LANXESS Deutschland GmbH

European Chemicals Agency
FI-00121 Helsinki Finland

Comments on the CLH report „Proposal for Harmonized Classification and Labelling based on Regulation (EC) No 1272/2008 (CLP Regulation), Thymol; 5-methyl-2-(propan-2-yl) phenol, Volume 1“ from February 2023

In this CLH proposal the Reference Member State (RSM) Spain proposes the classification of thymol as skin sensitizer, category 1 (H317).

We would like to provide for and on the behalf of the following registrants comments:

- LANXESS Deutschland GmbH, Kennedyplatz 1, 50569 Köln, Germany, and
- SYMRISE AG, Mühlenfeldstraße 1, 37603 Holzminden, Germany

While we support efforts to harmonize classification and ensure high protection of workers, consumers and environment, we are of the opinion that based on an evaluation of the scientific evidence provided in the CLH dossier, the proposed classification of thymol as Skin Sens. 1 is not justified, and we encourage the RAC not to reconsider the proposal made by the RSM.

Of critical importance, we would like to note that it was not sufficiently taken into account that thymol is classified as skin corrosive, category 1B. This normally grants a waiver for skin sensitization testing and explains why, currently, the REACH dossiers of the registrants report no or only historic data regarding skin sensitization.

Very recently we became aware of additional, new, *in silico* and *in vitro* studies not included in the current IUCLID dossier. We summarize these data below and intend to update the dossier in due time once data access for the REACH registrants is clarified.

Overall, as laid out below, neither the historic animal data, nor the human data or the newly generated *in vitro* data are sufficient for a classification of thymol as a skin sensitiser. Consequently, the CLH dossier should be rejected as not supporting a classification of thymol as Skin Sens. 1.

September 01, 2023

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Human data:

The studies on existing skin sensitization in humans do not warrant a skin sensitization classification.
In all three studies reporting on patch test results in unselected dermatitis patients (Itoh et al., 1988, Dohn, 1980, Meneghini at al. 1971), rates of positive reactions to thymol were clearly below 1%. In all studies with selected dermatitis patients (Berova et al., 1990, Djerassi and Berowa, 1966, Nethercott et al. 1989, Rantuccio and Meneghini, 1970) report rates of positive reactions to thymol were clearly below 2% with the exception of the oldest of these studies (Djerassi and Berowa, 1966). In the oldest study, 71% of stomatology office workers reacted to one or more of the patch tested materials, including 39 of 300 reactions that were positive towards thymol (Djerassi and Berowa, 1966). This study tested a very high concentration of 5% while all other patch test studies used 1% which suggests a high likelihood for confounding irritation reactions. Also, the many reactions to other substances could have provoked a false positive response to thymol ('angry back syndrome'). Without analyzing the individual patients' data this study is difficult to evaluate, especially since its result is not supported by any of the other studies. Taking together all human studies deemed reliable, the number of reported cases is eight (8) persons and thus, clearly smaller than 100. In summary, the human data do not support a classification conclusion according to CLP criteria.
Medical surveillance data on approx. 65 workers collected on one manufacturing plant at LANXESS Deutschland in Krefeld-Uerdingen, Germany between 2018 and 2022 did not reveal any health effects or symptoms indicating sensitization of skin potential (Currenta 2023; Annex 1).
The human data on thymol which is the major constituent of thyme oil, and on thyme oil itself were also evaluated by the Scientific Committee on Consumer Products (SCCS) in their "Opinion on Fragrance Allergens in Cosmetic Products (2012, SCCS/1459/11)". The SCCS has put thyme oil into the group of "Natural extracts with positive human data, which are, however, not sufficient to categorise as "established contact allergen in humans"". Consequently, they did not recommend to put thyme oil among fragrance allergens that should be labelled on consumer products for customer information.

[redacted]

Animal data:

The CLH dossier references animal studies with considerable methodological and qualitative doubts that question the usefulness for relevance for classification as Skin Sens. 1.

As reported in the CLH dossier, the listed animal studies are considered supportive information showing limitations regarding the level of reporting (dermal responses after application in preliminary and main study, clinical signs, body weights), absence of positive control (test substances were considered positive to humans) and test substance characterization, poorly described method and results and lack of information regarding GLP conditions.
Additionally, the studies are only cited from secondary literature with no (or limited) access to the original publication which significantly complicates full examination of the original data.
Due to insufficient documentation and absence of key information, the studies are inadequate for the evaluation process from our point of view.

Furthermore, all referenced animal studies are outdated (≥26 years old) and are based on experimental investigations in guinea pigs. The studies preceded the introduction of OECD test guidelines and GLP guidelines which severely reduce their ability to serve as a basis for a classification conclusion. The lack of more recent studies raises concerns about the validity and reliability of the studies when measured against today's standards. According to ECHA's "Guidance on the preparation of dossiers for harmonised classification and labeling", the animal method of first choice is the Local Lymph Node Assay (LLNA) using mice. Since the Guinea Pig Maximization Test (GPMT) is regarded as overpredictive due to its use of adjuvant, the potential of a substance being overestimated as weak sensitizer is higher in the GPMT than compared to the LLNA. Unfortunately, this methodological weakness has not been brought forward by the dossier submitter.

[redacted]

Furthermore, the CLH proposal selectively relies on a single weak positive GPMT (CTFA, 1997). This portrays an inaccurate and incomplete picture because it omits negative results mentioned in the CLH report for a weight-of-evidence determination. The guinea pig studies with negative results used different methods, i.e., open epicutaneous test, Draize test, maximisation test and Freund's complete adjuvant test. Especially the latter is considered to be even more sensitive than the GPMT. Unfortunately, the CLH report does not provide a rationale for the non-inclusion of these studies.

A further GPMT was published by Ishihara et al., 1986 and also reported negative results at 10% (for both induction and challenge).
Focussing on the GPMT amongst the guinea pig studies, a total of three GPMT are available:

- Escobar, A. (2006) secondary citation of a GPMT using 10% thymol for intradermal induction, 10% for topical induction and 5, 10 and 20% for challenge reported weak positive results only at 20%. A review of the respective study, which was limited to results table no 2 and

conclusion table no 1 of the study due to limited access to the original publication, has shown that skin reactions were reversible after 24 hours indicating a skin irritating rather than a skin sensitizing effect. A rechallenge would have provided clarity on the skin sensitizing potential of thymol, however, rechallenge was not performed leaving a doubt whether this study result should be considered positive.

- Ishihara et al. (1986) (copy of study attached; Annex 2) also used 10% for both induction and challenge, respectively, and reported that less than 30% of the animals showed a skin reaction, thus leading to a negative conclusion for the study result.

- Klecak et al. (1977) used 5% for intradermal induction, 25% for topical induction (which cause skin irritation) and <25% (not given) for challenge, and reported no effects.

Given that the corrosive properties of thymol make topical testing in animals very difficult and prone to interference by irritation effects, the different laboratories used similar thymol concentrations for induction and challenge in GPMTs. Also, since different guinea pig species, i.e., Dunkin-Hartley and Himalayan, were used in the studies, the study reporting weak positive findings cannot be attributed a higher weight of evidence than the other two studies. In conclusion, the animal studies have considerable methodological and reporting deficiencies and are considered not adequate as a basis for a classification conclusion. Ideally, a more objective analysis as provided by the LLNA would have provided stronger evidence. These major flaws do not provide support for the proposed classification and the evidence underlying the proposal are inappropriate to provide any meaningful information as to potential skin sensitizing effects of thymol.

In vitro/ in chemico and *in silico* data:

Annex VII Section 8.3 of REACH Regulation (EC) No 1907/2006 demands that skin sensitisation should be addressed primarily by *in vitro/in chemico* test methods. These data have very recently become available and are summarised below.

QSAR data were generated using the automated workflow of the OECD QSAR Toolbox. Results by QSAR were negative indicating that the substance is not sensitizing (LANXESS 2023; Annex 3).

- Thymol was negative in the direct peptide binding assay (OECD TG 442C) (robust study summary attached; Annex 4).
- Thymol was negative in the KeratinoSens™ assay (OECD TG 442D) (robust study summary attached; Annex 5).

- Thymol was positive in the human cell line activation test (OECD TG 442E) (robust study summary attached; Annex 6).

According to the 2-out-of-3 rule for interpreting the *in vitro* testing battery, the overall conclusion would be that thymol should not be considered a skin sensitizer and, thus, should not be classified according to CLP criteria (OECD TG 497).

Overall conclusion:

The CLP criteria require a weight-of-evidence assessment including all data that inform the skin sensitization endpoint and stipulate a threshold beyond which a chemical must be classified and labelled as a skin sensitizer with good reason. Single cases of allergic skin reactions can be found against virtually all organic and inorganic substances (for example even including metallic aluminium). However, classifying substances as Skin Sens. 1 based on just few reported human cases of skin sensitization while *in vitro* and animal data do not support such a classification is counterproductive with regard to public health as it would hamper identification, avoidance and replacement of truly relevant skin sensitizers.

The CLP regulation usually considers data from experimental studies as more reliable: “Evidence from animal studies is usually much more reliable than evidence from human exposure”. Evaluation of the human, animal and *in vitro* data clearly lead to the conclusion that the battery of *in vitro/ in chemico* tests, which yielded a negative conclusion for skin sensitization hazard, is the most reliable because it is the data required according to the REACH Annex, it constitutes the newest data set (from 2022-2023) and has been performed in accordance with the newest OECD testing guidelines.

[redacted]

The CLP guidance demands “In cases where evidence is available from both sources, and there is conflict between the results, the quality and reliability of the evidence from both sources must be assessed in order to decide on the classification on a case-by-case basis.”
A conflict between the animal and *in vitro* experimental data and the human data exists for thymol: The animal studies and the *in vitro* studies, when evaluated separately, each come to the conclusion that thymol should not be classified as a skin sensitizer. The human data indicate that cases of skin sensitization exist. Disregarding the one study that used a too high test concentration of 5%, the total number of human subjects that were identified with skin sensitization against thymol was eight (8) persons. The small number of cases of skin sensitization and the low frequency in both unselected and selected dermatitis patients are far below the ‘high frequency’ cut off values. Weighing the reliable and good quality *in vitro* data, supported by the animal and QSAR data, on the one hand side against the human data

which do not show sensitization in a substantial number of persons on the other hand side, lead to the conclusion that thymol does not meet the criteria for skin sensitization classification.

In summary, neither the historic animal data, nor the human data or the newly generated *in vitro/ in chemico* data are sufficient for a classification of thymol as skin sensitiser.

Therefore, the CLH dossier should be rejected as not supporting a classification of thymol as Skin Sens. 1.

Yours sincerely,

[redacted]

LANXESS Deutschland GmbH

[redacted]

References:

Itoh M, Hosono K, Kanthon H, Kinoshita M, Yamada K, Kurosaka R, et al., *Patch Tests Results with Cosmetic Ingredients Conducted between 1978 and 1986.* J Soc Cosmet Sci. 1988; 12:27-41.

Dohn W. *Dermatological patients not employed in handicraft or factories.* Contact Dermatitis. 1980; 6(2):148-50.

Meneghini CL, Rantuccio F, Lomuto M. *Additives, Vehicles, and Active Drugs of Topical Medicaments as Causes of Delayed-Type Allergic Dermatitis.* Dermatologica. 1971; 143:137-47.

Berova N, Stransky L, Krasteva M. *Studies on contact dermatitis in stomatological staff.* Dermatol Monatsschr. 1990; 176(1):15-8.

Djerassi E, Berowa N. *[Contact allergy in stomatology as an occupational problem].* Berufsdermatosen. 1966; 14(5):225-33.

Nethercott JR, Nield G, Holness DL. *A review of 79 cases of eyelid dermatitis.* J Am Acad Dermatol. 1989; 21(2 Pt 1):223-30.

Rantuccio F, Meneghini CL. *Results of patch testing with cosmetic components in consecutive eczematous patients.* Contact Dermatitis. 1970; Newsletter.

CTFA, *Delayed contact hypersensitivity study in guinea pigs of thymol.* December 09, 1997

Ishihara M, Ito M., Nishimura M., Kinoshita M., Kanto H., Nogami T., Yamada K. *Closed epicutaneous test.* 1986, Skin Res. 28 (2), 230-240

Escobar A. *Final Report on the Safety Assessment of Sodium p-Chloro-m-Cresol, p-Chloro-m-Cresol, Chlorothymol, Mixed Cresols, m-Cresol, o-Cresol, p-Cresol, Isopropyl Cresols, Thymol, o-Cymen-5-ol, and Carvacrol.* Int J Toxicol. 2006; 25 Suppl 1:29-127.

Klecak, G; Geleick, H; Frey, JR. *Screening of fragrance materials for allergenicity in the guinea pig I. Comparison of four testing methods.* Journal of Cosmetic Science, ISSN: 1525-7886, EISSN: 2689-5153

OECD. *OECD TG 497 Defined Approaches on Skin Sensitisation.* 2003, https://www.oecd-ilibrary.org/environment/guideline-no-497-defined-approaches-on-skin-sensitisation_b92879a4-en

Annex:

Annex 1:
Currenta GmbH & Co. OHG, Prof. Dr. Gabriele Leng. *Medical surveillance data on manufacturing plant personnel*. August 29, 2023

Annex 2:
Ishihara M, Ito M., Nishimura M., Kinoshita M., Kanto H., Nogami T., Yamada K. *Closed epicutaneous test.* 1986, Skin Res. 28 (2), 230-240

Annex 3:
LANXESS Deutschland GmbH, *QMRF Title: Skin sensitization for DASS.* Septmber 1, 2023, OECD QSAR Toolbox (version 4.6)

LANXESS Deutschland GmbH, *QSAR Toolbox prediction for single chemical.* September 1, 2023, OECD QSAR Toolbox (version 4.6)

Annex 4:
Direct Peptide Reactivity Assay (DPRA): Robust Study Summary

Annex 5
Keratinosens™: Robust Study Summary

Annex 6:
Human cell line activation test (h-CLAT): Robust Study Summary

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2023-08-29

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Thymol – CAS No. 89-83-8

Dear [redacted]

Enclosed please find my comments regarding the medical aspects concerning Thymol.

Medical surveillance data on manufacturing plant personnel

The HYD-Plant of LANXESS Deutschland GmbH in Krefeld-Uerdingen produces for many years Thymol.

Occupational medical surveillance of workers exposed to Thymol has been performed every year on a routine basis. The medical examination is based on german rules and includes e.g. medical history, physical examination, spirometry, ECG/Ergometry, vision-testing, audiometry, laboratory examinations of blood and urine. Special diagnostic tools are not available for Thymol.

The following results are based on ca. 65 employees, investigated every year, between 2018 and 2022.

Results:

The occupational medical surveillance has not revealed any health effects which could be ascribed to a possible Thymol exposure. Poisoning incidents did not occur and symptoms indicating sensitization of skin were not observed.

Conclusion:

Symptoms indicating sensitization of skin were not observed during the production of Thymol at the site of Lanxess Deutschland GmbH in Krefeld-Uerdingen.

Yours sincerely

[redacted]

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Ishihara,1986

RIFM - FEMA Database

Location 5601

Title Closed epicutaneous test.

Authors M.Ishihara, M.Itoh, M.Nishimura, M.Kinoshita, H.Kantoh, T.Nogami and K.Yamada

Journal Skin Research, 28(suppl 2), 230-240.

Keywords HYDROXY; SKN; SEN; SAL; METHOD; TERP; KET; SPECIES; GPG; EUG; COU; COMPARE; CINN; CET; ALD; ALC; HMN; MAX

Abstract [Article in Japanese] Representative animal sensitization test procedures are classified according to the method of induction & challenge. The guinea pig maximization test is compared with the human maximization test using 41 fragrance materials & it is confirmed that the guinea pig test is significantly more sensitive than the human test. In the induction phase of the Closed Epicutaneous Test(CET), test materials are occluded for 48 hr on the shaved nape & this procedure is repeated 3 times/wk for 2 wks. Following a 2 wk rest period, the test materials are occluded for 48 hr on the flank for challenge. The results of the CET using fragrance materials, dye intermediates & modifiers used for hair colorings, ammonium thioglycollate, rubber acclerators & antioxidants indicate that the CET is less sensitive than the guinea pig maximization test. However, there is a better degree of agreement between the CET & human maximization test results than between the guinea pig & human test results.

Sub-Reference	Study	Route	Species
01	Sensitization	skin	guinea pig
02	Sensitization	skin	guinea pig

RIFM - FEMA Database

Another Brief Reference

Another Location

5601

Closed Epicutaneous Test について

15

石原　勝　伊藤　正俊　西村　誠　木下三和子
関東　裕美　野上　哲則　山田　耕次

モルモットの感作試験の代表的なものを挙げ，感作誘導及び誘発により分類した。モルモットの maximization test (GMT) とヒトのそれ (HMT) の成績を比較すると前者が鋭敏度が明らかに優れることが41種の合成香料の感作試験の比較成績で示された。これに対し項部に被験物を2日間密封，週3回計2週間に亘りこれを反復し，その2週後に側腹部に同物質を密封する closed epicutaneous test (CET) を諸合成香料素材，フェニレンジアミン系染料，ゴムの加硫促進剤その他について施行したところ，CET は GMT より感度が劣るが，HMT との相関は GMT より CET が高いこと等の成績を得た。

キーワード：モルモット・マキシミゼーションテスト—ヒト・マキシミゼーションテスト—密封経皮テスト—フェニレンジアミン

モルモットの感作試験法

諸化学物質の経皮感作能は専ら感作試験により検討される。接触皮膚炎患者で施行される化学物質の診断用パッチテストの陽性率は化学物質の感作能のほか，使用頻度，製品中心の濃度，溶媒または基剤，その他の実際の使用状況，被験者などに種々左右され，感作能とは必ずしも平行しないことが屢々経験される。例えば合成香料素材の場合 benzyl salicylate の診断用パッチテストの陽性率の順位は本邦では欧米と異なり常に上位を占めているが，その感作能が低いことはヒトあるいはモルモットの感作試験の成績で明らかにされており，本邦における使用頻度あるいは濃度が高いことが高陽性率をもたらしていることが多分に推測される。Cadby[1] も使用頻度が諸合成香料のパッチテスト陽性率に大きく影響することを報告している。

ヒトと諸実験動物では化学物質の経皮吸収や代謝がかなり異なるため，化学物質の感作能はヒトで施行されることが最も望ましいと考えられており，Research Institute for Fragrance Materials (RIFM) では Kligman[2-4] が開発し，Kligman and Epstein[5] が改良したヒトの maximization test (以下 HMT と略記) で諸合成香料素材の経皮感作能を検討してきた。ヒトの感作法としてはこの他 repeat insult patch test 法も米国において屢々実施されている。

他方，本邦並びに欧州ではヒトの感作試験の実施が困難なため，化学物質の経皮感作能は通常モルモットで検討されている。モルモットの感作能を調べる方法として1959年の Draize 法以後種々の感作法[6-16] が開発，実施されてきた。第1図に代表的なモルモットの感作試験法を示す。この他，本邦では高瀬法[7] も報告されている。感作誘導 (induction) 並びに誘発 (challenge) のいずれも外用，皮下注射，外用と皮下注射の併用の3つに分類される。また外用も単純塗擦と密封法の相違，注射も Freund の complete adjuvant 使用の有無がある。Freund の complete adjuvant の注射並びに密封法は何れも感作率を多分に高めることが認められており，両者を併用する。Magnusson and Kligman の guinea pig maximization test (GMT) はモルモットの諸感作試験中感度が最も優れる点で高い評価を受けてきた。

GMT の場合は諸化学物質の感作能は感作成立の陽性

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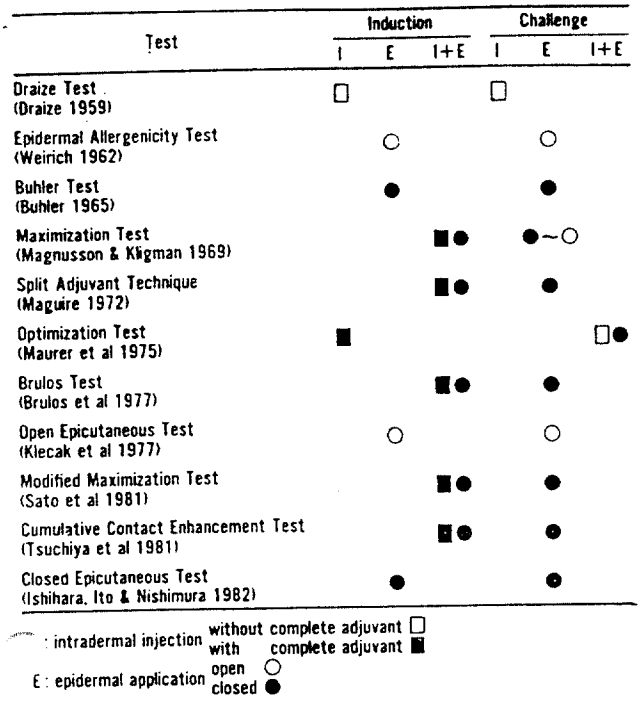


第1図　Animal Sensitization Test Procedures

率により，0～8％がⅠ (weak)，9～28％がⅡ (mild)，29～64％がⅢ (moderate)，65～80％がⅣ (strong)，81～100％がⅤ (extreme) と分類されるが，最近は紅斑（5段階）と浮腫（4段階）の程度の評価点 (mean score) の方がより重視されている。これは Hartley 系白色モルモットがかなり純系化され，以前は陽性率が低かった物質も最近のモルモットの試験では高陽性率を示す傾向があり，評価点の方が感作能の強弱の判定資料としてより有用と考えられているためである。ちなみに評価点が1.0以上のものは化粧品原料として通常不適当と評価されている。

合成香料のモルモットの maximization test の成績

以前 GMT と HMT とはほぼ同程度の成績が得られることがニッケルやホルムアルデヒドなどで報告[9, 18]されたが，近年施行された諸合成香料の GMT の成績と RIFM の HMT の成績は必ずしも一致せず，一般に GMT の陽性率が HMT のそれをかなり上廻ること，あるいは GMT では陽性でも HMT では陰性の例が少なくないことが認められている。

以下 GMT と HMT の比較成績を第1～5表に示す。なお GMT の成績は石原が米国の RIFM で講演する際高砂香料と資生堂の研究所の御好意で提供されたデーターを併せたものである。

12種のアルデヒド（第1表）の場合 HMT では citral と cinnamic aldehyde のみが陽性で他は全て陰性であるが，GMT では9種が陽性，かつ評価点が1.0以上のものが6種認められる。

アルコール（第2表）の場合，11種のうち HMT では cinnamic alcohol と farnesol のみが陽性があったが GMT では6種が陽性であった。但しアルコールの場合はアルデヒドと異なり評価点は陽性の6種いずれも1.0以下で感作能は低いと考えられた。

エステルとラクトン（第3表）の場合も HMT では8種全てが陰性であったのに対し GMT では6種が陽性で

第1表　Sensitization to Aldehydes

	materials	guinea pig maximization test				human maximization test			
		induction	challenge	rate	score	induction	challenge	rate	
5392-40-5	citral	10%	10%	V	3.4	6%	8%	III	(8/25) (2004)
104-55-2	cinnamic aldehyde	3%	3%	V	2.7	2%	2%	II	(11/25)
101-86-0	α-hexyl cinnamic aldehyde	10%	10%	IV	1.8	12%	12%	—	(0/25) (pending)
	undecyl aldehyde	10%	10%	V	2.4	5%	5%	—	(0/25)
106-23-0	citronellal	10%	10%	III	0.5	4%	4%	—	(0/25) (2004)
107-75-5	hydroxycitronellal	10%	10%	III	0.4	12%	12%	—	(0/25) (2004)
—	cyclamen aldehyde	10%	10%	V	2.2	12%	12%	—	(0/25)
—	lily aldehyde*	10%	10%	IV	2.0	4%	4%	—	(0/25)
121-33-5	vanillin	10%	10%	III	0.4	5%	5%	—	(0/25) (2004 / Dr Ro) !
	anisaldehyde	10%	10%	—	0	10%	10%	—	(0/25)
120-57-0	heliotropin**	10%	10%	—	0	6%	6%	—	(0/25) (2004)
—	citral dimethyl acetal	10%	10%	—	0	4%	4%	—	(0/25)

* p-tert-butyl-α-methyl hydroxy cinnamic aldehyde
** piperonal

第2表　Sensitization to Alcohols

materials	guinea pig maximization test				human maximization test		
	induction	challenge	rate	score	induction	challenge	rate
cinnamic alcohol	10%	10%	IV	0.7	10%	10%	II (11/86)
benzyl alcohol	10%	10%	III	0.8	10%	10%	— (0/25)
isobornylcyclohexanol	10%	10%	III	0.6	20%	20%	— (0/25)
santalol	10%	10%	II	0.2	20%	20%	— (0/25)
farnesol	10%	10%	III	0.7	12%	12%	II (13/100)
geraniol	10%	10%	III	0.5	6%	6%	— (0/25)
citronellol	10%	10%	—	0	6%	6%	— (0/25)
linalool	10%	10%	—	0	20%	20%	— (0/25)
l-menthol	10%	10%	—	0	8%	8%	— (0/25)
α-terpineol	10%	10%	—	0	12%	12%	— (0/25)
cedrol	10%	10%	—	0	8%	8%	— (0/25)

第3表　Sensitization to Esters and Lactones

materials	guinea pig maximization test				human maximization test		
	induction	challenge	rate	score	induction	challenge	rate
benzyl salicylate	10%	10%	V	1.6	30%	30%	— (0/25)
benzyl benzoate	10%	10%	III	0.4	30%	30%	— (0/25)
benzyl acetate	10%	10%	—	0	8%	8%	— (0/25)
methyl atrarate	10%	10%	III	0.6	10%	10%	— (0/25)
linalyl acetate	10%	10%	III	0.3	12%	12%	— (0/25)
ethylene brassylate	10%	10%	III	0.3	30%	30%	— (0/25)
geranyl acetate	10%	10%	—	0	4%	4%	— (0/25)
coumarin	10%	10%	IV	0.8	8%	8%	— (0/25)

第4表　Sensitization to Phenols and Ethers

materials	guinea pig maximization test				human maximization test		
	induction	challenge	rate	score	induction	challenge	rate
isoeugenol	2%	2%	V	2.5	10%	10%	IV (19/25)
					8%	8%	III (38/110)
eugenol	10%	10%	IV	1.1	8%	8%	— (0/25)
thymol	10%	10%	II	0.1	4%	4%	— (0/25)
anethol	10%	10%	—	0	2%	2%	— (0/25)

第5表　Sensitization to Ketones and Terpene Hydrocarbons

materials	guinea pig maximization test				human maximization test			
	induction	challenge	rate	score	induction	challenge	rate	
acetyl cedrene	10%	10%	V	2.6	30%	30%	−	(0/25)
8013-90-2 ionone	10%	10%	−	0	8%	8%	−	(0/25) (2mo4)
79-89-0 methyl ionone	10%	10%	−	0	10%	10%	−	(0/25) (2mo4)
cis-jasmone	10%	10%	−	0	8%	8%	−	(0/25)
127-91-3 β-pinene	10%	10%	−	0	12%	12%	−	(0/19) (2mo4)
d-limonene	10%	10%	−	0	8%	8%	−	(0/25)

あった。但しこの場合も benzyl salicylate を除く7種評価点はいずれも1.0以下であった。

フェノールとエーテル，ケトンとテルペンハイドロカーボンの成績を第4表並びに第5表に示す。これらの場合も GMT は HMT より感度が明らかに高い。

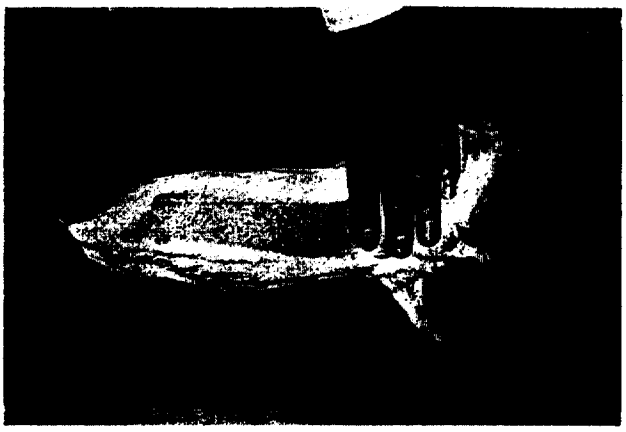
以上より，GMT は HMT より感度がかなり高く，HMT では陰性でも GMT 陽性の合成香料素材が少なからずみられること，GMT の場合陽性率よりも評価点の方が物質の感作能の程度をより反映することが結論として得られた。

Closed epicutaneous test の手技

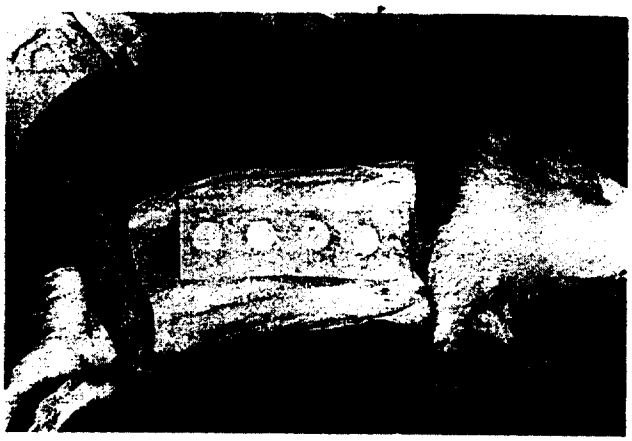
著者らは昭和54年（1979年）に現在 Closed epicutaneous test（以下 CET と略記）と呼称しているモルモットの感作法の研究に着手した。即ち感作誘導法として下注射は一切行わず，密封法を反復するのみとした。即ち本法では感作能を高める目的の Freund の complete adjuvant は使用せず，また単純塗擦のみで感作する Klecak ら[3] の Open epicutaneous test (OET) は感作率が低いことが考えられたので密封反復を施行した。密封を行う感作法として Bühler 法[8] が知られているが，一日6時間の密封を週1回ずつ3週間反復する方法で感作誘導を行うため，強力な感作物以外は陽性結果が得られない傾向がある。これに対し CET は被験物質を項部に48時間密封，週3回，2週間に亘りこれを反復して誘導する方法で，Bühler 法に比し誘導時の被験物質の皮膚接触時間を長くし，これにより感作率が高まることを期待したのである。

以下，実際の手技の大要を述べる。実験動物として Hartley 系白色モルモット（静岡実験動物農業協同組合）300〜350 g の雌を使用した。モルモットの両側腹部を電気バリカン（大東電気KK製，スライブモデル550，刃先0.05mm）で刈毛，さらに電気シェイバー（ブラウン製，ミクロン）で剃毛した（第2図）。次いで30〜0.1%の6段階濃度の試験試料（白色ワセリン基剤）約20mgをフィンチェンバー（EpitestFinn Chamber on Scanpol Tape）に塗布，両側腹部に貼布した（第3図）。その上を巾約5cm長さ約40cmの絆創膏（ニチバンKK製，キープシルク）2枚を用い胴体を巻き（第4図），フィンチェンバー部位を固定した。さらに巾約1.5cm長さ6cm厚さ1cmのレストンパッド（3M製）をフィンチェンバー部位の絆創膏の上に置き（第5図），次いで巾約7.5cm長さ約40cmの絆創膏でこれを固定，更に巾約5cm長さ約40cmの絆創膏2枚でその上を固定した（第6図）。48時間密封し，試料除去1時間後，24時間後，48時間後に刺激反応の有無を判定した。

一群10〜20匹のモルモットで感作試験を行った。項部を2.5cm四方に刈剃毛（第7図），最低刺激濃度あるいは最高無刺激濃度の試料約50mgをパッチテスト用絆創膏（鳥居製薬KK製，スモールサイズ）の布部分に均等に塗布し，項部に貼布した（第8図）。その上を巾約2.5cm長さ約30cmの絆創膏（ニチバンKK製，キープシルク）を巻いてパッチテスト用絆創膏を固定し（第9図），さらに巾約2cm四方のレストンパッドをパッチテスト用絆創膏部位の上に置き（第10図），その上を巾約2.5cm長さ約30cmの絆創膏で固定した（第11図）。この操作を週3回（月，水，金曜），2週間に亘り計6回反復した。次いで2週間の休止期後，刺激試験と同一の手技を用いて両側腹部に刺激濃度以下の諸濃度の試料を2日間密封し，惹起を行った（第12図）。

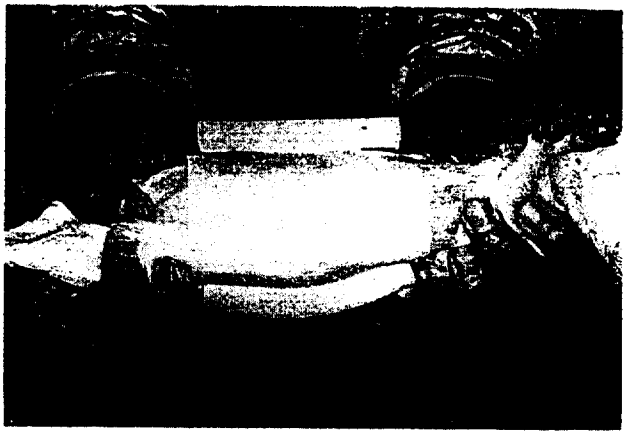


第2図　The flanks are clipped and shaved for the irritation test and for challenge.

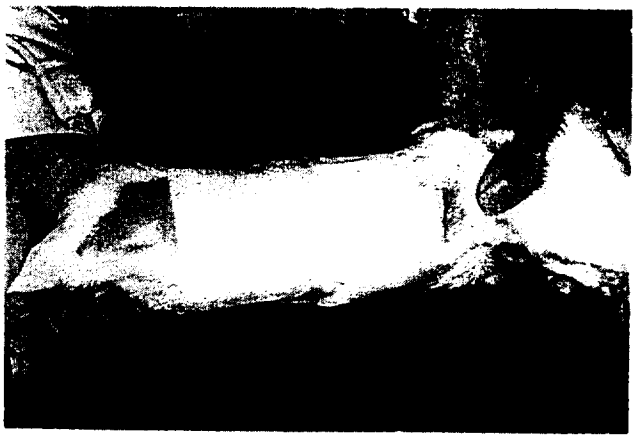


第3図　Finn chambers containing approx. 20 mg of test material are placed on the flank.

第4図　Two strips of adhesive tape are wraped around the animal completely covering the Finn chambers.



第5図　A Reston foam pad is placed over the Finn chambers.



第6図　A single strip and then two strips of adhesive tape are used to completely cover and secure the foam pad. After 48 hours, the test materials are removed.

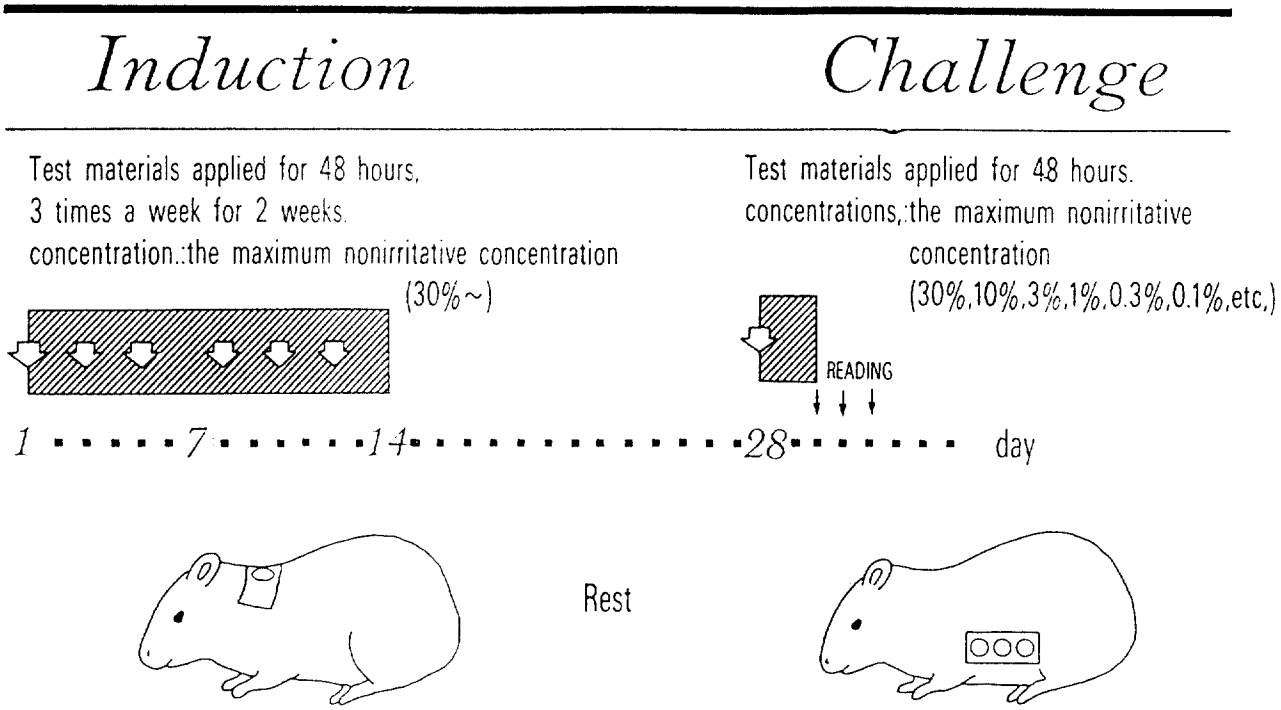
第7図　The nape is clipped and shaved for induction.

第8図　Round cloth of Torii's patch plaster (small size) containing 50 mg. of test material is applied on the nape.

第9図　Adhesive tape is wraped around the nape to secure the patch plaster.

第10図　A Reston foam pad is placed on the patched area.

第11図　A single strip of adhesive tape is used to secure the foam pad. This induction procedure is repeated three times per week for two weeks.



第 12 図

合成香料の Closed epicutaneous test の成績

第6表にフェノール系香料の刺激反応の成績を，第7表に7種のフェノール系合成香料，第8表に6種のサリチレート系合成香料，第9表にその他の9種の合成香料の CET 並びに HMT による感作成績の対比を示す。

フェノール系は HMT では isoeugenol のみが陽性であったが，CET では isoeugenol の他 acetyl isoeugenol, eugenol にも感作陽性例をみた[16]。サリチレート系合成香料は HMT では陽性のもの皆無であったが，CET

第6表　Frequency of Positive Irritation to Phenolic Type Compounds

compounds \ concentrations	10%	5%	2%	1%	0.5%	0.2%
eugenol	2	1	0	0	0	0
methyl eugenol	0	0	0	0	0	0
acetyl eugenol	0	0	0	0	0	0
isoeugenol	4	1	0	0	0	0
methyl isoeugenol	0	0	0	0	0	0
acetyl isoeugenol	0	0	0	0	0	0
benzyl isoeugenol	0	0	0	0	0	0

第7表　Sensitization Frequency of Some Phenolic Compounds

materials	guinea pig closed epicutaneous test			human maximization test		
	induction	challenge	rate	induction	challenge	rate
eugenol	10%	1%	II (4/20)	8%	8%	— (0/25)
methyl eugenol	10%	1%	— (0/20)	8%	8%	— (0/25)
acetyl eugenol	10%	1%	— (0/20)	20%	20%	— (0/25)
isoeugenol	10%	1%	IV (16/20)	10%	10%	IV (19/25)
				8%	8%	III (38/110)
methyl isoeugenol	10%	1%	— (0/20)	8%	8%	— (0/25)
acetyl isoeugenol	10%	1%	III (4/10)	10%	10%	— (0/23)
benzyl isoeugenol	10%	1%	— (0/9)	5%	5%	— (0/25)

第8表　Sensitization Frequency of Some Salicylate Compounds

materials	guinea pig closed epicutaneous test			human maximization test		
	induction	challenge	rate	induction	challenge	rate
methyl salicylate	30%	1%	— (0/10)	8%	8%	— (0/25)
ethyl salicylate	30%	1%	— (0/10)	12%	12%	— (0/25)
isoamyl salicylate	30%	1%	— (0/8)	10%	10%	— (0/25)
phenyl salicylate	30%	1%	— (0/10)	6%	6%	— (0/25)
phenylethyl salicylate	30%	1%	— (0/8)	8%	8%	— (0/25)
benzyl salicylate	30%	1%	II (3/20)	30%	30%	— (0/25)

第9表　Sensitization Frequency of Some Fragrance Materials

materials	guinea pig closed epicutaneous test			human maximization test		
	concentrations for induction	concentrations for challenge	rate	concentrations for induction	concentrations for challenge	rate
cinnamic aldehyde	0.5%	0.5%	III (3/5)	2%	2%	III (11/25)
cinnamic alcohol	10%	10%*	III (2/6)	4% 10%	4% 10%	— (0/25) II (11/86)
cinnamic acid	10%	1%	— (0/5)	4%	4%	— (0/25)
benzylideneacetone	10%	1%	V (5/5)	2%	2%	III (12/25)
hydroxycitronellal	10%	10%	II (2/12)	12%	12%	— (0/25)
citral	3%	1%	II (1/6)	8%	8%	III (8/25)
trans-2-hexenal	3%	1%	V (9/10)	4%	4%	— (0/25)
trans-2-hexenal DEA	10%	3%	IV (8/10)	8%	8%	III (16/25)
benzyl alcohol	30%	1%	— (0/10)	10%	10%	— (0/25)

* in acetone

では benzyl salicylate のみ濃度30%で誘発した際感作成立例が認められた[19]。その他の合成香料9種のうちHMTでは5種が，CETでは7種が感作の成立をみた。

著者らは現在，感作に及ぼす諸基剤の影響についても検討中で，その詳細は後日西村が報告する予定である。例えば isoeugenol の場合の感作率は w/o クリームが最も優れるが，白色ワセリンとの間には有意差をみない。他方流動パラフィン，トリエチルシトレート，ジイソブチルアジペートを基剤として使用すると isoeugenol の感作率が明らかに低下する。なお各種基剤の影響は合成香料素材でも異なる傾向がみられた。

その他の化学物質の Closed epicutaneous test の成績

合成香料以外にも諸種の化学物質の感作能を CET で検討してきた。コールドパーマネント第1液中の主剤チオグリコール酸アンモニウムは弱い感作能が認められた[20]。ゴムの加硫促進剤 DETBT, MMBT, TMTM, DMA，酸化防止剤の IPPD の感作能も本法により確認された[21]。フェニレンジアミン系化合物の CET の感作率についても比較検討し，その結果 p-PDA, o-PDA に比し m-PDA の感作率が明らかに低いこと，これらにニトロ基を添加すると夫々の感作能が有意に低下することを認めた[22-23]。

第10表　Comparison of Guinea Pig Closed Epicutaneous Test and Human Maximization Test

materials	guinea pig closed epicutaneous test			human maximization test		
	I (%)	C (%)	rate	I (%)	C (%)	rate
cinnamic aldehyde	0.5	0.5	Ⅲ	2	2	Ⅲ
cinnamic alcohol	10	10*	Ⅲ	10	10	Ⅱ
				4	4	—
cinnamic acid	10	1	—	4	4	—
benzylidene acetone	10	1	Ⅴ	2	2	Ⅲ
hydroxycitronellal	10	10	Ⅱ	12	12	—
citral	3	1	Ⅱ	8	8	Ⅲ
trans-2-hexenal	3	1	Ⅴ	4	4	—
trans-2-hexenal DEA	10	3	Ⅳ	8	8	Ⅲ
eugenol	10	1	Ⅱ	8	8	—
methyl eugenol	10	1	—	8	8	—
acetyl eugenol	10	1	—	20	20	—
isoeugenol	10	1	Ⅳ	10	10	Ⅳ
				8	8	Ⅲ
methyl isoeugenol	10	1	—	8	8	—
acetyl isoeugenol	10	1	Ⅲ	10	10	—
benzyl isoeugenol	10	1	—	5	5	—
methyl salicylate	30	1	—	8	8	—
ethyl salicylate	30	1	—	12	12	—
isoamyl salicylate	30	1	—	10	10	—
phenyl salicylate	30	1	—	6	6	—
phenylethyl salicylate	30	1	—	8	8	—
benzyl salicylate	30	1	Ⅱ	30	30	—
p-phenylenediamine	1	1	Ⅴ	10	0.5	Ⅴ
		0.1	Ⅴ			
tetramethylthiuram disulfide	0.8	0.8	—	25	5	Ⅱ
2-mercaptobenzothiazole	1.2	1.2	—	25	10	Ⅲ

I : concentrations for induction
C : concentrations for challenge
* in acetone

Closed epicutaneous test とヒトの maximization test の比較

第10表に21種の合成香料とその他3種の化学物質のモルモットの CET の成績並びに HMT の成績の対比を示す。合成香料のうち11種が CET 陽性，6種が HMT 陽性で5種の合成香料素材は CET のみが感作成立を認めた。他方 TMTD とMBT は HMT は陽性であるが CET は陰性であった。

以上を総括すると CET は GMT より感作力が明らかに劣るが，HMT の成績との相関が GMT よりかなり高いことが結論として考えられた。

謝　辞

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文　献

1. Cadby, P. A. : Reaction to perfume ingredients in relation to exposure patterns, 香粧会誌, 7 : 117—124, 1983.
2. Kligman, A. M. : The identification of contact allergens by human assay. Ⅰ. A critique of standard methods, J. Invest. Dermatol., 47 : 369—374, 1966.
3. Kligman, A. M. : The identification of contact allergens by human assay. Ⅱ. Factors influencing the induction and measurement of allergic contact dermatitis, J. Invest. Dermatol., 47 : 375—392, 1966.
4. Kligman, A. M. : The identification of contact allergens by human assay. Ⅲ. The maximization test. A procedure for screening and rating contact sensitizer, J. Invest. Dermatol., 47 : 393—409, 1966.
5. Kligman, A. M. and Epstein, W. : Updating the maximization test for identifying contact allergens, Contact Dermatitis, 1 : 231—239, 1975.
6. Draize, J. H. : Dermal toxicity. In appraisal of safty of chemical in foods, drugs and cosmetics,

Assoc. Foods and Drugs Official of the U.S., Austin, Texas St. Health, 46—59, 1959.

7. Weirich, E.G. : Zur Methodik der tierexperimentellen Prüfung kosmet. Dermatika auf ihre Hautverträglichkeit. Deutsch Gesellschaft für Fettwissenschaften, Düsseldorf, 1962. : 18) より引用.

8. Bühler, E.V. : Delayed contact hypersensitivity in the guinea pig, Arch. Dermatol., 91 : 171—175. 1965.

9. Magnusson, B. and Kligman, A.M. : The identification of contact allergens by animal assay. The guinea pig maximization test, J. Invest. Dermatol., 52 : 268—276, 1969.

10. Maguire Jr., H.C. and Chase, M.W. : Studies on the sensitization of animals with simple chemical compounds, J. Exp. Med., 135 : 357, 1972.

11. Maurer, T., Thoman, P., Weirich, E.G. and Hess, R. : The optimization test in the guinea pig. A model for the predictive evaluation of contact allergenicity of chemicals, Agents and Actions : 5, 174—179, 1975.

12. Brulos, M.F., Guillot, J.P., Marini, M.C. and Cotte, J. : The influence of perfume on the sensitizing potential of cosmetic bases. I. A technique for evaluating sensitizing potencial, J. Soc. Cosmet. Chem., 28 : 357—365, 1977.

13. Klecak, G., Geleick, H. and Hess, J.R. : Screening of fragrance materials for allergenicity in the guinea pig. I. Comparison of four testing methods, J. Soc. Cosmet. Chem., 28 : 53—64, 1977.

14. Sato, Y., Katsumura, Y., Ichikawa, H., Kobayashi, T., Kozuka, T., Morikawa, F. and Ohta, S. : A modified technique of guinea pig testing to identify delayed hypersensitivity allergens, Contact Dermatitis, 7 : 225—237, 1971.

15. Tuchiya, S., Kondo, M., Okamoto, K. and Takase, Y. : Studies on contact hypersensitivity in guinea pig. The cumulative contact enhancement test, Contact Dermatitis, 8 : 246—255, 1982.

16. Itoh, M. : Sensitization potency of some phenolic compounds—with special emphasis on the relationship between chemical structure and allergenicity —, J. Derm., 9 : 223—233, 1982.

17. 高瀬吉雄，小川秀興，岡本暉公彦：新しい皮膚の生理と安全性，第1版，清至書院，東京，1983，259—261.

18. Maurer, T. : Epidemiology of allergic contact dermatitis. Contact and photocontact allergens, lst Ed, Marcel Dekker New York, 1983, 24—25.

19. 伊藤正俊，石原　勝：Benzyl salicylate の接触アレルギー，治療，62，1858—1859，1980.

20. 伊藤正俊，石原　勝，細野久美子，関東裕美，西村誠：美容師の手の湿疹におけるアレルゲン，皮膚，27：510—520，1985.

21. 関東裕美，石原　勝，伊藤正俊，細野久美子，西村誠：ゴム製品中のアレルゲン，皮膚，27：501—509，1985.

22. Ishihara, M., Nogami, T., Itoh, M. and Nishimura. M. : Sensitization potency of dye intermediates and modifiers in guinea pig，皮膚，27：585—590，1985.

23. Ishihara, M., Nogami, T., Itoh, M., Hosono, K., Kantoh, H. and Nishimura, M. : Basic studies on contact dermatitis due to hair colorings and cold permanent wave solutions，香粧会誌，7：272—282，1983.

Closed Epicutaneous Test

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Key words : *guinea pig maximization test (GPMT)—human maximization test (HMT)—closed epicutaneous test (CET)*

Representative aminal sensitization test procedures are classified according to the method of induction and challenge. The guinea pig maximization test is compared with the human maximization test using 41 fragrance materials and it is confirmed that the guinea pig test is significantly more sensitive than the human test. In the induction phase of the Closed Epicutaneous Test (CET), test materials are occluded for 48 hours on the shaved naped and this procedure is repeated three times per week for two weeks. Following a two-week rest period, the test materials are occluded for 48 hours on the flank for challenge. The results of the CET using fragrance materials, dye intermediates and modifiers used for hair colorings, ammonium thioglycollate, rubber accelerators and antioxidants indicate that the CET is less senstive than the guinea pig maximization test. However, there is a better degree of agreement between the CET and human maximization test results than between the guinea pig and human test results.

QMRF Title: Skin sensitization for DASS

1.QSAR[1] identifier

1.1.QSAR identifier (title):

Skin sensitization for DASS

1.2.Other related models:

N/A

1.3.Software coding the model:

OECD QSAR Toolbox v.4.6

2.General information

2.1.Date of QMRF:

March 2023

2.2.QMRF author(s) and contact details:

Laboratory of Mathematical Chemistry, "Prof. As. Zlatarov" University, “Prof. Yakimov” Str.1, 8010 Burgas, Bulgaria
http://www.oasis-lmc.org

2.3.Date of QMRF update(s):

N/A

2.4.QMRF update(s):

N/A

2.5.Model developer(s) and contact details:

Laboratory of Mathematical Chemistry, "Prof. As. Zlatarov" University, “Prof. Yakimov” Str.1, 8010 Burgas, Bulgaria
http://www.oasis-lmc.org

European Chemicals Agency (ECHA)
P.O.Box 400, 00121 Helsinki, Finland
https://echa.europa.eu

Organization for Economic Co-operation and Development (OECD)
2, rue André Pascal, 75016 Paris, France
https://www.oecd.org

[1] This QMRF includes modifications to allow the same format to be used to report other in silico models (e.g. Structure Activity Relationship (SAR) models, expert systems, etc.) used as information sources in Defined Approaches for Skin Sensitisation (DASS). Not all information herein may be applicable to all in silico models.

2.6.Date of model development and/or publication:

March 2023

2.7.Reference(s) to main scientific papers and/or software package:

Guideline No. 497: Defined Approaches on Skin Sensitisation
https://www.oecd.org/chemicalsafety/guideline-no-497-defined-approaches-on-skin-sensitisation-b92879a4-en.htm

2.8.Availability of information about the model:

"Skin sensitization for DASS" model uses the "EC3 from LLNA or Skin sensitization from GPMT assays for defined approaches (SS AW for DASS)" automated workflow. The training set of the automated workflow includes chemicals from REACH Skin sensitization (normalized) v.2.1 and Skin sensitization v.1.8 databases.

2.9.Availability of another QMRF for exactly the same model:

N/A

3.Defining the endpoint - OECD Principle 1

3.1.Species:

Mouse; Guinea pig

3.2.Endpoint:

Skin sensitization

3.3. Comment on endpoint:

Using EC3 data from LLNA assay and/or Skin sensitization data from GPMT assay.

3.4.Endpoint units:

N/A

3.5.Dependent variable:

All available Skin sensitization data for the chemicals in the used training set were converted to positive/negative scale.

- The chemicals having LLNA/GPMT data specified as "Negative", "Nom Sensitizer" and LLNA EC3 data ≥ 50% are taken as Negatives.
- The chemicals having LLNA/GPMT data specified as "Positive", "Weakly positive", "Strongly positive", "Weak sensitizer", "Moderate sensitizer, "Strong sensitizer", "Category 1B", "Category 1A" and LLNA EC3 data < 50%are takes as Positives.

3.6.Experimental protocol:

OECD (2010), Test No. 429: Skin Sensitisation: Local Lymph Node Assay, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264071100-en

OECD (2022), Test No. 406: Skin Sensitisation, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264070660-en

3.7.Endpoint data quality and variability:

N/A

4.Defining the algorithm - OECD Principle 2

4.1.Type of model:

The model produces predictions from automated read-across and endpoint specific structural alerts.

4.2. Explicit algorithm:

Skin sensitization for DASS model aims to make mechanistically-justified read-across predictions. The analogues are identified by the same protein binding alert(s) (PBAs) found in the structure of the target chemical or its abiotic (autoxidation) or biotic (skin metabolism) degradation product. If no PABs are identified in the target, then the analogues are searched to have the same functional groups. In case no mechanistically or structurally similar analogues are found, the prediction is based on profiling result associated with presence or absence of PBAs in the target chemical and its metabolites/degradation products.

4.3.Descriptors in the model:

Profilers from the QSAR Toolbox

4.4.Descriptor selection:

Structural and mechanistically relevant profilers

4.5.Algorithm and descriptor generation:

The “algorithm” for each profiler is available within the QSAR Toolbox.

4.6.Software name and version for descriptor generation:

QSAR Toolbox v.4.6

4.7.Chemicals/Descriptors ratio:

N/A

5.Defining the applicability domain - OECD Principle 3

5.1.Description of the applicability domain of the model:

Based on the correctly predicted training set substances, three layers of applicability domain are automatically calculated by the Toolbox: 1) parametric; 2) structural and 3) mechanistic layers. Depending on the Toolbox prediction approach (read-across or profiling predictions) and prediction outcomes (positive or negative), one or more of these layers are taken into account to establish the overall Toolbox domain of the specific prediction. The applicability domain layers considered for different types of Toolbox predictions are summarized in the table here:

Toolbox DASS AW outcome		Applicability domain layer		
		Structural	Parametric	Mechanistic
Positive	Read-across	Not considered	Not considered	**Considered**
	Profiling	Not considered	Not considered	**Met by definition**
Negative	Read-across	Not considered	Not considered	**Considered**
	Profiling	**Considered**	**Considered**	**Met by definition**

Explanation and rationale for the use of different domain layers:

1) Positive predictions (both by read-across and profiling): the presence of an alert (which is the requirement for positive Toolbox prediction to be considered within in the mechanistic domain) is sufficient to consider the prediction to be within the Toolbox domain. Substances triggering an alert are considered as in domain because they contain the toxicophore that has been observed experimentally in skin sensitisers. No further checks are needed in this context to consider the prediction within the Toolbox *in silico d*omain.

2) Negative predictions by read-across: the structural and parametric domains are not taken into account because the Toolbox has already ensured some level of similarity with other substances in its training set that met the requirements to be selected as suitable analogues for read-across.

3) Negative prediction by profiling predictions: all domain layers are taken into account to ensure the highest possible reliability level for the Toolbox prediction. Stricter requirements are needed mainly for two reasons: 1. lack of alerts is not equal to proof of lack of sensitisation potential and 2. to apply a cautious approach since acceptance of negative predictions may lower the human health protection level risk in case of a false negative predictions

5.2.Method used to assess the applicability domain:

1) Parametric layer

Four physico-chemical parameters of the substances are taken into consideration: log Kow, molecular weight, vapour pressure and water solubility. The ranges of variation for the selected parameters are defined based on the training set substances that are correctly predicted by the original DASS AW. A substance is considered within the parametric domain if its physicochemical parameter values as calculated by the QSAR Toolbox fall into the ranges of variation given in the table below. It is noted that the ranges include parametric values calculated using EPISuite models implemented in QSAR Toolbox v. 4.5 that in some cases are wider than that covered by existing test methods.

Physico-chemical parameter	Calculated Parameter range
Log Kow	-9.66 ÷ 18.6
Molecular weight	16 Da ÷ 2290 Da
Vapour pressure*	0 Pa ÷ 3.45×10^{7} Pa
Water solubility	2.48×10^{-15} mg/L ÷ 1.00×10^{6} mg/L

*EPIWIN Vapor Pressure (Antoine method) is used for calculation

2) Structural layer

The structural layer is defined based on the atom centred fragments (ACF) derived from the structural characteristics of the TS substances that are correctly predicted by the original DASS AW.

The ACF are defined according to the following Toolbox default values for ACF:

- Any atom distance = 1
- Heteroatom distance = 1
- Extract C (sp3) fragments = YES
- Include whole aromatic rings = NO

For each substance, the following values are calculated:

- % Correct fragments: percentage of ACF occurring in correctly predicted structures in the training set
- % incorrect fragments: percentage of ACF occurring in incorrectly predicted structures in the training set
- % unknown fragments: percentage of ACF not occurring in the training set.

A substance is considered within the structural domain of the DASS AW if 100% of its ACF belong to the correct fragments.

3) Mechanistic layer

The predicted capability of a substance to interact with the skin proteins without and after (a)biotic activation is taken into consideration. The Toolbox endpoint-specific profiler Protein binding for skin sensitization by OASIS and two metabolic simulators – Autoxidation simulator and Skin metabolism simulator are used to predict such interaction. A positive prediction is considered within the mechanistic domain if the substance triggers “Protein binding for skin sensitization by OASIS” alerts without or after (a)biotic activation. A negative prediction is considered within the mechanistic domain if the substance does not permit expert review. These are best considered as out of domain for use if the substance does not trigger “Protein binding for skin sensitization by OASIS” without or after (a)biotic activation.

5.3.Software name and version for applicability domain assessment:

QSAR Toolbox v.4.6

5.4.Limits of applicability:

In order to belong to the model applicability domain a target structure must meet the requirements defined in all the applicable domain layers.

6.Internal validation - OECD Principle 4

6.1.Availability of the training set:

The training set (TS) consists of 2268 substances having LLNA and/or GPMT skin sensitisation experimental data. The TS substances are part of the following databases available in QSAR Toolbox v.4.5:

- Skin sensitization v.1.8;
- REACH Skin sensitisation (normalized) databases v.2.1.

6.2.Available information for the training set:

ID information for the chemicals from the TS (such as CAS RN, Chemical Name, SMILES, etc.) is available in the QSAR Toolbox software.

6.3.Data for each descriptor variable for the training set:

Details on the training set can be consulted in the QSAR Toolbox.

6.4.Data for the dependent variable for the training set:

Details on the training set can be consulted in the QSAR Toolbox.**6.5.Other information about the training set:**

The training set (TS) represents a dataset of 2268 unique chemicals available in *Skin sensitization* and *REACH Skin sensitisation (normalized)* databases used by the respective automated workflow for searching analogues. In case of multiple data the worst case scenario was applied. As a result, the training set consists of 1093 Positive and 1175 Negative chemicals.

6.6.Pre-processing of data before modelling:

In case of multiple data points for one substance of the TS, the most conservative scenario is taken into account (i.e. in case of positive and negative data for one chemical, the positive data is considered).

6.7.Statistics for goodness-of-fit:

6.8.Robustness - Statistics obtained by leave-one-out cross-validation:

6.9.Robustness - Statistics obtained by leave-many-out cross-validation:

6.10.Robustness - Statistics obtained by Y-scrambling:

6.11.Robustness - Statistics obtained by bootstrap:

6.12.Robustness - Statistics obtained by other methods:

7.External validation - OECD Principle 4

7.1.Availability of the external validation set:

A set of 168 chemicals having LLNA classifications, which were all agreed upon by the EG DASS and used to evaluate the performance of the DAs. Due to the availability of data, this dataset contains mainly cosmetic ingredients but also other types of chemicals that are used across sectors such as preservatives, dyes, or food ingredients.

7.2.Available information for the external validation set:

The external validation set is embedded in the software implementation of the model.
All available information on the reference set could be found in:
OECD (2016). Series on Testing & Assessment No. 256: Guidance Document On The Reporting Of Defined Approaches And Individual Information Sources To Be Used Within Integrated Approaches To Testing And Assessment (IATA) For Skin Sensitisation, Annex 1 and Annex 2. Organisation for Economic Cooperation and Development, Paris. Available at: [https://www.oecd.org/chemicalsafety/testing/series-testing-assessmentpublications-number.htm].

7.3.Data for each descriptor variable for the external validation set:

N/A

7.4.Data for the dependent variable for the external validation set:

N/A

7.2. Other information about the external validation set:

See section 7.2. above.

7.6. Experimental design of test set:

All of the 168 chemicals have curated LLNA reference data agreed upon by the expert group (EG DASS)

7.7. Predictivity - Statistics obtained by external validation:

N/A

7.8. Predictivity - Assessment of the external validation set:

N/A

7.9. Comments on the external validation of the model:

The results of the model are used in combination with in vitro (hCLAT) and in chemico (DPRA) data in ITSv2 defined approach for skin sensitization.
The predictive capacity of ITSv2 described in OECD TG 497 has been estimated using results from the specifically built automated workflow included in QSAR Toolbox v.4.5. Some discrepancies in the predicted or domain results provided by the automated workflow (or the respective (Q)SAR model), available in the subsequent QSAR Toolbox versions, could be expected due to possible modifications in the used data sources (databases, profiling schemes and metabolism simulators).

8. Providing a mechanistic interpretation - OECD Principle 5

8.1. Mechanistic basis of the model:

The “Skin sensitization for DASS” model uses the original respective automated workflow for defined approaches (DASS AW) that is running in the background. The DASS AW predicts skin sensitization effect based on LLNA and GPMT experimental data. It predicts skin sensitization by

applying the category approach and selecting analogues based on mechanistic or structural characteristics found in the target chemical. It automatically collects the analogous chemicals and removes dissimilar chemicals by using relevant mechanistic and structure-based profilers. The metabolic activation is taken into account in the prediction.

In case the DASSAW fails to make a read-across prediction (due to lack of enough analogues, data, etc.), a specifically developed profiler is applied. The profiler identifies the presence or absence of protein binding alerts in the parent chemical and predicted metabolites.

8.2. A priori or a posteriori mechanistic interpretation:

A priori mechanistic interpretation, with protein binding identified as the event responsible for the skin sensitisation potential of chemicals. All of the steps followed by the DASS AW in the background are provided as metadata accompanying the prediction. In case of interest to get more details on the specific features used for searching for analogues/subcategorization, the DASS AW could be executed separately.

8.3. Other information about the mechanistic interpretation:

The logic implemented in the original workflow for predicting skin sensitization is described in: Yordanova, D., Schultz, T.W., Kuseva, C., et. al. Automated and Standardized Workflows in the OECD QSAR Toolbox. *Comp. Toxicol.* 10, 2019, pp 89-104.
https://doi.org/10.1016/j.comtox.2019.01.006

9.Miscellaneous information

9.1.Comments:

The "Skin sensitization for DASS" model is included as an *in silico* data source for in ITSv2 defined approach. The ITSv2 combines three types of data – *in silico* (QSAR Toolbox), *in vitro* (h-CLAT) and *in chemico* (DPRA). Each of the data results for a target chemical is converted to a score, where the predicted data from QSAR Toolbox could have score of "1" in case of positive result and score of "0" in case of negative result. The skin sensitizing potential of a target chemical is predicted by calculation of a total battery score based on the individual scores for each of the three data types.

9.2.Bibliography:

OECD (2021), Guideline No. 497: Defined Approaches on Skin Sensitisation, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/b92879a4-en.

9.3.Supporting information:

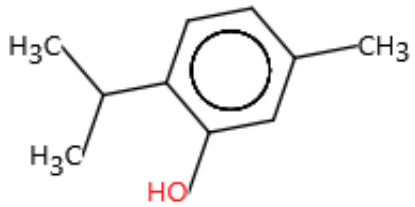
N/A

QSAR Toolbox prediction for single chemical

The password protection of this document has been removed by the user.
The user has not given explanation why.

Toolbox version: 4.6
Date: 1 Sept. 2023
Author(s): [redacted]
Contact details: Lanxess Deutschland GmbH

Target information

Structural information	Numerical identifiers	Chemical names
SMILES: CC(C)c1ccc(C)cc1O Structure 	CAS#: 89-83-8 Other: EC Number:2019448	2-isopropyl-5-methyl-phenol 5-methyl-2-(1-methylethyl)phenol 5-methyl-2-(propan-2-yl)phenol

Prediction summary

Predicted endpoint: Human Health Hazards -> Sensitisation -> Skin -> in Vivo -> GPMT <OR> LLNA -> EC3 <OR> S M W N <OR> Skin sensitisation

Predicted value: Negative [Skin sensitisation II (ECETOC)]

Data gap filling method: N/A, Automated workflow for EC3 from LLNA or Skin sensitization from GPMT assays for defined approaches (SS AW for DASS)

Applicability domain: In domain

Name: OECD / Skin sensitisation / 7.4.1, rel 2, WoE, in vitro skin sensitization DPRA, 2023 / Thymol / thymol / 89-83-8

Legal entity owner:

Printing date: 2023-09-01T13:18:14.478+02:00

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Thymol / 89-83-8 / 201-944-8 10

ENDPOINT_STUDY_RECORD: 7.4.1, rel 2, WoE, in vitro skin sensitization DPRA, 2023

UUID: fe301ce6-46a1-48be-bde9-3547007fe203
Dossier UUID:
Author: [redacted]
Date: 2023-09-01T13:17:58.447+02:00
Remarks:

Administrative data

EU: REACH

Endpoint
skin sensitisation: in chemico

Type of information
experimental study

Adequacy of study
weight of evidence

Robust study summary
true

Used for classification
false

Study period: start date
2023-04-03

End date
2023-04-11

Reliability
2 (reliable with restrictions)

Data source

Reference

Direct peptide reactivity assay (DPRA): Test report on THYMOL / Dr. A. Natsch / study report

Data access
data submitter has permission to refer Data owner gave permission per mail. We are in contact with the data owner regarding data sharing.

Data protection claimed
yes, but willing to share

Materials and methods

Test guideline

Qualifier
according to guideline

Guideline
OECD Guideline 442C (In Chemico Skin Sensitisation Assays addressing the Adverse Outcome Pathway key event on covalent binding to proteins)
from 18 June 2019

Version / remarks
2021

GLP compliance
no

Type of study
direct peptide reactivity assay (DPRA)

Test material

Test material information
Thymol / 89-83-8 / 201-944-8

Specific details on test material used for the study (confidential)
Batch number: VE00805765
Expiry Date: 19.10.2024
Product code: 9311001
Storage conditions: Ambient Temperature
Supplier: Givaudan Schweiz SA

In chemico test system

Details of test system
cysteine peptide, (Ac-RFAACAA-COOH)
lysine peptide (Ac-RFAAKAACOOH)

Details on the study design
PREPARATION OF TEST SOLUTION
The test substance was freely soluble in acetonitrile at 100 mM, and this preferred solvent according the SOP could thus be used.

PREPARATION OF CONTROLS
- Positive control: In each test cinnamic aldehyde is included as positive control (5 mM with the Cysteine peptide and 25 mM with the Lysine peptide).
- Negative (solvent/vehicle) control: Acetonitrile, concentration tested 25%
- Stability of vehicle control: A control experiment according to the DB-Alm protocol 154 to test whether Acetonitrile has no effect on peptide stability was performed. A 0.5 mM Cysteine solution was incubated for 24 h in Phosphate buffer / acetonitrile, and subsequently injected every 3 h for 48 h.

APPLICATION OF THE TEST CHEMICAL AND CONTROL SUBSTANCES
- Number of study: one
- Number of replicates: three
- The Lys-peptide Ac-RFAAKAA is incubated at a final concentration of 0.5 mM in an ammonium acetate buffer at pH 10.5 in presence of a final level of 25% acetonitrile and in presence of a 50-fold excess of the test substance (25 mM) dissolved in acetonitrile.
- The Cys-peptide Ac-RFAACAA is incubated at a final concentration of 0.5 mM in phosphate buffer at pH 7.5 in presence of a final level of 25% acetonitrile and in presence of a 10-fold excess of the test substance (5 mM) dissolved in acetonitrile.

- 24 h after start of the incubation the remaining peptide is quantified with HPLC-UV.

DATA EVALUATION
- Data evaluation is automatically performed by a standardized Excel template which forms part of the SOP.
- DAD-Detector: 220 nm

ENDPOINT VALUE
The endpoint is expressed as % peptide depletion.

PREDICTION MODEL
Based on the average peptide depletion values for both the Cysteine and the Lysine peptide, a reactivity class is attributed to the substances. Average depletion below 6.38% indicates minimal reactivity, substances in this class are predicted as non-sensitizers by the DPRA.
In case co-elution occurs with the Lysine peptide only, an alternative prediction model is proposed by the test guideline. In this Cysteine only model, chemicals are rated as sensitizers if they lead to >13.89% depletion of the Cysteine peptide.

PREDICTION MODEL TAKING BORDERLINE OUTCOMES INTO ACCOUNT AS IMPLEMENTED IN OECD TG 497
In order to define areas where lower confidence in the Defined Approach results may exist, borderline ranges (BRs) have been defined for output from the individual assays addressing the three KE of the 2o3 DA in OECD TG 497. The borderline ranges for DPRA are at an average depletion in the range of 4.95 % – 8.32 %. In addition, if the depletion is between 3 – 10% a repeated run is recommended. For the assessment of whether the outcome of repeated runs yields a positive, negative or borderline final outcome, the modified Prediction model applied (see Annex 1, Figure 1.1 in OECD TG 497).
This prediction model introduces a third outcome (borderline) to be used within the 2o3 DA, based on the same decision cut-offs of the prediction model described in TG 442C. Thus, a negative result in the original prediction model can only become negative or borderline, while a positive result from the original prediction model can only become positive or borderline.

ACCEPTANCE CRITERIA
- Test Chemical: The standard deviation for Cys-peptide depletion should be < 14.9% and for Lys-peptide depletion < 11.6%.
- Positive control: The mean Percent Peptide Depletion value of the three replicates for cinnamic aldehyde must fall within the ranges: cystein depletion (%): 60.8-100 (< 14.9% SD); lysine depletion (%): 40.2-69.4 (< 11.6% SD).
- Reference control: Mean peptide concentration in the reference samples A should be 0.5 ± 0.05 mM. The Coefficient of variation (CV) for the nine references B and C in acetonitrile must be below 15%. Mean peptide concentration in the reference samples C should be 0.5 ± 0.05 mM.
- System Suitability: The Calibration curves for both peptides were prepared. The R2 of the calibration curve must be >0.99
Mean peptide concentration in the reference samples A should be 0.5 ± 0.05 mM. The Coefficient of variation (CV) for the nine references B and C in Acetonitrile must be below 15%. Mean peptide concentration in the reference samples C should be 0.5 ± 0.05 mM.

Vehicle / solvent
acetonitrile

Positive control
cinnamic aldehyde
CAS 104-55-2

Results and discussion

Positive control results
Cinnamic aldehyde:
Mean cystein depletion: 72.3 % (0.6 % SD, 0.9 % CV)

Mean lysine depletion: 50.1 % (0.8 % SD, 1.5 % CV
All the acceptance criteria were fulfilled for the positive control cinnamic aldehyde.

In vitro / in chemico

Results

Key result
false

Group
test chemical

Run / experiment
mean

Parameter
other: mean cysteine/lysine depletion

Value

-0.5	%

Vehicle controls validity
valid

Negative controls validity
valid

Positive controls validity
valid

Remarks on result
no indication of skin sensitisation

Group
test chemical

Run / experiment
mean

Parameter
mean lysine depletion

Value

-0.7	mM

At concentration

25	mM

Vehicle controls validity
valid

Negative controls validity
valid

Positive controls validity
valid

Group
test chemical

Run / experiment
mean

Parameter
mean cystein depletion

Value

-0.3	%

At concentration

5	mM

Vehicle controls validity
valid

Negative controls validity
valid

Positive controls validity
valid

Group
test chemical

Run / experiment
run/experiment 1

Parameter
lysine depletion

Value

-0.7	mM

At concentration

25	mM

Vehicle controls validity
valid

Negative controls validity
valid

Positive controls validity
valid

Group
test chemical

Run / experiment
run/experiment 2

Parameter
lysine depletion

Value

-0.7 mM

At concentration

25 mM

Vehicle controls validity
valid

Negative controls validity
valid

Positive controls validity
valid

Group
test chemical

Run / experiment
run/experiment 3

Parameter
lysine depletion

Value

-0.7 mM

At concentration

25 mM

Vehicle controls validity
valid

Negative controls validity
valid

Positive controls validity
valid

Group
test chemical

Run / experiment
run/experiment 1

Parameter
cysteine depletion

Value

-2.4 %

At concentration

5 mM

Vehicle controls validity
valid

Negative controls validity
valid

Positive controls validity
valid

Group
test chemical

Run / experiment
run/experiment 2

Parameter
cysteine depletion

Value

-2.5	%

At concentration

5	mM

Vehicle controls validity
valid

Negative controls validity
valid

Positive controls validity
valid

Group
test chemical

Run / experiment
run/experiment 3

Parameter
cysteine depletion

Value

3.9	%

At concentration

5	mM

Vehicle controls validity
valid

Negative controls validity
valid

Positive controls validity
valid

Outcome of the prediction model
no or minimal reactivity [in chemico]

Any other information on results incl. tables

Test Substance overall results:

Cysteine depletion Average / SD (%)	Lysine depletion Average / SD (%)	Average cysteine/ lysine depletion (%)	Reactivity Class	Prediction
-0.3 / 3.7	- 0.7 / 0.0	- 0.5	Minimal	Non-sensitizer

Analysis for Borderline outcome:

Test substance	Average depletion 4.95-8.32	Outcome of run (Positive, Negative, Borderline)	Average Depletion 3-10%	Repetition of run recommended
Thymol	no	negative	no	no

Applicant's summary and conclusion

Conclusions
Average cysteine/lysine depletion (Outcome Prediction Model): - 0.5 %
Outcome Prediction Model Borderline: negative

Executive summary

The test substance gave -0.3 % depletion of the Cys-peptide and -0.7 % depletion of the Lys-peptide. The average peptide depletion is -0.5 %. This is below the threshold of 6.38%, and the substance is thus attributed to the “minimal” reactivity class, rating it as a non-sensitizer according to the DPRA prediction model. The test result was not in the borderline range. Thus the overall outcome is negative and the conclusion on the overall outcome does also apply if the test result is used within the 2o3 DA.

References

LITERATURE: Direct peptide reactivity assay (DPRA): Test report on THYMOL

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Dossier UUID:
Author: [redacted]
Date: 2023-08-30T11:12:47.859+02:00
Remarks:

General information

Reference Type
study report

Title
Direct peptide reactivity assay (DPRA): Test report on THYMOL

Author
[redacted]

Year
2023

Testing facility
Department of in vitro molecular screening
Givaudan Schweiz AG
Kemptpark 50
CH-8310
Kemptthal
Switzerland

Report date
2023-04-12

Report number
RCR 154'275

Study sponsor
Givaudan International SA 5, Chemin de la Parfumerie CH-1214 Vernier Switzerland

Study number
RCR 154'275

TEST_MATERIAL_INFORMATION: Thymol / 89-83-8 / 201-944-8

UUID: f22f3f32-0c03-4c42-8ffb-d18cc77f7db3
Dossier UUID:
Author: [redacted]
Date: 2023-08-30T22:45:58.295+02:00
Remarks:

Name
Thymol / 89-83-8 / 201-944-8

Composition

Composition

Type
Constituent

Reference substance
thymol / thymol / 89-83-8 / 201-944-8

EC number	EC name
201-944-8	EC Inventory
CAS number	**CAS name**
89-83-8	Phenol, 5-methyl-2-(1-methylethyl)-
IUPAC name	
thymol	

Other characteristics

Confidential details on test material
Batch number: VE00805765
Expiry Date: 19.10.2024
Product code: 9311001
Storage conditions: Ambient Temperature

Name: OECD / Skin sensitisation / 7.4.1, rel 2, WoE, in vitro skin sensitization KeratinoSens, 2023 / Thymol / thymol / 89-83-8

Legal entity owner:

Printing date: 2023-09-01T13:19:20.697+02:00

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ENDPOINT_STUDY_RECORD: 7.4.1, rel 2, WoE, in vitro skin sensitization KeratinoSens, 2023

UUID: ed39cd4d-ed70-4a4b-bfed-1bba3bb4cab3
Dossier UUID:
Author: [redacted]
Date: 2023-09-01T13:18:03.650+02:00
Remarks:

Administrative data

EU: REACH

Endpoint
skin sensitisation: in vitro

Type of information
experimental study

Adequacy of study
weight of evidence

Robust study summary
true

Used for classification
false

Study period: start date
2023-04-03

End date
2023-04-17

Reliability
2 (reliable with restrictions)

Data source

Reference

KeratinoSens™ assay: Test report on THYMOL / Dr. A. Natsch / study report

Data access
data submitter has permission to refer Data owner gave permission per mail. We are in contact with the data owner regarding data sharing.

Data protection claimed
yes, but willing to share

Materials and methods

Test guideline

Qualifier
according to guideline

Guideline
OECD Guideline 442D (In Vitro Skin Sensitisation: ARE-Nrf2 luciferase KeratinoSens™ test method) from 25 June 2018

Version / remarks
2018

GLP compliance
no

Type of study
ARE-Nrf2 luciferase KeratinoSens™ test method

Test material

Test material information
Thymol / 89-83-8 / 201-944-8

Specific details on test material used for the study (confidential)
Batch number: VE00805765
Expiry Date: 19.10.2024
Product code: 9311001
Storage conditions: Ambient Temperature
Supplier: Givaudan Schweiz SA

In vitro test system

Details on the study design
PREPARATION OF TEST SOLUTIONS
- The test substance was freely soluble in DMSO at 200 mM, and this preferred solvent according the SOP could thus be used.
- Positive control: Cinnamic aldehyde
- Negative (solvent/vehicle) control: Dimethylsulfoxide
- Luciferase substrate: The Luciferase substrate was prepared according to the following recipe: 20 m M Tricine; 2.67 mM MgSO4; 0.1 mM EDTA; 33.3 mM DTT; 270 µM Coenzyme A; 470 µM Luciferin; 530 µM ATP; pH 7.8

PREPARATION OF CULTURES
The KeratinoSens™ cell line was developed by the testing lab and stored on liquid nitrogen. It was grown in 10 cm petri dishes as described in the SOP to 80% confluency prior to testing for 3 – 4 days. Cells were counted using a counting chamber and adjusted to the desired density. During seeding into 96-well plates, the cell suspension was gently stirred and cell sedimentation was avoided by r epeatedly pipetting up and down to ensure homogeneous distribution of cells.

APPLICATION OF THE TEST CHEMICAL AND CONTROL SUBSTANCES
- Number of repetitions: three
- Number of replicates/repetition: three
- Highest concentration test chemical: 1000 µM
- Concentrations positive control : 4, 8, 16, 32, 64 µM
- Concentration negative control: 1%
- Positive control: In each test Cinnamic aldehyde is included as positive control. Cinnamic aldehyde was run in all three repetitions.
- Application procedure: Cells are grown for 24 h in 96-well plates. The medium is then replaced w ith medium containing a final level of 1% of the solvent DMSO containing the test substance. Each

compound is tested at 12 concentrations in the range from 0.98 to 2000 µM. Compounds such as an oil or extract without defined molecular weight are tested in the range from 0.2 to 400 µg/ml (which is equal to testing a molecule with MW = 200 at from 0.98 to 2000 µM). Each test plate contains six wells with the solvent control, 1 well with no cells for background value and 5 wells with a dose response of the positive control cinnamic aldehyde. In each repetition, three parallel replicate plates are run with this same set-up.

ENDPOINT VALUE
For Luciferase induction the maximal fold-induction over solvent control (Imax) and the concentration needed to reach a 1.5- and 3- fold induction (EC1.5 and EC3) are calculated. For cytotoxicity the IC50 value is extrapolated.

DATA EVALUATION
Data evaluation is automatically performed by a standardized Excel template which forms part of the SOP. The test plates are read by a plate reader, and the generated raw data are directly pasted into this template, and all data processing is performed automatically by this Excel sheet.
For both the MTT and the luciferase data, first the background value recorded in an empty well without added cells is subtracted.
For the MTT data the % viability is then calculated for each well in the test plate in relation to the average of the six solvent control wells.
For the luciferase data the average value of the six solvent control wells is set to 1, and for each well in the test plate the fold induction is calculated in relation to this value.

LUCIFERASE ACTIVITY MEASUREMENTS
Luminescence was read in a Promega Glomax Luminometer programmed to
i. add 50 µl of the luciferase substrate to each well, ii. to then wait for 1 second and iii. then to integrate the luciferase activity for 2 seconds.
For both the MTT and the luciferase data, first the background value recorded in an empty well without added cells is subtracted.

PREDICTION MODEL
Substances are rated positive if the following conditions are met:
• The Imax indicates > 1.5-fold gene induction, and this induction is statistically significant above the solvent control in a particular repetition as determined by Students T-test. The EC1.5 value is below 1000 µM (200 µg/ml in case no defined MW is available) in all three repetitions or in at least 2 repetitions. (If the Imax is exactly equal to 1.5, the substance is still rated negative and no EC1.5 value is calculated by the evaluation sheet.)
• At the lowest concentration with a gene induction above 1.5-fold (i.e. at the EC 1.5 determining value), the cellular viability is above 70%.
There is an apparent overall dose-response for luciferase induction, which is similar between the repetitions.

PREDICTION MODEL TAKING BORDERLINE OUTCOMES INTO ACCOUNT AS IMPLEMENTED IN OECD TG 497
In order to define areas where lower confidence in the Defined Approach results may exist, borderline ranges (BRs) have been defined for output from the individual assays addressing the three KE of the 2o3 DA in OECD TG 497. The borderline ranges for KeratinoSens™ are at a maximal induction (Imax) in the range of 1.35-fold – 1.67-fold. The prediction model of the KeratinoSens™ assay requires multiple runs. For the assessment of whether the outcome of repeated runs yields a positive, negative or borderline final outcome, the modified Prediction model is applied (see Annex 1, Figure 1.2 in OECD TG 497).
This prediction model introduces a third outcome (borderline) to be used within the 2o3 DA, based on the same decision cut-offs of the prediction model described in TG 442D. Thus, a negative result in the original prediction model can only become negative or borderline, while a positive result from the original prediction model can only become positive or borderline. A separate analysis is provided within this report to assess such borderline outcomes if the data are to be used within OECD TG 497.

ACCEPTANCE CRITERIA

Positive control: The targets are: (i) Average induction in the three replicates for cinnamic aldehyde at 64 µM should be between 2 and 8, and (ii) the EC 1.5 value should be between 7 µM and 30 µM. At least one of these two numerical criteria must be met in order to accept a repetition.

Vehicle / solvent control
DMSO

Negative control
other: DMSO

Positive control
cinnamic aldehyde [442D]

Results and discussion

Positive control results
Cinnamic aldehyde:
Average induction (4, 8, 16, 32 and 64 µM): 1.28, 1.27, 1.57, 1.83 and 2.62 µM, respectively
EC 1.5 (repetition 1, 2 and 3): 18.08, 9.86 and 14.46 µM, respectively
Average EC 1.5: 14.13 µM

Average induction in three replictes at 64 µM between 2 and 8: fulfilled
EC 1.5 between 7-30 µM: fulfilled
Both criteria were fulfilled in all three repetitions and thus all three repetitions were valid for the positive control.

In vitro / in chemico

Results

Group
test chemical

Run / experiment
mean

Parameter
EC 1.5 [442D]

Cell viability
389.59 µM (Geometric mean IC50)

Vehicle controls validity
valid

Positive controls validity
valid

Remarks on result
no indication of skin sensitisation

Group
test chemical

Run / experiment
run/experiment 1

Parameter
EC 1.5 [442D]

Cell viability
385.42 µM (IC50)

Vehicle controls validity
valid

Positive controls validity
valid

Remarks on result
no indication of skin sensitisation

Group
test chemical

Run / experiment
run/experiment 2

Parameter
EC 1.5 [442D]

Cell viability
395.66 µM (IC50)

Vehicle controls validity
valid

Positive controls validity
valid

Remarks on result
no indication of skin sensitisation

Group
test chemical

Run / experiment
run/experiment 3

Parameter
EC 1.5 [442D]

Cell viability
387.75 µM (IC50)

Vehicle controls validity
valid

Positive controls validity
valid

Remarks on result
no indication of skin sensitisation

Group
test chemical

Run / experiment
mean

Parameter
Imax [442D]

Value

1.25

Cell viability
389.59 µM (Geometric mean IC50)

Vehicle controls validity
valid

Positive controls validity
valid

Remarks on result
no indication of skin sensitisation

Group
test chemical

Run / experiment
run/experiment 1

Parameter
Imax [442D]

Value

1.26

Cell viability
385.42 µM

Vehicle controls validity
valid

Positive controls validity
valid

Remarks on result
no indication of skin sensitisation

Group
test chemical

Run / experiment
run/experiment 2

Parameter
Imax [442D]

Value

1.38

Cell viability
395.66 µM (IC50)

Vehicle controls validity
valid

Positive controls validity
valid

Remarks on result
positive indication of skin sensitisation

Group
test chemical

Run / experiment
run/experiment 3

Parameter
Imax [442D]

Value

1.12

Cell viability
387.75 µM (IC50)

Vehicle controls validity
valid

Positive controls validity
valid

Remarks on result
no indication of skin sensitisation

Outcome of the prediction model
negative [in vitro/in chemico]

Any other information on results incl. tables

Assessment of Borderline outcomes

Test substance	Imax 1.35-1.67	Induction above 1.35-fold statistically significant	Induction above 1.35-fold at concentration with 70% cell viability	Overall outcome of repetition
Thymol, Repetition 1	no	not applicable	not applicable	negative
Thymol, Repetition 2	yes	yes	yes	borderline
Thymol, Repetition 3	no	not applicable	not applicable	negative

Variability of the solvent control:

	% variability blanks	
Repetition 1	17.00	Accepted
Repetition 2	15.75	Accepted
Repetition 3	10.52	Accepted

Applicant's summary and conclusion

Conclusions
EC 1.5 & EC 3 (Prediction Model): no induction above a given threshold
Overall Outcome Prediction Model Borderline: negative

Executive summary

Thymol was weakly toxic to the KeratinoSens™ cells. In three independent repetitions, it did not induce the luciferase gene above the threshold of 1.5, indicating that it is a non-sensitizer in the standard prediction model of KeratinoSens ™. Analysis of the results for a potential borderline outcome indicates that the results do not fall into the borderline range for two repetitions, thus the negative outcome of the test can be used within the 2 out of 3 DA in TG 497.

References

LITERATURE: KeratinoSens™ assay: Test report on THYMOL

UUID: e0b5d6f9-f35f-4a46-9618-fcd2f56c700e
Dossier UUID:
Author: [redacted]
Date: 2023-08-30T22:51:23.072+02:00
Remarks:

General information

Reference Type
study report

Title
KeratinoSens™ assay: Test report on THYMOL

Author
[redacted]

Year
2023

Testing facility
Department of in vitro molecular screening
Givaudan Schweiz AG
Kemptpark 50
CH-8310
Kemptthal
Switzerland

Report date
2023-04-19

Report number
RCR 154'288

Study sponsor
Givaudan International SA 5, Chemin de la Parfumerie CH-1214 Vernier Switzerland

Study number
RCR 154'288

TEST_MATERIAL_INFORMATION: Thymol / 89-83-8 / 201-944-8

UUID: f22f3f32-0c03-4c42-8ffb-d18cc77f7db3
Dossier UUID:
Author: [redacted]
Date: 2023-08-30T22:45:58.295+02:00
Remarks:

Name
Thymol / 89-83-8 / 201-944-8

Composition

Composition

Type
Constituent

Reference substance
thymol / thymol / 89-83-8 / 201-944-8

EC number	EC name
201-944-8	EC Inventory
CAS number	**CAS name**
89-83-8	Phenol, 5-methyl-2-(1-methylethyl)-
IUPAC name	
thymol	

Other characteristics

Confidential details on test material
Batch number: VE00805765
Expiry Date: 19.10.2024
Product code: 9311001
Storage conditions: Ambient Temperature

Name: OECD / Skin sensitisation / in vitro skin sensitisation h-CLAT_2022_RL1 / non-REACH_THYMOL_89-83-8 / 2-isopropyl-5-methylphenol / 89-83-8

Printing date: 2023-08-30T18:43:54.830+02:00

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ENDPOINT_STUDY_RECORD: in vitro skin sensitisation h-CLAT_2022_RL1

UUID: 4f30b035-73f7-49a1-8e58-9fcf7fdb3faa
Dossier UUID:
Author: uivng
Date: 2023-08-30T18:43:05.486+02:00
Remarks:

Administrative data

EU: REACH

Endpoint
skin sensitisation: in vitro

Type of information
experimental study

Adequacy of study
weight of evidence

Robust study summary
true

Used for classification
false

Used for SDS
false

Study period
Feb-Jul 2022

Reliability
1 (reliable without restriction)

Rationale for reliability incl. deficiencies
guideline study
Reliability 1

Data source

Reference

HUMAN CELL LINE ACTIVATION TEST (H-CLAT) / A. Islas-Robles, H. King, J. Phillips / study report

Data access
data submitter has permission to refer

Data protection claimed
yes, but willing to share

Materials and methods

Test guideline

Qualifier
according to guideline

Guideline
OECD Guideline 442E (In Vitro Skin Sensitisation: human Cell Line Activation Test (h-CLAT)) before 9 October 2017

GLP compliance
no

Type of study
activation of dendritic cells

Test material

Test material information
Thymol CAS 89-83-8

In vitro test system

Details of test system
THP-1 cell line [442E]

Details on the study design
PREPARATION OF TEST SOLUTIONS
- Preparation of the test chemical stock solution
The solubility of the test articles was assessed in DMSO and 0.9% saline solution by preparing a top stock at 500 mg/mL for each solvent. The test article was determined to be fully soluble in DMSO at the tested concentration. For the test article the physical description in DMSO was a clear colorless non-viscous solution.

- Preparation of the test chemical serial dilutions
The same solvent used in the dose range finding assay will be used to dissolve the test article in the definitive assays. The test article will be prepared as stock concentrations corresponding to 100-fold (for saline) or 500-fold (for DMSO). Seven serial dilutions using a dilution factor of 1.2 to 1.5 will be made using the same solvent to obtain 8 serial dilutions. These dilutions will then be further diluted 50-fold (for test articles diluted in saline) or 250-fold (for test articles diluted in DMSO) in the culture medium (2X dosing dilutions). These dosing dilutions are prepared at 2X the desired final concentr ation so that when 500 µL of each dosing dilution are added to 500 µL of cell suspension in the 24-w ell plate, a 1X final dose concentration is achieved. The test article dilutions should be exposed to the cells within one hour of preparation.

- Preparation of the positive controls
The positive control will be DNCB prepared at a stock concentration of 2 mg/ml in DMSO. The work ing solution of DNCB will be prepared by making an 8 µg/ml dilution of the stock in culture medium. The working solution of DNCB will be dosed on the cell in the same manner as the test article.

- Preparation of the solvent, vehicle and negative controls
The solvent control will be culture medium for test articles diluted in saline, or DMSO in culture medi um for test articles diluted in DMSO. A single concentration of the solvent control(s) will be prepared in culture medium and dosed on the cel ls in the same manner as the test article so that the final concentration of saline on the cells is 1% and DMSO is 0.2%

- Log Kow of the test chemical
3.4

- Other:

Culture Medium (RPMI-1640 with 10% heat inactivated Fetal Bovine Serum and 0.05 mM 2-mercaptoethanol)

DOSE RANGE FINDING ASSAY:
A preliminary dose range finding assay was performed to determine the viability of the THP-1 cells after 24 ± 0.5 hour exposure to 8 test article concentrations. The CV75, which is the calculated test article concentration leading to 75% cell viability, was calculated for the test article, if possible.

- Highest concentration used
1000 µg/ml

- Solubility in solvents
>500 mg/mL in DMSO
- Results of selecting appropriate concentration and determination of cytotoxicity e.g. CV75
Seven serial doses using a typical dilution factor of 1.2 to 1.5 will be prepared such that 8 doses will be tested in the definitive assay. If there was insufficient cytotoxicity in the dose finding assay (i.e. CV75 > highest prepared dose), the highest soluble concentration of test article, up to a maximum stock concentration of 500 mg/ml in either saline or DMSO may be selected. At the Study Director's discretion and justification, the range of doses and the dilution factor to be used in the definitive assay may be modified.

- Final concentration range selected on basis of:
1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8 µg/ml

APPLICATION OF THE TEST CHEMICAL AND CONTROL SUBSTANCES
- Number of replicates
One

- Number of repetitions
Two

- Test chemical concentrations
84.0, 70.0, 58.3, 48.6, 40.5, 33.8, 28.1, 23.4 µg/ml based on CV75 of 70.0 µg/ml determined in pre-test

- Application procedure
The 2X dosing dilutions will be applied to the cells by adding 500 µL of each of the 2X dosing dilutions, drop-wise under gentle agitation of the plate by hand, to the appropriate wells containing 500 µl of cell suspension. The treated plates will be sealed with plate sealers (to avoid evaporation or cross-contamination of volatile test articles) and the plate will be shaken by hand prior to incubation for 24 ± 0.5 hours at standard culture conditions. For each test article, one experiment is needed to derive a prediction. Each experiment consists of at least two independent runs. In the case of incongruent results and/or at the Study Director's discretion, a third run (or more) may be completed.

- Exposure time
24 hours

- Study evaluation and decision criteria used
Negative results are acceptable only for test articles exhibiting cell viability <90% at the highest dose tested. Negative results with cell viabilities of ~90% at the highest dose tested are not valid, and may require retesting at higher doses, unless the highest allowable doses were tested (i.e., up to 5000 µg/mL in saline, 1000 µg/mL in DMSO, or the highest soluble concentration).
Based on the OECD TG 442E, a negative result for test materials with Log Kow (octanol-water partition coefficient) >3.5 should not be considered.

- Description on study acceptance criteria
The assay will be accepted if all of the following acceptance criteria are met:

1) The cell viability values of the solvent control(s) are >90%.
2) For the solvent control(s), RFI values of both CD86 and CD54 are less than the positive criteria (CD86 RFI <150 and CD54 RFI <200).
3) For the positive control (DNCB), RFI values of both CD86 and CD54 are predicted to be positive (CD86 RFI ~150 and CD54 RFI ~200), and cell viability is >50%.
4) For the medium and solvent controls, the MFI ratio of both CD86 and CD54 to isotype control should be >105%.
5) The cell viability of the test article-treated cultures should be >50% in at least four doses.

SEEDING AND INCUBATION
- Seeding conditions (passage number and seeding density)
Cells will be routinely passaged every 2 to 3 days and seeded at a density of 0.1x10e6 to 0.2x10e6 cells/ml. The cells will routinely be maintained at densities ranging from 0.1 to 0.8x10e6 cells/ml. The cell density should not exceed 1.0x10e6 cells/ml. Cells can be propagated up to 2 months after thawing but not in excess of 30 passages post thawing.
At least 2 weeks after thawing, the cells will undergo a reactivity check. Only the cells which pass the reactivity check will be used in subsequent studies. Routine cell culture activities and reactivity check assay will be documented in the cell culture records and briefly summarized in the study report.
Prior to an assay, cells will be seeded in culture flasks at densities of 0.1 to 0.2x10e6 cells/ml and pre-cultured for 72 ± 4 or 48 ± 4 hours, respectively. The culture conditions and cell density defined for this pre-assay culture conditioning should be maintained as consistently as possible to ensure optimal CD54 and CD86 induction and expression. On the day of testing, cells will be harvested from the culture flasks and seeded into 24-well plates.
On the day of dosing, cells will be collected by centrifugation (200 to 300 g, in a centrifuge set to 5 minutes at room temperature). The cells will be resuspended in fresh culture medium to a density of 2.0x10e6 cells/ml , and 500 µl of the cell suspension will be seeded into the appropriate wells of a 24-well plate (resulting in l.0x106 cells/well). The plates will be maintained at standard culture conditions.

- Incubation conditions
The 2X dosing dilutions will be applied to the cells by adding 500 µl of each of the 2X dosing dilutions, drop-wise under gentle agitation of the plate by hand, to the appropriate wells containing 500 µl of cell suspension. The treated plates will be sealed with plate sealers (to avoid evaporation or cross-contamination of volatile test articles) and the plate will be shaken by hand prior to incubation for 24 ± 0.5 hours at standard culture conditions.
MEASUREMENT OF CELL SURFACE EXPRESSION/LUCIFERASE ACTIVITY
- Flow cytometry used
MACSQuant Analyzer (Miltenyi)

- Propidium iodide staining/cytotoxicity measurements
After 24 ± 0.5 hours of exposure, the samples will be removed from the 24-well plates and added to labeled micro-centrifuge tubes.
Note: After the appropriate exposure time and cell collection, the cell suspension(s) and reagents used to process the cell suspensions, including the FACS buffer, blocking buffer, and antibody mixture, will be maintained in cold conditions (for example, using benchtop cooler racks, ice packs and/or ice).
The cells will be collected by centrifugation (200 to 300 g, in a centrifuge set for 5 minutes and 4 °C). The supernatants will be carefully decanted into a waste container. The remaining cell pellets will be resuspended with 1 ml of FACS buffer and centrifuged again using the above centrifuge settings and decanting the supernatant. The rinsing process is performed 2 additional times using 1 ml of FACS buffer.
After the three rinses, each cell pellet will be resuspended in 600 µl of FACS buffer and 200 µl of the suspension will be transferred to the appropriate wells of a 96-well round-bottom plate. Propidium Iodide will be added to the appropriate samples of the 96-well plate to make a final concentration of 0.625 µg/ml of PI in the plate.
The PI uptake will be analyzed using flow cytometry. Cells stained with PI represent the non-viable cell population and will be gated out to identify the viable populations. Approximately 10,000 living (PI negative) cells will be acquired. When the cell viability is low, up to approximately 30,000 cells including dead cells can be acquired. Alternatively, the data acquisition can be finished one minute

after the initiation. The cell viability will be calculated (e.g. PI negative events versus total events). T he CV75 value, a concentration expected to result in 75% cell viability, will be calculated.

- Preparation for CD54 and/or CD86 expression measurements/cell staining

After 24 ± 0.5 hours of exposure, the samples will be placed into labeled microcentrifuge tubes and the cells will be collected by centrifugation as described above.

Note: After the appropriate exposure time and cell collection, the cell suspension(s) and reagents used to process the cell suspensions, including the FACS buffer, blocking buffer, and antibody mi xture, will be maintained in cold conditions (for example, using benchtop cooler racks, ice packs and/ or ice).

The supernatants will be carefully decanted into a waste container. The remaining cell pellets will be resuspended with 1 ml of FACS buffer and centrifuged. The rinsing process is performed two addi tional times using 1 ml of FACS buffer. Finally, cells will be resuspended in 600 µL of 0.01% (w/v) blocking suspension (prepared in FACS buffer from a 1% (w/v) stock suspension immediately before use) and incubated at 2 to 8 •c for 15 ± 1 minutes. After the blocking step, the samples will be divided into 3 aliquots of 180 µLeach into the designated wells of a 96-well round-bottom plate. The cells will be collected by centrifugation as described in § 7.4.5 and the supernatants will be aspirated without disturbing the cell pellet. A master mixture of each antibody (CD54, CD86 and mouse IgG isotype control) will be prepared based on the number of samples needing to be stained with each antibody so that each sample receives 50 µL of the appropriate antibody dose. For each test article dilution or control there will be 3 cell populations each treated with a different antibody mixture. There will be a separate cell population treated with FITC anti-CD54, FITC anti-CD86, and FITC isotype control. The antibody mixtures will be prepared in FACS buffer using the following ratios: 3 µL of CD54 to 50 µL total; 6 µL of CD86 to 50 µL total; 3 µL of isotype control to 50 µL total.

Fifty microliters of each antibody mixture will be added to the appropriate wells of the 96-well plate. The plate will be gently agitated by hand to mix the reagents and then incubated in the dark at 2 to 8 2c for 30 ± 1 minutes under gentle agitation. Following incubation, 150 µL of FACS buffer will be added to each well and the plate will be centrifuged as described. The wash step is repeated twic e with 200 µL of FACS buffer. Finally, cells will be resuspended in 200 µL of FACS buffer. PI will be added to the appropriate wells of the 96-well plate to make a final concentration of 0.625 µg/ml of PI in the plate.

The expression of CD54, CD86, isotype control and PI uptake will be analyzed using flow cytometry. Cells stained with PI will be gated out to identify the viable populations. Approximately 10,000 livin g (PI negative) cells will be acquired. When the cell viability is low, up to 30,000 cells including dead cells can be acquired. Alternatively, the data acquisition can be finished one minute after the initiati on. The cell viability will be calculated (e.g. PI negative events versus total events). In addition the MFI of the antibody stained cell populations will be calculated. The MFI values will be used to calculate the RFI values to determine skin sensitization predictions.

DATA EVALUATION

- Cytotoxicity assessment

The following plots are prepared using the flow cytometry software (MACSQuantify™ Version 2.10 / MACSQuant® Analyzer used for operation and data collection and Flowlogic 7.2.1 for data analysis):

• Side Scatter (SSC) versus Forward Scatter (FSC)

FSC is a measure of cell size. SSC is a measure of cell granularity. This plot is created to confirm a single population of cells is present without excessive debris.

• 2 Histogram Plots (Cell Count versus PI) (Cell Count versus FITC)

These plots are used to determine the percentage of each cell population expressing PI (for cell viability) or FITC (for upregulation of CD54 and CD86).

A gate will be placed between the peak of the PI negative fraction and the PI positive fraction on the histogram using the DNCB-treated isotype control cells. The PI negative fraction corresponds to liv ing cells which are used for subsequent analysis. The MFI of the living populations of each cell sa mple is determined by the software and used to determine the RFI values for each test article treated sample.

The isotype controls consist of the same test article concentrations tested for the CD54 and CD86 staining, but these samples will be treated with isotype control consisting of mouse IgG. Use of the i sotype control will allow for the distinction between specific CD54 and CD86 antibody binding and no n-specific background antibody binding.

- Prediction model used
Each test article will be tested in at least two independent definitive assays to derive a single predict ion (POSITIVE or NEGATIVE) for the potential to activate dendritic cells. The definitive assays may be performed on the same day provided that for each assay:
a) independently harvested cells will be used (i.e. cells collected from different culture flasks), and
b) independent fresh stock solutions of the 2X dosing dilutions of the test articles and antibodies will be prepared.
If the RFI of CD86 is equal to or greater than 150% at any tested dose with ~50% cell viability in at least 2 independent assays and/or if the RFI of CD54 is equal to or greater than 200% at any test ed dose with ~50% cell viability in at least 2 independent assays, the prediction will be considered as positive. Otherwise, the prediction will be considered as negative. In case the first 2 independent assays are not concordant, a third assay will be performed and typically the final prediction will be based on the mode of the conclusions from the 3 individual runs (i.e. 2 out of 3).
Test articles with limited solubility may still be tested at lower soluble concentrations. In such a case, a negative result will be considered inconclusive, whereas a positive result will be used to support the identification of the test article as a potential skin sensitizer.
For test articles considered to be positives, 2 effective concentrations (EC) values, the EC150 for CD86 and EC200 for CD54 will be calculated. Two consecutive concentrations starting from the lowest dose will be used in the EC calculations. The EC values represent the calculated test article concentration at which an RFI of 150 or 200 is achieved.

Vehicle / solvent control
DMSO

Positive control
dinitrochlorobenzene (DNCB) [442E]

Results and discussion

Positive control results
DNCB results:
viability Exp1: 74.1% Exp2: 93.56%
CD54 RFI Exp1: 1352.70% Exp2: 5035.00%
CD86 RFI Exp1: 328.96% Exp2: 457.46%

DMSO controls:
viability Exp1: 98.46%, Exp2: 96.55%
CD54 RFI Exp1: 108.82%, Exp2: 18.69%
CD86 RFI Exp1: 92.37%, Exp2: 82.00%

In vitro / in chemico

Results

Key result
false

Group
test chemical

Run / experiment
run/experiment 1

Parameter
RFI CD86>200 [442E]

Value

127.31 %

Cell viability
61.28%

Vehicle controls validity
valid

Negative controls validity
not applicable

Positive controls validity
valid

Remarks on result
no indication of skin sensitisation RFI at highest tested concentration. RFI at highest concentration (70.0 µg/ml) with viability >50% was 109.24

Key result
false

Group
test chemical

Run / experiment
run/experiment 2

Parameter
RFI CD86>200 [442E]

Value

109.59	%

Cell viability
95.49%

Vehicle controls validity
valid

Negative controls validity
not applicable

Positive controls validity
valid

Remarks on result
no indication of skin sensitisation RFI at highest tested concentration (58.3 µg/ml) with viability >50%. Max. RFI was 121.45% at 48.6 µg/ml.

Key result
false

Group
test chemical

Run / experiment
run/experiment 1

Parameter
RFI CD54>150 [442E]

Value

291.13	%

Cell viability
94.56%

Vehicle controls validity
valid

Negative controls validity
not applicable

Positive controls validity
valid

Remarks on result
positive indication of skin sensitisation lowest concentration with RFI >200. RFI showed positive dose response up to highest concentration tested (84.0 µg/ml) with viability 61.87% and RFI 1 058.72%.

Key result
false

Group
test chemical

Run / experiment
run/experiment 2

Parameter
RFI CD54>150 [442E]

Value

240.88 %

Cell viability
97.76%

Vehicle controls validity
valid

Negative controls validity
not applicable

Positive controls validity
valid

Remarks on result
positive indication of skin sensitisation lowest concentration (33.8 µg/ml) with RFI >200. RFI showed positive dose response up to concentrations reducing viability below 75% (58.3 µg/ml) with viability 95.89% and RFI 1048.99%.

Any other information on results incl. tables

RESULTS

PRELIMINARY CYTOTOXICITY ASSESSMENT

Table 1: Results of preliminary cytotoxicity assessment

TA ID	Well ID	Well Name	Living #Events	Living %Parent	Concentrations

					on the cells (µg/mL)			
Controls	D1	Media	10017	98.21		**Potential Definitive Trial Dose Ranges Based on CV75**		
	C1	DMSO	10018	97.63		**100X/500X in Primary Solvent (mg/mL)**	**2X in Media (µg/mL)**	**1X on the Cells (µg/mL)**
	B1	DNCB	20185	68.67				
Thymol	A6	TA5D1	3809	39.74	1000.0	42.0	168.0	84.0
	B6	TA5D2	1435	56.70	500.0	35.0	140.0	70.0
	C6	TA5D3	1412	10.37	250.0	29.2	116.7	58.3
	D6	TA5D4	3734	8.25	125.0	24.3	97.2	48.6
	E6	TA5D5	10072	88.05	62.5	20.3	81.0	40.5
	F6	TA5D6	10037	95.95	31.3	16.9	67.5	33.8
	G6	TA5D7	10030	96.50	15.6	14.1	56.3	28.1
	H6	TA5D8	10035	96.63	7.8	11.7	46.9	23.4

The CV75 value was determined to be 70 µg/mL.

Table 2: RFI CD86, RFI CD54 and rel. viability. Mean values and standard deviations of 1st experiment.

Well ID	**Well Name**	**Final Test Article Concentration (µg/mL)**	**Viable Events**	**Positive Events**	**% Viable**	**Living FITC Geometric Mean**	**Calculated RFI**
A10	TA3D1 CD54	84.0	9972	9960	61.87	43.78	1058.72
B10	TA3D2 CD54	70.0	9992	9956	88.18	31.03	684.40
C10	TA3D3 CD54	58.3	9995	9959	93.34	19.35	349.85

D10	TA3D4 CD54	48.6	9988	9956	94.56	17.59	291.13
E10	TA3D5 CD54	40.5	9996	9962	95.55	13.78	197.86
F10	TA3D6 CD54	33.8	9996	9973	96.28	12.83	168.50
G10	TA3D7 CD54	28.1	9998	9964	96.31	12.03	149.24
H10	TA3D8 CD54	23.4	9998	9978	97.39	14.81	234.25
A11	TA3D1 CD86	84.0	9985	9967	61.28	25.15	127.31
B11	TA3D2 CD86	70.0	9993	9983	88.05	22.37	109.24
C11	TA3D3 CD86	58.3	9998	9986	93.34	21.99	112.10
D11	TA3D4 CD86	48.6	9990	9988	93.62	23.96	126.51
E11	TA3D5 CD86	40.5	9993	9989	96.33	22.48	120.78
F11	TA3D6 CD86	33.8	9995	9988	96.17	22.97	124.60
G11	TA3D7 CD86	28.1	9997	9995	96.97	23.61	131.05
H11	TA3D8 CD86	23.4	9997	9992	96.83	21.29	112.58
A12	TA3D1 Isotype Control	84.0	9983	9926	62.62	9.16	
B12	TA3D2 Isotype Control	70.0	9992	9911	87.93	8.65	
C12	TA3D3 Isotype Control	58.3	9996	9940	93.77	7.91	

D12	TA3D4 Isotype Control	48.6	9990	9946	93.86	8.07	
E12	TA3D5 Isotype Control	40.5	9992	9945	96.39	7.31	
F12	TA3D6 Isotype Control	33.8	9999	9954	96.95	7.32	
G12	TA3D7 Isotype Control	28.1	9997	9948	96.85	7.15	
H12	TA3D8 Isotype Control	23.4	9997	9950	96.37	7.15	

Table 3: RFI CD86, RFI CD54 and rel. viability. Mean values and standard deviations of 2nd experiment.

A4	TA1D1 CD54	84.0	5741	5728	20.89	30.38
B4	TA1D2 CD54	70.0	6032	6023	24.53	35.87
C4	TA1D3 CD54	58.3	9997	9981	95.98	39.59
D4	TA1D4 CD54	48.6	9998	9984	96.49	33.96
E4	TA1D5 CD54	40.5	9999	9978	97.57	19.31
F4	TA1D6 CD54	33.8	9995	9975	97.76	14.90
G4	TA1D7 CD54	28.1	9995	9974	98.14	12.79
H4	TA1D8 CD54	23.4	9993	9953	96.93	11.69
A5	TA1D1 CD86	84.0	7471	7457	18.70	27.32
B5	TA1D2 CD86	70.0	8138	8130	23.79	29.99
C5	TA1D3 CD86	58.3	9997	9994	95.49	25.91
D5	TA1D4 CD86	48.6	9995	9990	96.22	27.63

E5	TA1D5 CD86	40.5	9998	9994	97.69	26.18
F5	TA1D6 CD86	33.8	10000	9997	98.30	25.74
G5	TA1D7 CD86	28.1	9997	9997	98.25	26.05
H5	TA1D8 CD86	23.4	9995	9986	97.55	22.19
A6	TA1D1 Isotype Control	84.0	7086	7065	21.23	7.35
B6	TA1D2 Isotype Control	70.0	7547	7526	26.10	7.99
C6	TA1D3 Isotype Control	58.3	9994	9957	95.53	8.54
D6	TA1D4 Isotype Control	48.6	10000	9954	96.86	8.38
E6	TA1D5 Isotype Control	40.5	9997	9961	97.32	8.06
F6	TA1D6 Isotype Control	33.8	9999	9970	97.86	7.77
G6	TA1D7 Isotype Control	28.1	10000	9947	98.45	7.75
H6	TA1D8 Isotype Control	23.4	9996	9964	98.21	7.71

The EC150 (the concentration resulting in a RFI of 150%) for CD86 could not be determined as no value was higher than 150%.

The EC200 (the concentration resulting in a RFI of 200) for CD54 was calculated from the results as 40.7 and 30.5 µg/ml for experiments 1 and 2, respectively.

Applicant's summary and conclusion

Interpretation of results
other: positive indication of skin sensitisation

Conclusions
In summary, after 24 hours of exposure to test substance, CD54 expression, but not CD86 ex pression, was induced in THP-1 cells beyond the threshold of a positive response according to OECD 442E in two independent experiments. From this it has to be concluded that test substance induces dendritic cell activation.

References

LITERATURE: HUMAN CELL LINE ACTIVATION TEST (H-CLAT)

UUID: a26bc36a-21d6-4d17-8740-4ef55ad07976
Dossier UUID:
Author: uivng
Date: 2023-08-30T14:29:55.003+02:00
Remarks:

General information

Reference Type
study report

Title
HUMAN CELL LINE ACTIVATION TEST (H-CLAT)

Author
[redacted]

Year
2022

Testing facility
Institute for In Vitro Sciences, Gaithersburg, MD, USA

Report date
2022-09-22

Report number
AK75.177000

TEST_MATERIAL_INFORMATION: Thymol CAS 89-83-8

UUID:	0d4eaaae-8da2-496d-a923-7ef7b9430b76
Dossier UUID:	
Author:	uivng
Date:	2023-08-30T13:56:10.405+02:00
Remarks:	

Name
Thymol CAS 89-83-8

Composition

Other characteristics

Test material form
solid: crystalline