10.8: estimated concentration for an upregulation of CD86 by 10.8 MFI

ECΔ300: estimated concentration for an upregulation of CD54 by 300 MFI

EDTA: Ethylenediaminetetraacetic acid

FACS: fluorescence-activated cell sorting
FCS: fetal calf serum
FITC: fluorescein isothiocyanate
HEPES: 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
IATA: integrated approaches to testing and assessment
MFI: mean fluorescence intensity
PBS: phosphate buffered saline
PI: propidium iodide
RT: room temperature
TC: test chemical
SDS: sodium dodecyl sulfate

Last Update: 17/04/2019.

Part B. Technical Description

Procedure Details, Latest Version: 10 November 2016.

Protocol Name: Cocultured activation test (COCAT)

The protocol is based on the Standard Operating Procedure (SOP) used in the optimization study on the COCAT.

Contact person

Prof. Dr. Brunhilde Blömeke
University Trier
Department of Environmental Toxicology
Universitätsring 15
54296 Trier
Germany
email: bloemeke@uni-trier.de
telephone: +49 651 201 3781
fax: +49 651 201 3780

Dr. Jennifer Hennen
University Trier
Department of Environmental Toxicology
Universitätsring 15
54296 Trier
current address:
Chemservice S.A.
5, an de Laengten
6776 Grevenmacher
Luxembourg
email: luxembourg@chemservice-group.com
telephone: +352 270776 1
fax: +352 270776 75

Materials and Preparations

CELL OR EXPERIMENTAL SYSTEM

The immortalized keratinocyte cell line HaCaT can be purchased from Cell Lines Service GmbH, #300493. HaCaT cells at passages 4 to 16 have been used in COCAT.

The monocytic cell line THP-1 can be purchased e.g. from ATCC, #TIB-202. THP-1 cells have been used in COCAT up to a maximum passage of 25.

EQUIPMENT

Fixed Equipment

Standard cell culture laboratory equipment including an incubator with CO₂ supply, biological safety cabinet and centrifuge.

In addition:

Multi-channel pipette

Flow cytometer equipped with two lasers (excitation at 488 nm and 635 nm; preferred model is FACSVerse™ from Becton Dickinson)

Consumables

96 well plates, flat bottom (e.g. Sarstedt, 83.3924)

96 well plates, V-bottom (e.g. Sarstedt, 83.3926.500) or U-bottom (e.g. Sarstedt, 83.3925.500)

96 well plates, deep well (e.g. Greiner bio-one, 786261)

Tissue culture flask, 75 cm² (e.g. Sarstedt, 83.1813.002)

Sample tube, 50 mL (e.g. Sarstedt, 62.547.254)

Pipette tips

5 ml Tubes 75 x 12 mm (e.g. Sarstedt, 55.1579)

MEDIA, REAGENTS, SERA, OTHERS

DMEM (Sigma Aldrich, D5671)

RPMI 1640 (Sigma Aldrich, R8758)

Foetal bovine serum (FBS) Superior, heat inactivated at 56 °C for 30 min (Biochrom, S0615)

L-glutamine (Sigma Aldrich, G7513)

Antibiotic-antimycotic solution (penicillin 10000 U/mL, Streptomycin sulfate 10 mg/mL, Amphotericin B 25 µg/mL; Sigma Aldrich, A5955)

HEPES (Sigma Aldrich, H4034)

β-Mercaptoethanol (Sigma Aldrich, M7522)

Trypsin-EDTA solution (0.05 % trypsin, 0.02 % EDTA; Sigma Aldrich, T3924)

Phosphate-buffered saline (Sigma Aldrich, D8537)

Trypan blue solution, 0.4 % (Sigma Aldrich, T8154)

Dimethylsulfoxide (Carl Roth, A994.2)

FITC-labeled mouse anti-human CD86 (BD, 555657)

APC-labeled mouse anti-human CD54 (BD, 559771)

APC-labeled mouse IgG1, kappa (BD, 555751)

FITC-labeled mouse IgG1, kappa (BD, 555748)

Propidium iodide (Sigma Aldrich, 81845)

PREPARATIONS

Media and Endpoint Assay Solutions

Maintenance medium for HaCaT cells

Each bottle of DMEM (500 mL) is freshly supplemented with 5 mL L-glutamine (200 mM, final concentration: 2 mM) and 5 mL antibiotics (final concentration: 1 %) one day prior to its first usage. This supplemented medium must be stored at 4 °C.

FBS is freshly added prior to usage (5 mL FCS per 45 mL DMEM, final concentration: 10 %). The required volume of complete culture medium must be prewarmed to 37 °C before use.

Maintenance medium for THP-1 cells

Each bottle of RPMI (500 mL) is freshly supplemented with HEPES (6.25 mL of 2 M solution, final concentration: 25 mM), 5 mL L-glutamine (200 mM, final concentration: 4 mM) and 5 mL antibiotics (final concentration: 1 %) one day prior to its first usage. This supplemented medium must be stored at 4 °C.

FBS (5 mL FCS per 45 mL RPMI, final concentration: 10 %) and β -mercaptoethanol (250 μ L stock solution per 50 mL complete medium, final concentration: 50 μ M) freshly added prior to usage. The required volume of complete culture medium must be prewarmed to 37 °C before use.

Medium for coculture and exposure to test chemicals

Same medium as used for maintenance of THP-1 cells (RPMI supplemented with 25 mM HEPES, 10 % FCS and 1 % antibiotics), but do not add β -mercaptoethanol.

HEPES

To prepare 2 M stock solutions, dissolve 100 g HEPES in a final volume of 209.8 mL sterile water. Prepare aliquots à 6.25 mL, store at 4 °C protected from light.

PI

To prepare PI stock solution with final concentration of 1 mg/mL, dissolve 25 mg in 25 mL H₂O. Prepare aliquots à 1 mL in dark tubes, store at 4 °C protected from light.

β -Mercaptoethanol

To prepare a 10 mM stock solution, add 7 μ L β -mercaptoethanol to 10 mL RPMI, store at 4 °C protected from light.

Test Compounds

Solvent selection:

Solubility of each chemical is tested and evaluated by visual inspection before day 1 of the experiment. If no precipitates or droplets/cloudiness occur in DMSO after 30 min or in cell culture medium after 5 min, the solvent is considered appropriate. DMSO should be used as solvent whenever possible.

1. Test chemicals are ideally dissolved in DMSO in a final concentration of 2 M.
2. If not soluble in DMSO at 2 M within 30 min, try to dissolve in culture medium at a final concentration of 16 mM.

3. If a chemical is also not soluble in cell culture medium at 16 mM within 5 min, try to dissolve in cell culture medium at 8 mM. In this case, THP-1 cells must be seeded in 90 μ L instead of 135 μ L on day 3 (see section "routine procedures", page 10), then test chemicals are added in 90 μ L per well instead of 45 μ L (resulting in a dilution step of 2 instead of 4).
4. If a chemical is not soluble in cell culture medium at 8 mM, try to dissolve in DMSO at 1 M, then 0.5 M, or in medium at 4 mM, and so on. In this case, chemicals cannot be tested up to 4000 μ M, thus a negative result must be considered inconclusive.

Preparation of stock solutions for exposure of the cells on day 3:

Freshly prepare stock solutions for each run on the day of exposure.

For test chemicals dissolved in DMSO, prepare 2 M stock solution before harvesting THP-1 cells. In contrast if cell culture medium is used as solvent, dissolve test chemical after seeding THP-1 cells in the 96 well plate to avoid prolonged interaction of chemical with proteins contained in complete medium before exposure of the cells.

Positive Control(s)

2,4-Dinitrochlorobenzene (DNCB) is used as positive control at a final concentration of 20 μ M. DNCB is dissolved in DMSO to a final concentration of 50 mM. This solution is further diluted by addition of 10 μ L of this 50 mM solution to 40 μ L DMSO (final concentration: 10 mM).

Negative Control(s)

Sodium dodecyl sulfate is used as negative control at a final concentration of 144 μ M. A 20 % (=693.5 mM) stock solution is further diluted by addition of 10 μ L of this 20 % solution to 857 μ L complete THP-1 medium (final concentration: 8 mM).

Method

EXPERIMENTAL SYSTEM PROCUREMENT

Cell stocks are to be prepared by the test lab from the cells obtained from a reliable cell bank.

ROUTINE PROCEDURES

Maintenance of HaCaT cells

HaCaT cells are maintained in DMEM supplemented with 10 % FCS and 1 % antibiotics solution in the presence of 5 % CO₂ at 37 °C. Cells are harvested at 70 – 80 % confluence. Cell culture medium is withdrawn, cells are washed twice with prewarmed PBS and detached by incubation with trypsin-EDTA (2 mL per cell culture flask (75 cm²)) at 37 °C for 5 min. Cells are transferred to 50 mL sample tubes and centrifuged (300 x g, 5 min, RT). Supernatant is withdrawn and cell pellet is resuspended in prewarmed fresh culture medium (0.5 mL / cell culture flask (75 cm²)). For counting using a Neubauer hemacytometer, 10 μ L of cell suspension is mixed with 90 μ L trypan blue solution. Cells are seeded in an initial density of 6 and 4 x 10³ cells/cm², for reaching 70 – 80 % confluence after 3 or 4 days, respectively.

Maintenance of THP-1 cells

THP-1 cells are maintained in RPMI supplemented with 10 % FCS, 25 mM HEPES, 4 mM L-glutamine and 1 % antibiotics solution in the presence of 5 % CO₂ at 37 °C. For harvesting, cells are transferred from cell culture flasks to 50 mL sample tubes and centrifuged (300 x g, 5 min, RT). Supernatant is withdrawn, and cell pellet is resuspended in 10 mL prewarmed PBS. Upon centrifugation (300 x g, 5 min, RT), cells are resuspended in prewarmed fresh culture medium (2 mL / cell culture flask (75 cm²)). For counting using a Neubauer hemacytometer, 10 µL of cell suspension is mixed with 90 µL trypan blue solution. Cells are seeded in an initial density of 2 and 1 x 10⁵ cells/mL in a final volume of 30 mL per 75 cm² cell culture flask for routine culture every 3 or 4 days, respectively. Density of THP-1 cells should always be below 1 x 10⁶ cells/mL.

Seeding of HaCaT cells for testing (day 1)

HaCaT cells are harvested on day 1 (Monday or Tuesday). HaCaT cells are harvested and counted as described above, and seeded with an appropriate number of cells to reach 100 % confluence within 48 h (based on our experience: 2.5 x 10⁴ cells/well). To seed 2.5 x 10⁴ cells in 200 µL culture medium per well in two 96 well plates (at least 6 rows on one plate and 4 rows on another plate), an excess volume of cell suspension sufficient for e.g. 130 wells is prepared. Here, the estimated volume of HaCaT cell suspension comprising 3.25 x 10⁶ cells (i.e. 130 x 2.5 x 10⁴ cells), are transferred to a sample tube with a final volume of 22 mL (i.e. 130 x 200 µL) of complete HaCaT culture medium. Upon resuspension, cells are distributed by adding 200 µL per well. Care has to be taken to ensure even distribution of HaCaT cells in the sample tube during seeding.

Addition of THP-1 cells for testing (day 3)

THP-1 cells are harvested and counted as described above. To seed 8 x 10⁴ cells in 135 µL culture medium per well (or in 90 µL if the test chemical can only be dissolved in cell culture medium at 8 mM, see above), an excess volume of cell suspension sufficient for e.g. 130 wells is being prepared by transferring the estimated volume of THP-1 cell suspension containing 10.4 x 10⁶ cells (i.e. 130 x 8 x 10⁴ cells) to a sample tube with a final volume of 17.55 mL (or 11.7 mL, i.e. 130 x 135 µL or 90 µL) complete THP-1 medium. The 96 well plate containing confluent HaCaT cells is carefully inspected microscopically, HaCaT monolayer should be 100 % confluent. HaCaT medium is withdrawn and 100 - 200 µL of complete THP-1 medium per well is added in order to wash residual HaCaT medium. To avoid running dry of HaCaT cells, handle a reasonable number of wells simultaneously (or use multichannel pipettes). After having changed culture medium in the 96 well plates, withdraw culture medium from wells and add the appropriate volume of cell culture medium containing 8 x 10⁴ THP-1 cells of the prepared THP-1 cell suspension. Again, avoid running dry of HaCaT cells and sedimentation of THP-1 cells in the sample tube.

After each well contains confluent HaCaT cells and THP-1 cells in 135 µL (or in 90 µL if the test chemical can only be dissolved in cell culture medium at 8 mM, see above) complete THP-1 medium, continue with the addition of test chemicals after letting cells settle down.

TEST MATERIAL EXPOSURE PROCEDURES

After addition of THP-1 cells, proceed with test chemicals. Freshly prepare the stock solution in pure DMSO (or in complete THP-1 medium if not soluble in DMSO) after addition of THP-1 cells.

If the test chemical is dissolved in DMSO, proceed as follows:

Prepare 2-fold dilutions in pure DMSO. Use a U bottom 96-well plate ("plate I") for 2-fold serial dilutions of test chemicals (TC), one row per chemical. Add >60 μL of the stock solution in second well of each row, then pipette 40 μL solvent (DMSO or if not soluble in DMSO use culture medium) in 9 wells per row, beginning in well 3 of each row. For each test chemical, serial dilutions are prepared by transferring 40 μL from column 1 to column 2, mix by repeated rigorous pipetting in column 2, then transfer 40 μL from column 2 to column 3 and so on. In column 12, add 40 μL of pure DMSO as solvent control in row A, add 40 μL of the positive control 10 mM DNCB stock solution (see below) in row B, add 180 μL of 8mM SDS in row C (dissolved in medium) and 20 μL of DMSO in row C (1.1-fold dilution, 7.2 mM SDS).

Plate I

	1	2	3	4	5	6	7	8	9	10	11	12
A		TC 1, 2 M	TC 1, 1 M	TC 1, 0.5 M	TC 1, 0.25 M	TC 1, 0.125 M	TC 1, 0.063 M	TC 1, 0.031 M	TC 1, 0.016 M	TC 1, 0.008 M	TC 1, 0.004 M	DMSO
B		TC 2, 2 M	TC 2, 1 M	TC 2, 0.5 M	TC 2, 0.25 M	TC 2, 0.125 M	TC 2, 0.063 M	TC 2, 0.031 M	TC 2, 0.016 M	TC 2, 0.008 M	TC 2, 0.004 M	DNCB, 10 mM
C		TC 3, 2 M	TC 3, 1 M	TC 3, 0.5 M	TC 3, 0.25 M	TC 3, 0.125 M	TC 3, 0.063 M	TC 3, 0.031 M	TC 3, 0.016 M	TC 3, 0.008 M	TC 3, 0.004 M	180 μL SDS + 20 μL DMSO
D-H												

Prepare a fresh deep-well 96 well plate ("plate II"): Pipette 496 μL of complete THP-1 medium into rows A-C (11 wells in rows A and B, 10 wells in row C) Add 230 μL of complete THP-1 medium into row C, well 12.

Plate II

	1	2	3	4	5	6	7	8	9	10	11	12
A	496 μL complete THP-1 medium in each well											
B	496 μL complete THP-1 medium in each well											
C	496 μL complete THP-1 medium in each well										230 μL complete THP-1 medium	
D-H												

Pipette 4 μL from row A of plate I (containing serial dilutions of test chemical 1 in DMSO, and DMSO as solvent control in column 12) into row A of the deep-well plate (Plate II, 125-fold dilution), mix by repeated pipetting, then transfer 3x 45 μL each to rows B-D of a 96 well plate containing cells in 135 μL medium ("Plate III", 4-fold dilution). Proceed with row B and C of plate II accordingly. Transfer 20 μL of 200 μL from plate I row D, well 11 to plate II row C, well 12 (12.5-fold dilution), mix by repeated pipetting, then transfer 3x 45 μL on a 96 well plate containing HaCat and THP-1 cells in 135 μL medium (exposure plate, 4-fold dilution). Plates are then incubated for 24 h in the CO_2 incubator.

Overview of the dilutions and addition of test chemicals to the cells(if test chemical is soluble in DMSO):

Plate I:

	1	2	3	4	5	6	7	8	9	10	11	12
A		TC 1, 2 M	TC 1, 1 M	TC 1, 0.5 M	TC 1, 0.25 M	TC 1, 0.125 M	TC 1, 0.063 M	TC 1, 0.031 M	TC 1, 0.016 M	TC 1, 0.008 M	TC 1, 0.004 M	100% DMSO
B		TC 2, 2 M	TC 2, 1 M	TC 2, 0.5 M	TC 2, 0.25 M	TC 2, 0.125 M	TC 2, 0.063 M	TC 2, 0.031 M	TC 2, 0.016 M	TC 2, 0.008 M	TC 2, 0.004 M	DNCB, 10 mM
C		TC 3, 2 M	TC 3, 1 M	TC 3, 0.5 M	TC 3, 0.25 M	TC 3, 0.125 M	TC 3, 0.063 M	TC 3, 0.031 M	TC 3, 0.016 M	TC 3, 0.008 M	TC 3, 0.004 M	7.2 mM SDS in 10% DMSO
D-H												



125-fold dilution in culture medium
(transfer 4 µL from plate I to plate II containing 496 µL or 230 µL complete THP-1 medium respectively)

Plate II:

	1	2	3	4	5	6	7	8	9	10	11	12
A		TC 1, 16 mM	TC 1, 8 mM	TC 1, 4 mM	TC 1, 2 mM	TC 1, 1 mM	TC 1, 500 µM	TC 1, 250 µM	TC 1, 125 µM	TC 1, 62.5 µM	TC 1, 31.3 µM	0.8% DMSO
B		TC 2, 16 mM	TC 2, 8 mM	TC 2, 4 mM	TC 2, 2 mM	TC 2, 1 mM	TC 2, 500 µM	TC 2, 250 µM	TC 2, 125 µM	TC 2, 62.5 µM	TC 2, 31.3 µM	DNCB, 80 µM
C		TC 3, 16 mM	TC 3, 8 mM	TC 3, 4 mM	TC 3, 2 mM	TC 3, 1 mM	TC 3, 500 µM	TC 3, 250 µM	TC 3, 125 µM	TC 3, 62.5 µM	TC 3, 31.3 µM	576 µM SDS in 0.8% DMSO
D-H												



4-fold dilution in culture medium
(transfer 3x45 µL from plate II into exposure plate)

Exposure Plates (containing HaCaT and THP-1 cells):

	1	2	3	4	5	6	7	8	9	10	11	12
A		TC 1, 4000 µM	TC 1, 2000 µM	TC 1, 1000 µM	TC 1, 500 µM	TC 1, 250 µM	TC 1, 125 µM	TC 1, 62.5 µM	TC 1, 31.3 µM	TC 1, 15.6 µM	TC 1, 7.8 µM	0.2% DMSO
B		TC 1, 4000 µM	TC 1, 2000 µM	TC 1, 1000 µM	TC 1, 500 µM	TC 1, 250 µM	TC 1, 125 µM	TC 1, 62.5 µM	TC 1, 31.3 µM	TC 1, 15.6 µM	TC 1, 7.8 µM	0.2% DMSO
C		TC 1, 4000 µM	TC 1, 2000 µM	TC 1, 1000 µM	TC 1, 500 µM	TC 1, 250 µM	TC 1, 125 µM	TC 1, 62.5 µM	TC 1, 31.3 µM	TC 1, 15.6 µM	TC 1, 7.8 µM	0.2% DMSO
D		TC 2, 4000 µM	TC 2, 2000 µM	TC 2, 1000 µM	TC 2, 500 µM	TC 2, 250 µM	TC 2, 125 µM	TC 2, 62.5 µM	TC 2, 31.3 µM	TC 2, 15.6 µM	TC 2, 7.8 µM	20 µM DNCB
E		TC 2, 4000 µM	TC 2, 2000 µM	TC 2, 1000 µM	TC 2, 500 µM	TC 2, 250 µM	TC 2, 125 µM	TC 2, 62.5 µM	TC 2, 31.3 µM	TC 2, 15.6 µM	TC 2, 7.8 µM	20 µM DNCB
F		TC 2, 4000 µM	TC 2, 2000 µM	TC 2, 1000 µM	TC 2, 500 µM	TC 2, 250 µM	TC 2, 125 µM	TC 2, 62.5 µM	TC 2, 31.3 µM	TC 2, 15.6 µM	TC 2, 7.8 µM	20 µM DNCB
G-H												

	1	2	3	4	5	6	7	8	9	10	11	12
A		TC 3, 4000 µM	TC 3, 2000 µM	TC 3, 1000 µM	TC 3, 500 µM	TC 3, 250 µM	TC 3, 125 µM	TC 3, 62.5 µM	TC 3, 31.3 µM	TC 3, 15.6 µM	TC 3, 7.8 µM	144 µM SDS in 0.2% DMSO
B		TC 3, 4000 µM	TC 3, 2000 µM	TC 3, 1000 µM	TC 3, 500 µM	TC 3, 250 µM	TC 3, 125 µM	TC 3, 62.5 µM	TC 3, 31.3 µM	TC 3, 15.6 µM	TC 3, 7.8 µM	144 µM SDS in 0.2% DMSO
C		TC 3, 4000 µM	TC 3, 2000 µM	TC 3, 1000 µM	TC 3, 500 µM	TC 3, 250 µM	TC 3, 125 µM	TC 3, 62.5 µM	TC 3, 31.3 µM	TC 3, 15.6 µM	TC 3, 7.8 µM	144 µM SDS in 0.2% DMSO
D-H												

If the test chemical is dissolved in culture medium at 16 mM, proceed as follows:

Prepare 2-fold dilutions in complete THP-1 culture medium. Use a deep-well 96 well plate ("plate I") for 2-fold serial dilutions of test chemicals (TC), one row per chemical. Add >200 μ L of the stock solution in second well of each row, then pipette 180 μ L complete THP-1 culture medium in 9 wells per row, beginning in well 3 of each row. For each test chemical, serial dilutions are prepared by transferring 180 μ L from column 1 to column 2, mix by repeated rigorous pipetting in column 2, then transfer 180 μ L from column 2 to column 3 and so on. In column 12, add 180 complete THP-1 culture medium in row A, add 4 μ L of the positive control 10 mM DNCB stock solution (see below) in row B containing 496 μ L complete THP-1 medium, add 18 μ L of 8mM SDS in row C (dissolved in medium) containing 232 μ L medium. Then transfer 3x 45 μ L to the plates containing HaCat and THP-1 cells in 135 μ L culture medium (exposure plate, 4-fold dilution). Plates are then incubated for 24 h in the CO₂ incubator.

Plate I:

	1	2	3	4	5	6	7	8	9	10	11	12
A		TC 1, 16 mM	TC 1, 8 mM	TC 1, 4 mM	TC 1, 2 mM	TC 1, 1 mM	TC 1, 500 μ M	TC 1, 250 μ M	TC 1, 125 μ M	TC 1, 62.5 μ M	TC 1, 31.3 μ M	Medium
B		TC 2, 16 mM	TC 2, 8 mM	TC 2, 4 mM	TC 2, 2 mM	TC 2, 1 mM	TC 2, 500 μ M	TC 2, 250 μ M	TC 2, 125 μ M	TC 2, 62.5 μ M	TC 2, 31.3 μ M	DNCB, 80 μ M
C		TC 3, 16 mM	TC 3, 8 mM	TC 3, 4 mM	TC 3, 2 mM	TC 3, 1 mM	TC 3, 500 μ M	TC 3, 250 μ M	TC 3, 125 μ M	TC 3, 62.5 μ M	TC 3, 31.3 μ M	576 μ M SDS
D-H												



4-fold dilution in culture medium
(transfer 3x45 μ L from plate II into exposure plate containing 135 μ L cells)

Exposure Plates

	1	2	3	4	5	6	7	8	9	10	11	12
A		TC 1, 4000 μ M	TC 1, 2000 μ M	TC 1, 1000 μ M	TC 1, 500 μ M	TC 1, 250 μ M	TC 1, 125 μ M	TC 1, 62.5 μ M	TC 1, 31.3 μ M	TC 1, 15.6 μ M	TC 1, 7.8 μ M	Medium
B		TC 1, 4000 μ M	TC 1, 2000 μ M	TC 1, 1000 μ M	TC 1, 500 μ M	TC 1, 250 μ M	TC 1, 125 μ M	TC 1, 62.5 μ M	TC 1, 31.3 μ M	TC 1, 15.6 μ M	TC 1, 7.8 μ M	Medium
C		TC 1, 4000 μ M	TC 1, 2000 μ M	TC 1, 1000 μ M	TC 1, 500 μ M	TC 1, 250 μ M	TC 1, 125 μ M	TC 1, 62.5 μ M	TC 1, 31.3 μ M	TC 1, 15.6 μ M	TC 1, 7.8 μ M	Medium
D		TC 2, 4000 μ M	TC 2, 2000 μ M	TC 2, 1000 μ M	TC 2, 500 μ M	TC 2, 250 μ M	TC 2, 125 μ M	TC 2, 62.5 μ M	TC 2, 31.3 μ M	TC 2, 15.6 μ M	TC 2, 7.8 μ M	20 μ M DNCB
E		TC 2, 4000 μ M	TC 2, 2000 μ M	TC 2, 1000 μ M	TC 2, 500 μ M	TC 2, 250 μ M	TC 2, 125 μ M	TC 2, 62.5 μ M	TC 2, 31.3 μ M	TC 2, 15.6 μ M	TC 2, 7.8 μ M	20 μ M DNCB
F		TC 2, 4000 μ M	TC 2, 2000 μ M	TC 2, 1000 μ M	TC 2, 500 μ M	TC 2, 250 μ M	TC 2, 125 μ M	TC 2, 62.5 μ M	TC 2, 31.3 μ M	TC 2, 15.6 μ M	TC 2, 7.8 μ M	20 μ M DNCB
G-H												

	1	2	3	4	5	6	7	8	9	10	11	12
A		TC 3, 4000 μ M	TC 3, 2000 μ M	TC 3, 1000 μ M	TC 3, 500 μ M	TC 3, 250 μ M	TC 3, 125 μ M	TC 3, 62.5 μ M	TC 3, 31.3 μ M	TC 3, 15.6 μ M	TC 3, 7.8 μ M	144 μ M SDS
B		TC 3, 4000 μ M	TC 3, 2000 μ M	TC 3, 1000 μ M	TC 3, 500 μ M	TC 3, 250 μ M	TC 3, 125 μ M	TC 3, 62.5 μ M	TC 3, 31.3 μ M	TC 3, 15.6 μ M	TC 3, 7.8 μ M	144 μ M SDS
C		TC 3, 4000 μ M	TC 3, 2000 μ M	TC 3, 1000 μ M	TC 3, 500 μ M	TC 3, 250 μ M	TC 3, 125 μ M	TC 3, 62.5 μ M	TC 3, 31.3 μ M	TC 3, 15.6 μ M	TC 3, 7.8 μ M	144 μ M SDS
D-H												

If the test chemical is dissolved in culture medium at 8 mM, proceed as follows: Prepare 2-fold dilutions in complete THP-1 culture medium. Use a deep-well 96 well plate ("plate I") for 2-fold serial dilutions of test chemicals (TC), one row per chemical. Add >350 μ L of the stock solution in second well of each row, then pipette 300 μ L complete THP-1 culture medium in 9 wells per row, beginning in well 3 of each row. For each test chemical, serial dilutions are prepared by transferring 300 μ L from column 1 to column 2, mix by repeated rigorous pipetting in column 2, then transfer 300 μ L from column 2 to column 3 and so on. In column 12, add 300 complete THP-1 culture medium in row A, add 2 μ L of the positive control 10 mM DNCB stock solution (see below) in row B containing 498 μ L complete THP-1 medium, add 9 μ L of 8mM SDS in row C (dissolved in medium) containing 241 μ L medium. Then transfer 3x 90 μ L to the plates containing HaCat and THP-1 cells in 90 μ L culture medium (exposure plate, 2-fold dilution). Plates are then incubated for 24 h in the CO₂ incubator.

Plate I:

	1	2	3	4	5	6	7	8	9	10	11	12
A		TC 1, 8 mM	TC 1, 4 mM	TC 1, 2 mM	TC 1, 1 mM	TC 1, 500 μ M	TC 1, 250 μ M	TC 1, 125 μ M	TC 1, 62.5 μ M	TC 1, 31.3 μ M	TC 1, 15.6 μ M	Medium
B		TC 2, 8 mM	TC 2, 4 mM	TC 2, 2 mM	TC 2, 1 mM	TC 2, 500 μ M	TC 2, 250 μ M	TC 2, 125 μ M	TC 2, 62.5 μ M	TC 2, 31.3 μ M	TC 2, 15.6 μ M	DNCB, 40 μ M
C		TC 3, 8 mM	TC 3, 4 mM	TC 3, 2 mM	TC 3, 1 mM	TC 3, 500 μ M	TC 3, 250 μ M	TC 3, 125 μ M	TC 3, 62.5 μ M	TC 3, 31.3 μ M	TC 3, 15.6 μ M	288 μ M SDS
D-H												



2-fold dilution in culture medium
(transfer 3x90 μ L from plate II into exposure plate containing 90 μ L cells)

Exposure Plates

	1	2	3	4	5	6	7	8	9	10	11	12
A		TC 1, 4000 μ M	TC 1, 2000 μ M	TC 1, 1000 μ M	TC 1, 500 μ M	TC 1, 250 μ M	TC 1, 125 μ M	TC 1, 62.5 μ M	TC 1, 31.3 μ M	TC 1, 15.6 μ M	TC 1, 7.8 μ M	Medium
B		TC 1, 4000 μ M	TC 1, 2000 μ M	TC 1, 1000 μ M	TC 1, 500 μ M	TC 1, 250 μ M	TC 1, 125 μ M	TC 1, 62.5 μ M	TC 1, 31.3 μ M	TC 1, 15.6 μ M	TC 1, 7.8 μ M	Medium
C		TC 1, 4000 μ M	TC 1, 2000 μ M	TC 1, 1000 μ M	TC 1, 500 μ M	TC 1, 250 μ M	TC 1, 125 μ M	TC 1, 62.5 μ M	TC 1, 31.3 μ M	TC 1, 15.6 μ M	TC 1, 7.8 μ M	Medium
D		TC 2, 4000 μ M	TC 2, 2000 μ M	TC 2, 1000 μ M	TC 2, 500 μ M	TC 2, 250 μ M	TC 2, 125 μ M	TC 2, 62.5 μ M	TC 2, 31.3 μ M	TC 2, 15.6 μ M	TC 2, 7.8 μ M	20 μ M DNCB
E		TC 2, 4000 μ M	TC 2, 2000 μ M	TC 2, 1000 μ M	TC 2, 500 μ M	TC 2, 250 μ M	TC 2, 125 μ M	TC 2, 62.5 μ M	TC 2, 31.3 μ M	TC 2, 15.6 μ M	TC 2, 7.8 μ M	20 μ M DNCB
F		TC 2, 4000 μ M	TC 2, 2000 μ M	TC 2, 1000 μ M	TC 2, 500 μ M	TC 2, 250 μ M	TC 2, 125 μ M	TC 2, 62.5 μ M	TC 2, 31.3 μ M	TC 2, 15.6 μ M	TC 2, 7.8 μ M	20 μ M DNCB
G-H												

	1	2	3	4	5	6	7	8	9	10	11	12
A		TC 3, 4000 μ M	TC 3, 2000 μ M	TC 3, 1000 μ M	TC 3, 500 μ M	TC 3, 250 μ M	TC 3, 125 μ M	TC 3, 62.5 μ M	TC 3, 31.3 μ M	TC 3, 15.6 μ M	TC 3, 7.8 μ M	144 μ M SDS
B		TC 3, 4000 μ M	TC 3, 2000 μ M	TC 3, 1000 μ M	TC 3, 500 μ M	TC 3, 250 μ M	TC 3, 125 μ M	TC 3, 62.5 μ M	TC 3, 31.3 μ M	TC 3, 15.6 μ M	TC 3, 7.8 μ M	144 μ M SDS
C		TC 3, 4000 μ M	TC 3, 2000 μ M	TC 3, 1000 μ M	TC 3, 500 μ M	TC 3, 250 μ M	TC 3, 125 μ M	TC 3, 62.5 μ M	TC 3, 31.3 μ M	TC 3, 15.6 μ M	TC 3, 7.8 μ M	144 μ M SDS
D-H												

In each run, each concentration and control is tested in triplicates. Each test chemical is analyzed in three individual runs using cells from different passages and independently prepared stock solutions of the test chemical.

ENDPOINT MEASUREMENT

After 24h exposure, THP-1 cells are carefully resuspended in the 96 well plate, then cells are equally divided to 2 V-bottom 96 well plates (one for staining with anti-CD86 and anti-CD54 antibodies, and the other one for isotype controls). Remaining THP-1 cells are collected by washing the wells with 180 μ L PBS which is again equally distributed to the same wells of those 2 V-bottom plates. Efficient collection of all THP-1 cells is assured by microscopic inspection of the wells. Care has to be taken not to touch adherent HaCaT cells with pipette tips. V-bottom plates are centrifuged (250 x g, 5 min), cells are washed with 200 μ L PBS per well and centrifuged again (250 x g, 5 min) and supernatant discarded.

Antibody solutions are prepared by addition of 2 μ L of antibody in 50 μ L of PBS per well, i.e. a solution of 5 mL PBS containing 200 μ L (100 x 2 μ L) of anti-CD86 and 200 μ L of anti-CD54 antibodies, or containing 200 μ L of each corresponding isotype control.

50 μ L of these solutions are added to each well of the corresponding plate. Carefully mix by tapping at the sides by hand, then cells are incubated at 4 °C for 30 min in the dark.

Upon staining with antibodies, 150 μ L of PBS per well is added. V-bottom plates are centrifuged (250 x g, 5 min), supernatants are withdrawn and 200 μ L of PBS is added to each well.

Expression of CD86 and CD54 is analyzed via flow cytometry. For analysis on FACSVerse™ flow cytometer, cells are transferred to FACS tubes. Constant instrument settings are used to monitor any changes in the autofluorescence of the THP-1 cells. For each sample, 5 x 10³ cells are analyzed.

Immediately prior to analysis, 2 μ L of PI solution (final concentration of 10 μ g/mL) is added to each tube. Raw data are acquired using BD FACSuite v1.0.5. software.

ACCEPTANCE CRITERIA

Cell viability of THP-1 cells of DMSO controls should be > 75 %.

Cells exposed to the positive control DNCB should be > 50 % viable and Δ MFI of CD86 and CD54 should exceed to thresholds for positivity (10.8 and 300 for CD86 and CD54, respectively).

Cells exposed to the negative control SDS should be > 50 % viable and Δ MFI of CD86 and CD54 should not exceed to thresholds for positivity (10.8 and 300 for CD86 and CD54, respectively).

Data Analysis

Flow cytometric analysis

Calculate Δ MFI as indicator for upregulation of CD86 and CD54 as follows for each chemical:

corrected MFI = MFI of anti-CD86 or anti-CD54 stained subsample – MFI of corresponding isotype control stained subsample

Δ MFI = corrected MFI of chemical-treated cells – corrected MFI of solvent-treated cells

For each concentration of every chemical, the cell viability is recorded from the isotype control cells.

Estimation of EC Δ 10.8 and EC Δ 300

Additionally, if the threshold for positivity has been reached at a cell viability > 50 %, EC Δ 10.8 (CD86) and EC Δ 300 (CD54) can be estimated according to the following equation (similar to the EC3 derived from the local lymph node assay):

$$EC\Delta 10.8 = c + [(10.8 - d) / (b - d)] \times (a - c)$$

where:

a = the lowest concentration resulting in Δ MFI of CD86 \geq 10.8

b = the actual Δ MFI caused by concentration a

c = the highest concentration failing to produce a Δ MFI of CD86 of 10.8

d = the actual Δ MFI caused by concentration c

and

$$EC\Delta 300 = c + [(300 - d) / (b - d)] \times (a - c)$$

where:

a = the lowest concentration resulting in Δ MFI of CD54 \geq 300

b = the actual Δ MFI caused by concentration a

c = the highest concentration failing to produce a Δ MFI of CD54 of 300

d = the actual Δ MFI caused by concentration c

It should be noted that the vehicle control data (Δ MFI = 0) should not be used for coordinates c and d.

If the Δ MFI exceeds the threshold for positivity already at the lowest concentration, EC Δ 10.8 (CD86) or EC Δ 300 (CD54) is calculated by log-linear extrapolation using the two lowest concentrations according to the following equation:

$$EC\Delta 10.8 = 2^{\lceil \log_2 a + (\log_2 c - \log_2 a) / (b - d) * (10.8 - b) \rceil}$$

where:

a = the lowest concentration resulting in Δ MFI of CD86 \geq 10.8

b = the actual Δ MFI caused by concentration a

c = the second lowest concentration resulting in Δ MFI of CD86 > 10.8

d = the actual Δ MFI caused by concentration c

and

$$EC\Delta 300 = 2^{\left[\log_2 a + \frac{(\log_2 c - \log_2 a)}{(b - d)} * (300 - b) \right]}$$

where:

a = the lowest concentration resulting in Δ MFI of CD54 \geq 300

b = the actual Δ MFI caused by concentration a

c = the second lowest concentration resulting in Δ MFI of CD54 $>$ 300

d = the actual Δ MFI caused by concentration c

Prediction Model

Each chemical is tested in triplicates in three individual runs. If Δ MFI for CD86 and/or CD54 exceeds the threshold for positivity (Δ MFI \geq 10.8 for CD86, Δ MFI \geq 300 for CD54) at a concentration with cell viability $>$ 50 % in at least two runs, the test chemical is predicted as sensitizer.

Note:

If a chemical is not soluble in cell culture medium at 8 mM or in DMSO at 2 M, this chemical cannot be tested up to 4000 μ M. Thus, a negative result must be considered as inconclusive.

Annexes

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