



Development of an *in vitro* skin sensitization test based on ROS production in THP-1 cells

Kazutoshi Saito*, Masaaki Miyazawa, Yuko Nukada, Hitoshi Sakaguchi, Naohiro Nishiyama

Safety Science Research Laboratories, Kao Corporation, 2606 Akabane, Ichikai-Machi, Haga-Gun, Tochigi 321-3497, Japan

ARTICLE INFO

Article history:

Received 3 September 2012

Accepted 24 December 2012

Available online 31 December 2012

Keywords:

Reactive oxygen species (ROS)

Skin sensitization

In vitro testing

THP-1 cells

Animal testing alternatives

ABSTRACT

Recently, it has been reported that reactive oxygen species (ROS) produced by contact allergens can affect dendritic cell migration and contact hypersensitivity. The aim of the present study was to develop a new *in vitro* assay that could predict the skin sensitizing potential of chemicals by measuring ROS production in THP-1 (human monocytic leukemia cell line) cells. THP-1 cells were pre-loaded with a ROS sensitive fluorescent dye, 5-(and 6-)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA), for 15 min, then incubated with test chemicals for 30 min. The fluorescence intensity was measured by flow cytometry. For the skin sensitizers, 25 out of 30 induced over a 2-fold ROS production at more than 90% of cell viability. In contrast, increases were only seen in 4 out of 20 non-sensitizers. The overall accuracy for the local lymph node assay (LLNA) was 82% for 50 chemicals tested. A correlation was found between the estimated concentration showing 2-fold ROS production in the ROS assay and the EC3 values (estimated concentration required to induce positive response) of the LLNA. These results indicated that the THP-1 cell-based ROS assay was a rapid and highly sensitive detection system able to predict skin sensitizing potentials and potency of chemicals.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Allergic contact dermatitis resulting from skin sensitization is a common occupational and environmental health problem (Peiser et al., 2012). Presently, the identification and evaluation of unknown skin sensitizers rely on animal testing, such as LLNA (Kimber et al., 2002). However, in addition to the ethical issues concerning the use of animal testing, the 7th amendment to the cosmetic directive (Directive 76/768/EEC) will impose a ban on marketing cosmetics in Europe after March 2013 if the finished product or its ingredients have been tested using animals. Thus,

in vitro tests that can evaluate the skin sensitization potential of chemicals will need to be developed.

During the induction phase of skin sensitization, dendritic cells (DCs) play an important role in processing and presenting antigens after exposure to skin sensitizers (Kimber et al., 2000). Following an encounter with skin sensitizers, DCs become activated and differentiate into mature DCs by up-regulating the expression of various surface markers (e.g., CCR7, CD54 and CD86) and secreting several cytokines (e.g., IL-1 β) (Sasaki and Aiba, 2007) and chemokines (Toebak et al., 2006). After migration to draining lymph nodes, mature DCs are able to effectively present antigens to naive T cells (Toebak et al., 2009). More recent studies have demonstrated that a variety of changes induced by sensitizers in the DC or DC-like cell lines in cultures, including activation of intracellular kinases (Mitjans et al., 2010), expression of selected genes (Hooberghs et al., 2008), expression of several cell surface molecules (Yoshida et al., 2003; Azam et al., 2006) and production of selected chemokines (Mitjans et al., 2008; Nukada et al., 2008) can be used for the development of DC-based assays. Among them, the most promising assays are the human Cell Line Activation Test (h-CLAT) (Ashikaga et al., 2010) and the Myeloid U937 Skin Sensitization Test (MUSST) (Python et al., 2007), which focus on the expression of the DC surface markers (e.g., CD86). These tests have examined approximately 100 chemicals and have been shown to have an overall accuracy of more than 80% as compared to the LLNA data. At the present time, they are formally undergoing pre-validation

Abbreviations: BH, 1-bromohexane; BKC, benzalkonium chloride; CB, chlorobenzene; CM-H₂DCFDA, 5-(and 6-)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester; DCs, dendritic cells; DCV5, concentration showing 5% decrease of cell viability relative to vehicle control; DEP, diethyl phthalate; DMSO, dimethylsulfoxide; DNCB, 2,4-dinitrochlorobenzene; DNFB, 1-fluoro-2,4-dinitrobenzene; ECVAM, European Centre for the Validation of Alternative Methods; HBSS, Hank's Balanced Salt Solution; HCA, hexylcinnamaldehyde; h-CLAT, human Cell Line Activation Test; LA, lactic acid; LLNA, local lymph node assay; MDBGN, methylidibromo glutaronitrile; MFI, mean fluorescence intensity; MS, methyl salicylate; OA, octanoic acid; OXA, oxazolone; PA, phthalic anhydride; PG, propyl gallate; PI, propidium iodide; PPD, *p*-phenylenediamine; ROS, reactive oxygen species; ROS EC₂, estimated concentration which showed 2-fold ROS production; ROS *I*_{max}, maximal ROS induction level; TMTD, tetramethylthiuram disulfide; TrxR, thioredoxin reductase.

* Corresponding author. Tel.: +81 285 68 7342; fax: +81 285 68 7452.

E-mail address: saitou.kazutoshi@kao.co.jp (K. Saito).

under the European Centre for the Validation of Alternative Methods (ECVAM) (Aeby et al., 2010). However, it has been suggested that a cellular redox imbalance induced by reactive oxygen species (ROS) can trigger a variety of changes that occur in the DCs after an *in vitro* exposure to sensitizers (Sasaki and Aiba, 2007). In fact, it has been reported that both DC migration and contact hypersensitivity, induced by the skin sensitizer trinitrochlorobenzene, were inhibited in a transgenic mouse over-expressing extracellular superoxide dismutase (Na et al., 2007). Since superoxide dismutase is one of the major tissue defense enzymes that protect tissue against the toxic effect of ROS, it was expected that ROS production could be involved in the early phase of the skin sensitization processes, including DC activation and migration. Moreover, Nukada et al. (2011) also demonstrated that selected skin sensitizers induced significant ROS production in THP-1 cells, which are known to show DC-like phenotypes when exposed to skin sensitizers (Yoshida et al., 2003; Miyazawa et al., 2007; Lambrechts et al., 2009). Similar ROS production has been observed in human monocyte-derived DCs (Byamba et al., 2010) and in the human DC-like cell line, U937 cells (Migdal et al., 2010a). Consequently, it was hypothesized that ROS production in DC could be a novel and universal marker for identifying the skin sensitizing potential of chemicals.

In this study, we used THP-1 cells to develop a new *in vitro* assay based on ROS production (ROS assay) that could identify skin sensitizers. In the first step, we optimized the ROS assay protocol. Subsequently, we then evaluated the predictive performance of the ROS assay with chemicals that have been demonstrated to have *in vivo* sensitizing properties.

2. Materials and methods

2.1. Cell line and medium

THP-1 cells from the American Type Culture Collection (Manassas, VA, USA) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Invitrogen Corp., Carlsbad, CA, USA), 0.05 mM 2-mercaptoethanol and 1% penicillin–streptomycin (Invitrogen Corp.).

2.2. Test chemicals and preparation of chemicals

We selected test chemicals based on the skin sensitizing properties reported in the literature (Gerberick et al., 1992, 2005; Kimber et al., 1998), their diverse chemical structures, and availability from commercial sources. The 31 sensitizers employed in this study were oxazolone (OXA), 5-chloro-2-methyl-4-isothiazolin-3-one solution, 1-fluoro-2,4-dinitrobenzene (DNFB), 2,4-dinitrochlorobenzene (DNCB), 4-nitrobenzyl bromide, glutaraldehyde, 1,4-dehydroquinone, *p*-phenylenediamine (PPD), phthalic anhydride (PA), benzoyl peroxide, propyl gallate (PG), formaldehyde, methyl-dibromo glutaronitrile (MDBGN), isoeugenol, 2-mercaptobenzothiazole, 3-dimethylaminopropylamine, ethylenediamine, cinnamic aldehyde, tetramethylthiuram disulfide (TMTD), diethylenetriamine, 4-chloroaniline, 1-bromohexane (BH), hexylcinnamaldehyde (HCA), citral, abietic acid, lilial, cyclamen aldehyde, imidazolidinyl urea, ethylene glycol dimethacrylate, butyl glycidyl ether and hydroxycitronellal. The 20 non-sensitizers used were acetanilide, benzalkonium chloride (BKC), 1-bromobutane, 1-butanol, chlorobenzene (CB), diethyl phthalate (DEP), dimethyl formamide, glycerol, hexane, 4-hydroxybenzoic acid, isopropanol, lactic acid (LA), methyl salicylate (MS), octanoic acid (OA), propylene glycol, saccharin, salicylic acid, vanillin, zinc sulfate and sodium lauryl sulfate. This collection includes reference chemicals recommended by ECVAM and the European Cosmetic Association

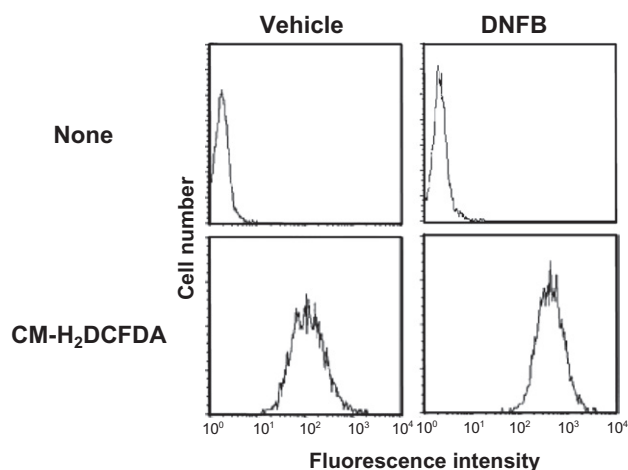


Fig. 1. DNFB treatment induced ROS production in THP-1 cells. Cells pre-loaded with HBSS alone or CM-H₂DCFDA were incubated for 30 min with 2.5 µg/mL DNFB under serum free conditions. The samples were then examined for fluorescence profiles.

for developing *in vitro* skin sensitization assays (Casati et al., 2009). With the exception of lilial, all tested chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). Lilial was purchased from Wako Pure Chemicals (Osaka, Japan). All tested chemicals were initially prepared in saline, dimethylsulfoxide (DMSO; Sigma–Aldrich) or acetone (Sigma–Aldrich) and then added to Hanks' Balanced Salt Solution (HBSS; Invitrogen Corp.). The maximum final concentration of DMSO and acetone in the culture medium was 2.5%, which had no effect on either the ROS production or the cell viability (data not shown).

2.3. Preliminary experiments to determine the standard protocol for the ROS assay

A cell-permeable indicator, 5-(and 6-)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA; Invitrogen Corp.) was used to indirectly measure the intracellular ROS generation. This dye was retained within the cells after removal of the acetate group by cellular esterase, with oxidation then converting the molecule into a green-fluorescent form. This ROS indicator is photosensitive, so all the procedure was performed under dark conditions. THP-1 cells (2×10^6 cells/mL) were preloaded with 2 µM CM-H₂DCFDA for 15 min at 37 °C in HBSS. After washing, THP-1 cells (1×10^6 cells/mL) were exposed to the test chemicals for 30 min at 37 °C in 5 mL tubes (BD Biosciences, San Jose, CA, USA) covered with aluminum foil to prevent any exposure to light.

To determine the standard protocol for the ROS assay, we conducted the following two experiments under different chemical exposure conditions. The first experiment was designed to examine the effect of FBS on ROS production (Fig. 2). In this step, THP-1 cells were exposed to 2.5 µg/mL DNFB or 150 µg/mL OXA for 30 min in the absence or presence of serum (0%, 5% and 10%). In the second experiment, we examined ROS production by exposing the THP-1 cells to each of four selected sensitizers (OXA, MDBGN, TMTD and HCA) (Fig. 3a) and four selected non-sensitizers (BKC, BC, glycerol and MS) (Fig. 3b) for 30 min under serum free conditions. After the exposure to the chemicals, the fluorescence intensity of a total of 5000 cells was measured by the FACS Calibur and CellQuest Pro software (BD Biosciences). Cell viability was determined by propidium iodide (PI; Sigma–Aldrich) staining. The final concentration of PI was set at 0.625 µg/mL.

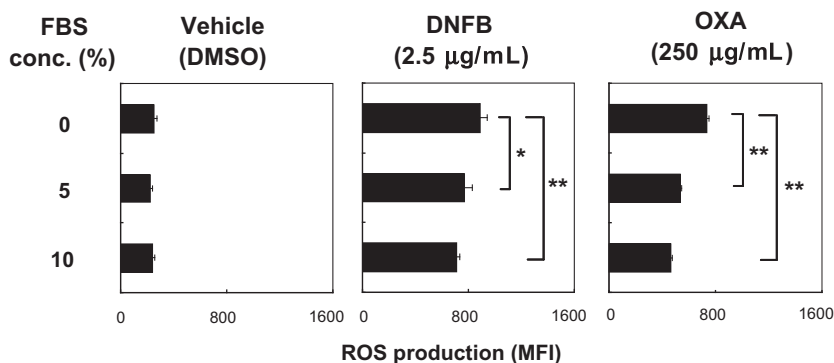


Fig. 2. FBS suppresses the ROS production induced by sensitizers. Cells were treated with each sensitizer (DNFB: 2.5 µg/mL, OXA: 150 µg/mL) for 30 min at the indicated serum concentrations (0%, 5% or 10%). Results are expressed as mean $\pm$ SEM of three independent experiments ($n = 1$). Statistical analysis was performed using Dunnett's multiple comparison test with * $P < 0.05$, ** $P < 0.01$ versus serum 0%.

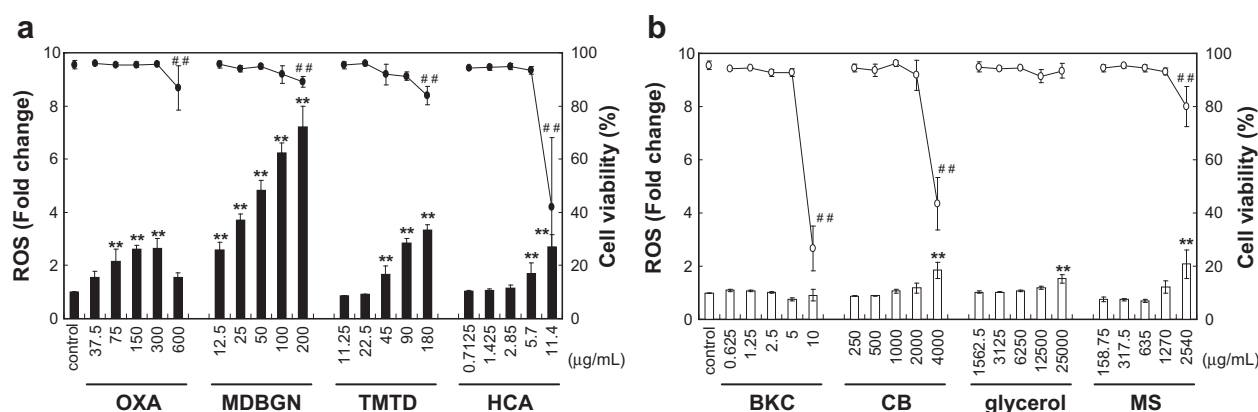


Fig. 3. (a and b) ROS production induced by the four selected sensitizers and four selected non-sensitizers. Cells were treated with the indicated sensitizers and non-sensitizers for 30 min under serum free conditions. ROS (fold change; black bars indicate sensitizers (a) and outline bars indicate non-sensitizers (b)) and cell viability (black dots indicate sensitizers (a) and outline dots indicate non-sensitizers (b)) were determined. Results are expressed as mean $\pm$ SEM of three independent experiments ($n = 1$). Statistical analysis was performed using Dunnett's multiple comparison test with **** $P < 0.01$ versus control.

2.4. ROS assay standard protocol

2.4.1. Dose finding study

A dose finding study was conducted to determine the concentration that would cause a 5% decrease of the cell viability relative to vehicle control (DCV5) for each chemical. THP-1 cells (2×10^6 cells/mL) were preloaded with 2 µM CM-H₂DCFDA for 15 min at 37 °C in HBSS. THP-1 cells (1×10^6 cells/mL) were washed and then exposed to the test chemicals at five concentrations ($1/2^4$, $1/2^3 \times$, $1/2^2 \times$, $1/2 \times$ and $1 \times 12,500$ µg/mL) in the absence of serum for 30 min at 37 °C in 5-mL tubes. PI (at a final concentration of 0.625 µg/mL) was added to each tube, with the number of living cells then measured. After the DCV5 was calculated by linear regression, five concentrations ($1/2^3 \times$, $1/2^2 \times$, $1/2 \times$, $1 \times$ and $2 \times$ DCV5) were selected for the main study. If a test chemical was non-toxic and the DCV5 could not be calculated, five basic concentrations ($1/2^4 \times$, $1/2^3 \times$, $1/2^2 \times$, $1/2 \times$ and $1 \times 25,000$ µg/mL) were used for the main study.

2.4.2. Main study

THP-1 cells (2×10^6 cells/mL) were preloaded with 2 µM CM-H₂DCFDA for 15 min in HBSS. After washing, THP-1 cells (1×10^6 cells/mL) were exposed to the test chemicals at the five designated concentrations for 30 min under FBS-free conditions in a 5-mL tube. PI was added to each tube (final concentration of 0.625 µg/mL) and the fluorescence intensity was measured by FACS Calibur. ROS production relative to the vehicle control (fold change) was

determined in three independent studies, with the average ROS production then calculated. Any chemical that induced more than a 2-fold average ROS production at over 90% cell viability was defined as being positive. The maximal ROS induction level (ROS I_{max}) was determined using the data from the chemicals that had tested concentrations with over 90% cell viability. Based on the average ROS production of three independent experiments, the estimated concentration which showed a 2-fold ROS production (ROS EC₂) at over 90% cell viability was calculated using linear extrapolation from the values above and below the induction threshold (2-fold ROS production).

2.5. Statistics

Dunnett's multiple comparison test was used to evaluate the statistical significance. P -values less than or equal to 0.05 and 0.01 were considered to be statistically significant.

3. Results

3.1. Effect of FBS on ROS production induced by skin sensitizers

In the first step, we examined the amount of ROS production that was inducible by sensitizers in the THP-1 cells by indirectly measuring the level of oxidative stress with a ROS sensitive dye CM-H₂DCFDA. Briefly, THP-1 cells were pre-loaded with

CM-H₂DCFDA for 15 min, washed extensively, and then incubated with 2.5 µg/mL DNFB or vehicle alone. The brief exposure time (30 min) used for the test chemicals was selected based on the results of a study by Migdal et al. (2010b). This previous study demonstrated that the potent skin sensitizer, DNCB, induced a significant and rapid ROS production within 30 min in the human monocyte-derived dendritic cells. In our study, treatment with DNFB induced a marked increase of the fluorescence intensity in the THP-1 cells (Fig. 1). Using mean fluorescence intensity (MFI) as a parameter, we next examined the effect of FBS on the ROS production induced by two potent skin sensitizers (DNFB and OXA). As seen in Fig. 2, both DNFB (2.5 µg/mL) and OXA (150 µg/mL) showed elevated ROS production (891 in DNFB and 736 in OXA) compared to vehicle (222 in DMSO) under serum free condition. FBS inhibited sensitizer-induced ROS production in a dose-dependent manner, while it did not affect ROS production in the vehicle controls. The test concentration of each chemical failed to induce cytotoxicity in either the absence or presence of FBS. These data indicated that FBS needs to be removed in order to be able to detect sensitizer-induced ROS production.

3.2. ROS production by sensitizers and non-sensitizers

We next examined ROS production after treatment with either four sensitizers with different sensitizing potencies, or four non-sensitizers. Based on the results shown in Fig. 2, we decided to conduct the subsequent chemical treatment experiments under FBS-free conditions. As seen in Fig. 3a, the four sensitizers dose-dependently induced significant ROS production, which was upregulated over 1.5-fold even at cell viability levels (non-toxic concentrations) comparable to that in the vehicle controls. Fig. 3b shows the ROS production induced by the four non-sensitizers. BKC did not induce ROS production irrespective of cytotoxicity. Conversely, while CB and MS were able to induce significant ROS production, this only occurred when toxic concentrations were present (43.4% cell viability for 4000 µg/mL CB, and 80.1% for 2540 µg/mL MS). On the other hand, glycerol induced significant but slight (1.5-fold) ROS production at 25,000 µg/mL, which was the highest test concentration used during these experiments. These data suggest that ROS production should be able to be detected at non-toxic concentrations (cell viabilities comparable to that seen in the vehicle controls), which will make it possible to distinguish between sensitizers and non-sensitizers.

3.3. Predictive performance of ROS assay with 50 chemicals

A total of 50 test chemicals (30 sensitizers and 20 non-sensitizers) were evaluated in accordance with the standard ROS assay protocol described in the Materials and Methods. Table 1 shows the chemical vehicles, the DCV5, the ROS $I_{\max}$, the ROS assay judgments (positive: P or negative: N) and the ROS EC2 for the 50 test chemicals. Based on the results in Fig. 3, the ROS $I_{\max}$ and ROS EC2 were determined from the ROS production at over 90% cell viability. The ROS $I_{\max}$ values, which were dependent on test chemicals used, varied from 0.50 (acetanisole) to 7.92 (benzoyl peroxide). There was an over 2-fold ROS production induced by 25 of the 30 tested sensitizers and by 4 out of the 20 non-sensitizers, which demonstrates there was a relatively high sensitivity (83%), specificity (80%) and accuracy (82%) for the LLNA results (Table 2). Compared with other cut-off values (positive criteria for 1.5-fold or 2.5-fold ROS production), the overall accuracy for the 2-fold ROS production was higher (Table 2). Eight out of 10 tested sensitizers classified as weak by the LLNA (citraol, abietic acid, linal, cyclamen aldehyde, imidazolidinyl urea, ethylene glycol dimethacrylate, butyl glycidyl ether and hydroxycitronellal) were also judged as positive (Table 1). Three strong sensitizers (PPD, PA and PG) and two

weak sensitizers (BH and HCA) were judged as being false negatives and four non-sensitizers (DEP, OA, saccharin and vanillin) were judged as being false positives (Table 1). The ROS EC2 values for the 29 tested chemicals varied from 0.20 (4-nitrobenzylbromide) to 1420 (DEP), with lower scores observed in the extreme and strong sensitizers as compared to the moderate, weak and non-sensitizers (Table 1). For the seven chemicals that had ROS EC2 values lower than 10 µg/mL, all were classified as “Extreme” or “Strong” sensitizers by LLNA ($EC3 < 1$). For the 9 out of the 11 chemicals that had ROS EC2 values between 10 and 100 µg/mL, all were classified as “Moderate” or “Weak” by LLNA ($1 \leq EC3 \leq 100$) (Table 3).

3.4. Scatter plot of the linear fit result of the Log (ROS EC2) versus Log (LLNA EC3)

To examine the relationship between the LLNA EC3 and ROS EC2, scatter plot analysis of Log (ROS EC2) versus Log (LLNA EC3) was conducted using the 25 chemicals judged as being positive in both the LLNA and the ROS assay (Fig. 4). Although the Pearson correlation coefficient (r) was 0.65 for the entire group, when OXA was removed from the analysis, $r = 0.79$ for the remaining 24 chemicals.

4. Discussion

In the present study, we attempted to develop a novel *in vitro* assay based on ROS production (ROS assay) to detect the sensitizing potential of chemicals in THP-1 cells. We initially optimized the assay protocol based on the following two findings: (1) FBS inhibited ROS production induced by sensitizers; and (2) significant ROS production by sensitizers was induced at concentrations resulting in over 90% of cell viability. Next, we evaluated the predictive performance of this ROS assay with 50 selected test chemicals. Our results revealed that the ROS assay displayed an overall accuracy of 82% for the LLNA. A good correlation was also obtained between the ROS EC2 and the LLNA EC3 values. These findings indicated the utility of the ROS assay as an *in vitro* assay for rapidly detecting the skin sensitizing potential of chemicals.

Our current results showed that FBS inhibited sensitizer-induced ROS production (Fig. 2). In general, most sensitizers have been shown to have a high binding ability to proteins (Divkovic et al., 2005). It has been reported that selected sensitizers can also bind to FBS proteins as well as to cellular ones *in vitro* (Hopkins et al., 2005). These previous reports additionally suggested that the apparent amount of sensitizers exposed to the cells might be reduced in the presence of FBS. Taken together, these findings suggest that FBS exclusion from the assay could contribute to an increased sensitivity for the sensitizers.

When compared to the non-sensitizers, the skin sensitizers induced significant ROS production at relatively low and non-toxic concentrations (Fig. 3). Usually, ROS is constitutively produced in the mitochondrial respiratory chain and neutralized by cellular detoxification enzymes such as catalase and glutathione/glutathione peroxidase (Feissner et al., 2009; Poyton et al., 2009). In addition, some ROS-producing enzymes like NADPH oxidase have also been shown to exist in mammalian cells (Dikalov, 2011). Nordberg et al. (1998) reported that DNCB induced NADPH oxidase activity through the irreversible inhibition of thioredoxin reductase (TrxR) by binding to the Cys residue of TrxR. Other investigators have reported that the known sensitizer, cinnamic aldehyde, inhibited TrxR activity via a similar mechanism (Chew et al., 2010). Since it is well known that sensitizers bind to electrophilic residues of amino acids (e.g., Cys, Lys) in proteins (Divkovic et al., 2005), it is likely that inhibition of TrxR activity may be one of the possible

Table 1

Summary of the ROS assay data for the 50 chemicals tested.

Chemical name	LLNA	LLNA	Vehicle	DCV5 ($\mu\text{g/mL}$)	ROS I_{max}		Judgment (2-fold) ^c	ROS EC2 ($\mu\text{g/mL}$)
	Potency category ^b	EC3 (%)			Average	SEM		
Oxazolone ^a	Extreme	0.003	DMSO	299	2.66	0.21	P	64.6
5-Chloro-2-methyl-4-isothiazolin-3-one solution		0.005	Saline	95.5	4.31	0.20	P	1.10
2,4-Dinitrochlorobenzene ^a		0.05	DMSO	292	5.98	0.27	P	7.20
4-Nitrobenzyl bromide ^a		0.05	DMSO	743	3.87	0.38	P	0.20
Glutaraldehyde	Strong	0.1	Saline	506	6.33	1.15	P	1.40
1,4-Dehydroquinone		0.11	Saline	2.50	2.40	0.19	P	0.60
p-Phenylenediamine ^a		0.16	DMSO	6770	1.05	0.02	N	n.i.
Phthalic anhydride		0.16	DMSO	475	1.29	0.07	N	n.i.
Benzoyl peroxide		0.3	DMSO	15.5	7.92	1.11	P	3.5
Propyl gallate		0.32	DMSO	1320	1.77	0.05	N	n.i.
Formaldehyde		0.61	Saline	8220	3.94	0.14	P	63.0
Methyldibromoglutaronitrile ^a		0.9	DMSO	102	6.24	0.22	P	8.80
Isoeugenol ^a	Moderate	1.2	DMSO	223	2.42	0.11	P	181
2-Mercaptobenzothiasole ^a		1.7	DMSO	552	3.94	0.18	P	113
3-Dimethylaminopropylamine		2.2	Saline	67.6	2.48	0.16	P	53.6
Ethylenediamine		2.2	Saline	69.1	2.31	0.14	P	54.9
Cinnamic aldehyde ^a		3	DMSO	389	2.89	0.06	P	23.0
Tetramethylthiuramdisulfide ^a		5.2	DMSO	90.9	2.84	0.10	P	57.6
Diethylenetriamine		5.8	Saline	127	2.14	0.07	P	110
4-Chloroaniline		6.5	DMSO	604	2.60	0.18	P	381
1-Bromohexane	Weak	10	DMSO	971	1.36	0.13	N	n.i.
Hexylcinnamaldehyde		11	DMSO	5.70	1.71	0.23	N	n.i.
Citral		13	DMSO	185	2.49	0.13	P	35.6
Abietic acid		15	DMSO	62.8	3.47	0.28	P	11.6
Lilial		19	DMSO	251	3.94	0.22	P	21.2
Cyclamen aldehyde		22	DMSO	523	3.41	0.23	P	93.8
Imidazolidinyl urea		24	Saline	4670	2.89	0.09	P	365
Ethyleneglycol dimethacrylate		28	DMSO	2960	3.12	0.08	P	61.5
Butyl glycidyl ether		31	DMSO	5500	3.17	0.18	P	776
Hydroxycitronellal		33	DMSO	2531	3.52	0.33	P	373
Acetoanisole	Non-sensitizer	Not calculated	DMSO	1390	0.50	0.10	N	n.i.
Benzalkonium chloride			Saline	5.00	1.10	0.03	N	n.i.
1-Bromobutane			DMSO	5080	1.41	0.16	N	n.i.
1-Butanol			Saline	6910	1.23	0.03	N	n.i.
Chlorobenzene			DMSO	1970	1.18	0.11	N	n.i.
Diethyl phthalate			DMSO	13,300	2.32	0.15	P	1420
Dimethyl formamide			Saline	25,000<	1.73	0.03	N	n.i.
Glycerol ^a			Saline	25,000<	1.52	0.10	N	n.i.
4-Hydroxybenzoic acid			DMSO	840	1.50	0.05	N	n.i.
Hexane			Acetone	14,300	1.10	0.01	N	n.i.
Isopropanol			Saline	25,000<	1.22	0.03	N	n.i.
Lactic acid ^a			Saline	454	1.26	0.12	N	n.i.
Methyl salicylate			DMSO	1270	1.22	0.14	N	n.i.
Octanoic acid			DMSO	658	2.42	0.14	P	168
Propylene glycol			Saline	25,000<	1.44	0.06	N	n.i.
Saccharin			DMSO	1670	2.92	0.21	P	542
Salicylic acid ^a			DMSO	552	1.50	0.06	N	n.i.
Vanillin			DMSO	2620	3.32	0.48	P	321
Zinc sulfate			Saline	160	1.24	0.06	N	n.i.
Sodium lauryl sulfate ^a			Saline	15.4	1.91	0.06	N	n.i.

Characters highlighted in bold indicate average ROS I_{max} of the chemicals with over a 2-fold induction. n.i. = no significant induction above threshold.^a Reference chemicals recommended by ECVAM and the European Cosmetic Association for developing an *in vitro* skin sensitization assay.^b Refers to paper published by Gerberick et al. (2005).^c Based on the ROS assay. "P" indicates positive while "N" indicates negative assay results.

mechanisms involved in the ROS production by sensitizers. On the other hand, it has been reported that cellular stress conditions like hypoxia and hyperosmolality cause increased intracellular ROS

levels in human leukemia cell lines (Aquilano et al., 2007; Kim et al., 2010). Although the precise mechanism responsible for this action remains unclear, it is possible that ROS production by

Table 2

Predictive performances for different positive criteria.

Positive criteria	Sensitivity	Specificity	Accuracy
1.5-Fold ROS	90% (27/30)	60% (12/20)	78% (39/50)
2.0-Fold ROS	83% (25/30)	80% (16/20)	82% (41/50)
2.5-Fold ROS	67% (20/30)	90% (18/20)	76% (38/50)

The chemicals were judged as positive when the ROS I_{max} of each chemical exceeded the indicated positive criteria.

Table 3

Classification of LLNA potency category based on ROS EC2.

LLNA potency category	ROS EC2 ($\mu\text{g/mL}$)		
	<10	≥ 10 to <100	≥ 100
Extreme or Strong	7	2	0
Moderate or Weak	0	9	7
Non-sensitizers	0	0	4

A total of 29 chemicals judged as positive in the ROS assay were applied to the analysis. Bold letters indicate relatively high classification capability of ROS EC2 for LLNA potency categories.

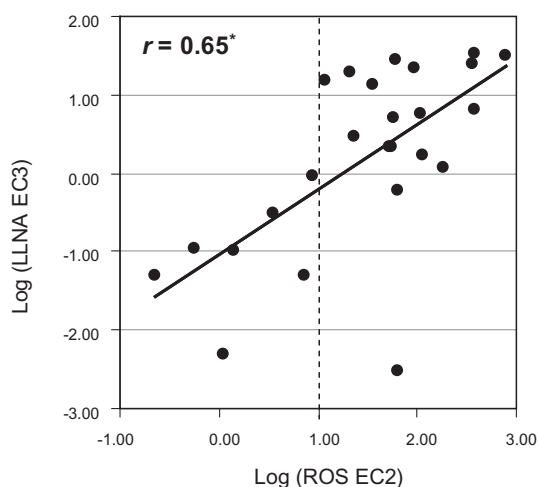


Fig. 4. Scatter plot of the linear fit result of Log (ROS EC2) versus Log (LLNA EC3) for 25 skin sensitizers judged as positive in both the LLNA and the ROS assay. *When OXA was removed from the analysis, Pearson correlation coefficient; $r = 0.79$ for the remaining 24 skin sensitizers.

non-sensitizers at high and/or toxic concentrations may be affected by nonspecific cellular stresses.

When the optimized ROS assay standard protocol was used to test 50 chemicals, the sensitivity, specificity and accuracy of the ROS assay were 83%, 80% and 82%, respectively (Table 2). Additionally, the predictivity for the 10 weak sensitizers (categorized as “weak” by the LLNA) was 80% in the ROS assay (Table 1). These values were comparable with those found for the *in vitro* skin sensitization assays, which have been prevalidated by ECVAM (Aeby et al., 2010). These findings suggest that the ROS assay has a high predictive ability and therefore, can be used to evaluate the sensitizing potential of chemicals.

As seen in Table 1 some discordant results were noted for some chemicals between the ROS assay and the LLNA. In particular, five compounds were found to have false negative results and are indicative of the limitations of this assay. LLNA showed PPD to be a strong sensitizer ($EC3 = 0.16$) and it has been reported to act as a pre-hapten (Karlberg et al., 2008). It has been found that some oxidation products of PPD, (although not PPD itself), exhibit a DC activation property *in vitro* (Aeby et al., 2009). Thus, a 30-min treatment in the ROS assay might be insufficient for oxidizing PPD. PG has been reported to be a strong ROS scavenger (Chen et al., 2007), as well as a strong sensitizer (LLNA $EC3 = 0.32$). In the ROS assay, while PG induced ROS production ($ROS I_{max} = 1.77$), the results did not meet the positive criteria. Thus, it is likely that the false negative result for PG in the ROS assay might be due to the scavenging effect. In addition, although LLNA showed PA was a strong sensitizer ($EC3 = 0.16$), it failed to induce ROS production. More importantly, the other cell-based tests also could not detect PA as a sensitizer (Natsch and Emter, 2008; Ashikaga et al., 2010). It has been reported that the sensitizing potential of trimellitic anhydride failed to induce DC activation *in vitro* (Mitjans et al., 2008). The results for these anhydrides suggested there was transformation of the chemical structure due to hydrolysis in the medium. LLNA has shown that both HCA ($EC3 = 11.0$) and BH ($EC3 = 10.0$) are weak sensitizers. As seen in Fig. 3a, HCA dose-dependently induced ROS production, but did not reach to the 2-fold ROS production at over 90% cell viability ($ROS I_{max} = 1.71$). A similar dose response was also observed for BH (data not shown). These results suggested that the ROS assay may not have had enough sensitivity to detect these weak sensitizers. Based on the false negative outcomes, some chemicals, such as the pre-haptens, ROS

scavengers, and some of the weak sensitizers, might not be detected when using this ROS assay.

Conversely, DEP, OA, saccharin and vanillin were judged to be false positives in the ROS assay. Among them, it has been previously reported that vanillin showed allergic skin reaction in both guinea pigs (Watanabe et al., 2001) and humans (Ferguson and Beck, 1995). Thus, it appears that the ROS assay correctly evaluated the sensitizing potential of vanillin. OA is the medium-chain fatty acid that has been reported to be oxidized via β -oxidation (Guo et al., 2006). Since β -oxidation has been shown to be linked to ROS generation (Yamagishi et al., 2001), this suggests that OA-mediated ROS production may be associated with β -oxidation and that this mechanism might also be different from that induced by the skin sensitizers.

Since the $EC3$ of the LLNA has been allowed for use in estimating the sensitizing potency of chemicals (Basketter et al., 2000; Schneider and Akkan, 2004), $EC3$ has also been used to determine the risk assessment of several skin sensitizers (e.g., fragrances and preservatives) (Loveless et al., 2010). Therefore, it is likely that predictions on the sensitizing potency of chemicals will also be a requirement in non-animal testing. As shown in Table 3, all of the seven chemicals that had a ROS $EC2$ value lower than $10 \mu\text{g/mL}$ were classified as “Extreme” or “Strong” sensitizers by LLNA ($EC3 < 1$). In addition, 9 out of the 11 chemicals (82%) with ROS $EC2$ values between 10 and $100 \mu\text{g/mL}$ were classified as “Moderate” or “Weak” by LLNA ($1 \leq EC3 \leq 100$). Furthermore, when OXA was excluded from the analysis, there was a good correlation between the ROS $EC2$ of the 24 sensitizers and the $EC3$ of LLNA (Fig. 4). These results suggest that the ROS $EC2$ values could be useful for estimating the sensitizing potency of chemicals. It has been suggested that some of the DC-based assays could be used to estimate skin sensitization potency. For example, the estimated concentrations exceeding the positive criteria for the surface molecules on the THP-1 cells exhibited a good correlation with the $EC3$ of the LLNA for the 16 selected sensitizers (Sakaguchi et al., 2009). Lambrechts et al. (2010) additionally reported that the estimated concentrations that exhibited a certain amount of cytotoxicity in the CD34⁺ progenitor-derived dendritic cells showed a good correlation with the $EC3$ of the LLNA for 15 selected sensitizers. Moreover, the ROS $EC2$ may also be a novel indicator that can be used to predict the sensitizing potency of chemicals. Although LLNA showed OXA was an extreme sensitizer ($EC3 = 0.003$), the ROS $EC2$ value was much larger (ROS $EC2 = 64.6$). This result demonstrates that if OXA were a new chemical with unknown sensitizing potential, the sensitizing potency would have been underestimated. A similar underestimation of the sensitizing potency of OXA has also been observed in other cell-based assays such as the DC-based assay VITOSENS (Lambrechts et al., 2010) and the keratinocyte-based assay KeratinoSens (Emter et al., 2010). Thus, this suggests that this discrepancy is not limited to just the ROS assay. Natsch et al. (2010) reported that OXA rapidly and completely reacted with the Lys model peptides, although only a minority of strong sensitizers depleted the Lys peptides by >50%. Even though this unique reactivity might explain why the sensitizing potency of OXA was underestimated in the ROS assay, further studies will need to be performed to definitively clarify these findings.

In conclusion, the THP-1 cell-based ROS assay proved to be a rapid and highly sensitive detection system that can be used to predict the skin sensitizing potential and potency of chemicals. It is expected that this ROS assay will enable us to evaluate the skin sensitization potential of various chemicals more effectively.

Conflict of interest

None declared.

References

- Aeby, P., Sieber, T., Beck, H., Gerberick, G.F., Goebel, C., 2009. Skin sensitization to p-phenylenediamine: the diverging roles of oxidation and N-acetylation for dendritic cell activation and the immune response. *J. Invest. Dermatol.* 129, 99–109.
- Aeby, P., Ashikaga, T., Bessou-Touya, S., Schepky, A., Gerberick, F., Kern, P., Marrec-Fairley, M., Maxwell, G., Ovigne, J.M., Sakaguchi, H., Reisinger, K., Tailhardat, M., Martinozzi-Teissier, S., Winkler, P., 2010. Identifying and characterizing chemical skin sensitizers without animal testing: Colipa's research and method development program. *Toxicol. In Vitro* 24, 1465–1473.
- Aquilano, K., Filomeni, G., Di Renzo, L., Vito, M., Stefano, C., Salimei, P.S., Ciriolo, M.R., Marfè, G., 2007. Reactive oxygen and nitrogen species are involved in sorbitol-induced apoptosis of human erythroleukaemia cells K562. *Free Radic. Res.* 41, 452–460.
- Ashikaga, T., Sakaguchi, H., Sono, S., Kosaka, N., Ishikawa, M., Nukada, Y., Miyazawa, M., Ito, Y., Nishiyama, N., Itagaki, H., 2010. A comparative evaluation of in vitro skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA). *Altern. Lab. Anim.* 38, 275–284.
- Azam, P., Peiffer, J.L., Chamoussat, D., Tissier, M.H., Bonnet, P.A., Vian, L., Fabre, I., Ourlin, J.C., 2006. The cytokine-dependent MUTZ-3 cell line as an in vitro model for the screening of contact sensitizers. *Toxicol. Appl. Pharmacol.* 212, 14–23.
- Basketter, D.A., Balikie, L., Dearman, R.J., Kimber, I., Ryan, C.A., Gerberick, G.F., Harvey, P., Evans, P., White, I.R., Rycroft, R.J., 2000. Use of the local lymph node assay for the estimation of relative contact allergenic potency. *Contact Dermatitis* 42, 344–348.
- Byamba, D., Kim, T.G., Kim, D.H., Je, J.H., Lee, M.G., 2010. The roles of reactive oxygen species produced by contact allergens and irritants in monocyte-derived dendritic cells. *Ann. Dermatol.* 22, 269–278.
- Casati, S., Aebly, P., Kimber, I., Maxwell, G., Ovigne, J.M., Roggen, E., Rovida, C., Tosti, L., Basketter, D., 2009. Selection of chemicals for the development and evaluation of in vitro methods for skin sensitisation testing. *Altern. Lab. Anim.* 37, 305–312.
- Chen, C.H., Liu, T.Z., Chen, C.H., Wong, C.H., Chen, C.H., Lu, F.J., Chen, S.C., 2007. The efficacy of protective effects of tannic acid, gallic acid, ellagic acid, and propyl gallate against hydrogen peroxide-induced oxidative stress and DNA damages in IMR-90 cells. *Mol. Nutr. Food Res.* 51, 962–968.
- Chew, E.H., Nagle, A.A., Zhang, Y., Scarmagnani, S., Palaniappan, P., Bradshaw, T.D., Holmgren, A., Westwell, A.D., 2010. Cinnamaldehydes inhibit thioredoxin reductase and induce Nrf2: potential candidates for cancer therapy and chemoprevention. *Free Radic. Biol. Med.* 48, 98–111.
- Dikalov, S., 2011. Cross talk between mitochondria and NADPH oxidases. *Free Radic. Biol. Med.* 51, 1289–1301.
- Divkovic, M., Pease, C.K., Gerberick, G.F., Basketter, D.A., 2005. Hapten-protein binding: from theory to practical application in the in vitro prediction of skin sensitization. *Contact Dermatitis* 53, 189–200.
- Emter, R., Ellis, G., Natsch, A., 2010. Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers in vitro. *Toxicol. Appl. Pharmacol.* 245, 281–290.
- Feissner, R.F., Skalska, J., Gaum, W.E., Sheu, S.S., 2009. Crosstalk signaling between mitochondrial Ca^{2+} and ROS. *Front Biosci.* 14, 1197–1218.
- Ferguson, J.E., Beck, M.H., 1995. Contact sensitivity to vanilla in a lip salve. *Contact Dermatitis* 33, 352.
- Gerberick, G.F., House, R.V., Fletcher, E.R., Ryan, C.A., 1992. Examination of the local lymph node assay for use in contact sensitization risk assessment. *Fundam. Appl. Toxicol.* 19, 438–445.
- Gerberick, G.F., Ryan, C.A., Kern, P.S., Schlatter, H., Dearman, R.J., Kimber, I., Patlewicz, G.Y., Basketter, D.A., 2005. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. *Dermatitis* 16, 157–202.
- Guo, W., Xie, W., Han, J., 2006. Modulation of adipocyte lipogenesis by octanoate: involvement of reactive oxygen species. *Nutr. Metab. (London)* 3, 30.
- Hooyberghs, J., Schoeters, E., Lambrechts, N., Nelissen, I., Witters, H., Schoeters, G., Van Den Heuvel, R., 2008. A cell-based in vitro alternative to identify skin sensitizers by gene expression. *Toxicol. Appl. Pharmacol.* 231, 103–111.
- Hopkins, J.E., Naisbitt, D.J., Kitteringham, N.R., Dearman, R.J., Kimber, I., Park, B.K., 2005. Selective haptenation of cellular or extracellular protein by chemical allergens: association with cytokine polarization. *Chem. Res. Toxicol.* 18, 375–381.
- Karlberg, A.T., Bergström, M.A., Börje, A., Luthman, K., Nilsson, J.L., 2008. Allergic contact dermatitis – formation, structural requirements, and reactivity of skin sensitizers. *Chem. Res. Toxicol.* 21, 53–69.
- Kim, D., Kim, Y.J., Koh, H.S., Jang, T.Y., Park, H.E., Kim, J.Y., 2010. Reactive oxygen species enhance TLR10 expression in the human monocytic cell line THP-1. *Int. J. Mol. Sci.* 11, 3769–3782.
- Kimber, I., Hilton, J., Dearman, R.J., Gerberick, G.F., Ryan, C.A., Basketter, D.A., Lea, L., House, R.V., Ladics, G.S., Loveless, S.E., Hastings, K.L., 1998. Assessment of the skin sensitization potential of topical medicaments using the local lymph node assay: an interlaboratory evaluation. *J. Toxicol. Environ. Health A* 53, 567–579.
- Kimber, I., Cumberbatch, M., Dearman, R.J., Bhushan, M., Griffiths, C.E., 2000. Cytokines and chemokines in the initiation and regulation of epidermal Langerhans cell mobilization. *Br. J. Dermatol.* 142, 401–412.
- Kimber, I., Dearman, R.J., Basketter, D.A., Ryan, C.A., Gerberick, G.F., 2002. The local lymph node assay: past, present and future. *Contact Dermatitis* 47, 315–328.
- Lambrechts, N., Verstraelen, S., Lodewyckx, H., Felicio, A., Hooyberghs, J., Witters, H., Tendeloo, V., Cauwenberge, P., Nelissen, I., Heuvel, R., Schoeters, G., 2009. THP-1 monocytes but not macrophages as a potential alternative for CD34⁺ dendritic cells to identify chemical skin sensitizers. *Toxicol. Appl. Pharmacol.* 236, 221–230.
- Lambrechts, N., Vanheel, H., Nelissen, I., Witters, H., Van Den Heuvel, R., Van Tendeloo, V., Schoeters, G., Hooyberghs, J., 2010. Assessment of chemical skin-sensitizing potency by an in vitro assay based on human dendritic cells. *Toxicol. Sci.* 116, 122–129.
- Loveless, S.E., Api, A.M., Crevel, R.W., Debruyne, E., Gamer, A., Jowsey, I.R., Kern, P., Kimber, I., Lea, L., Lloyd, P., Mehmood, Z., Steiling, W., Veenstra, G., Woolhiser, M., Hennes, C., 2010. Potency values from the local lymph node assay: application to classification, labelling and risk assessment. *Regul. Toxicol. Pharmacol.* 56, 54–66.
- Migdal, C., Foggia, L., Tailhardat, M., Courtellemont, P., Haftek, M., Serres, M., 2010a. Sensitization effect of thimerosal is mediated in vitro via reactive oxygen species and calcium signaling. *Toxicology* 274, 1–9.
- Migdal, C., Tailhardat, M., Courtellemont, P., Haftek, M., Serres, M., 2010b. Responsiveness of human monocyte-derived dendritic cells to thimerosal and mercury derivatives. *Toxicol. Appl. Pharmacol.* 246, 66–73.
- Mitjans, M., Viviani, B., Lucchi, L., Galli, C.L., Marinovich, M., Corsini, E., 2008. Role of p38 MAPK in the selective release of IL-8 induced by chemical allergen in naive THP-1 cells. *Toxicol. In Vitro* 22, 386–395.
- Mitjans, M., Galbiati, V., Lucchi, L., Viviani, B., Marinovich, M., Galli, C.L., Corsini, E., 2010. Use of IL-8 release and p38 MAPK activation in THP-1 cells to identify allergens and to assess their potency in vitro. *Toxicol. In Vitro* 24, 1803–1809.
- Miyazawa, M., Ito, Y., Yoshida, Y., Sakaguchi, H., Suzuki, H., 2007. Phenotypic alterations and cytokine production in THP-1 cells in response to allergens. *Toxicol. In Vitro* 21, 428–437.
- Na, K., Kim, K.E., Park, S.T., Kim, T.Y., 2007. EC-SOD suppresses contact hypersensitivity in mouse skin by impairing Langerhans cell migration. *J. Invest. Dermatol.* 127, 1930–1937.
- Natsch, A., Emter, R., 2008. Skin sensitizers induce antioxidant response element dependent genes: application to the in vitro testing of the sensitization potential of chemicals. *Toxicol. Sci.* 102, 110–119.
- Natsch, A., Gfeller, H., Kuhn, F., Granier, T., Roberts, D.W., 2010. Chemical basis for the extreme skin sensitization potency of (E)-4-(ethoxymethylene)-2-phenylloxazol-5(4H)-one. *Chem. Res. Toxicol.* 23, 1913–1920.
- Nordberg, J., Zhong, L., Holmgren, A., Arnér, E.S., 1998. Mammalian thioredoxin reductase is irreversibly inhibited by dinitrohalobenzenes by alkylation of both the redox active selenocysteine and its neighboring cysteine residue. *J. Biol. Chem.* 273, 10835–10842.
- Nukada, Y., Miyazawa, M., Kosaka, N., Ito, Y., Sakaguchi, H., Nishiyama, N., 2008. Production of IL-8 in THP-1 cells following contact allergen stimulation via mitogen-activated protein kinase activation or tumor necrosis factor- α production. *J. Toxicol. Sci.* 33, 175–185.
- Nukada, Y., Ito, Y., Miyazawa, M., Sakaguchi, H., Nishiyama, N., 2011. The relationship between CD86 and CD54 protein expression and cytotoxicity following stimulation with contact allergen in THP-1 cells. *J. Toxicol. Sci.* 36, 313–324.
- Peiser, M., Tralau, T., Heidler, J., Api, A.M., Arts, J.H., Basketter, D.A., English, J., Diepgen, T.L., Fuhlbrigge, R.C., Gaspari, A.A., Johansen, J.D., Karlberg, A.T., Kimber, I., Lepoittevin, J.P., Liebsch, M., Maibach, H.I., Martin, S.F., Merk, H.F., Platzeck, T., Rustemeyer, T., Schnuch, A., Vandebriel, R.J., White, I.R., Luch, A., 2012. Allergic contact dermatitis: epidemiology, molecular mechanisms, in vitro methods and regulatory aspects. Current knowledge assembled at an international workshop at BfR, Germany. *Cell Mol. Life Sci.* 69, 763–781.
- Poyton, R.O., Ball, K.A., Castello, P.R., 2009. Mitochondrial generation of free radicals and hypoxic signaling. *Trends Endocrinol. Metab.* 20, 332–340.
- Pythou, F., Goebel, C., Aebly, P., 2007. Assessment of the U937 cell line for the detection of contact allergens. *Toxicol. Appl. Pharmacol.* 220, 113–124.
- Sakaguchi, H., Ashikaga, T., Miyazawa, M., Kosaka, N., Ito, Y., Yoneyama, K., Sono, S., Itagaki, H., Toyoda, H., Suzuki, H., 2009. The relationship between CD86/CD54 expression and THP-1 cell viability in an in vitro skin sensitization test-human cell line activation test (h-CLAT). *Cell Biol. Toxicol.* 25, 109–126.
- Sasaki, Y., Aiba, S., 2007. Dendritic cells and contact dermatitis. *Clin. Rev. Allergy Immunol.* 33, 27–34.
- Schneider, K., Akkan, Z., 2004. Quantitative relationship between the local lymph node assay and human skin sensitization assays. *Regul. Toxicol. Pharmacol.* 39, 245–255.
- Toebak, M.J., Pohlmann, P.R., Sampat-Sardjoepersad, S.C., von Blomberg, B.M., Bruynzeel, D.P., Schep, R.J., Rustemeyer, T., Gibbs, S., 2006. CXCL8 secretion by dendritic cells predicts contact allergens from irritants. *Toxicol. In Vitro* 20, 117–124.
- Toebak, M.J., Gibbs, S., Bruynzeel, D.P., Schep, R.J., Rustemeyer, T., 2009. Dendritic cells: biology of the skin. *Contact Dermatitis* 60, 2–20.
- Watanabe, K., Matsuda, M., Furuhashi, S., Kimura, T., Matsunaga, T., Yamamoto, I., 2001. Skin reaction induced by aldehydes for food flavoring agents. *J. Health Sci.* 47, 327–329.
- Yamagishi, S.I., Edelstein, D., Du, X.L., Kaneda, Y., Guzmán, M., Brownlee, M., 2001. Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A. *J. Biol. Chem.* 276, 25096–25100.
- Yoshida, Y., Sakaguchi, H., Ito, Y., Okuda, M., Suzuki, H., 2003. Evaluation of the skin sensitization potential of chemicals using expression of co-stimulatory molecules, CD54 and CD86, on the naive THP-1 cell line. *Toxicol. In Vitro* 17, 221–228.