Archives of Toxicology (2018) 92:3103–3115
https://doi.org/10.1007/s00204-018-2287-8

IMMUNOTOXICOLOGY

Advancing the predictivity of skin sensitization by applying a novel HMOX1 reporter system

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Received: 11 May 2018 / Accepted: 13 August 2018 / Published online: 21 August 2018

Abstract

Reporter cell lines are a particularly useful tool to screen for the skin sensitization potential of chemicals. Current cell models based on Keap1-Nrf2 mimic induction by conducting antioxidant response element-luciferase plasmids. However, plasmid-based reporters may ignore comprehensive aspects of induction, thus affecting the accuracy of hazard identification. Herein, we developed a novel HaCaT-based reporter system, EndoSens, whereby luciferase was specifically inserted into the cassette for heme oxygenase (decycling) 1 (HMOX1, the most consistent marker induced by skin sensitizers) by CRISPR/Cas9. Testing data from 20 coded substances showed an accuracy of 90%, sensitivity of 91.7%, and specificity of 87.5%, which exceeded the OECD requirement. Among the 35 chemicals examined, predictivity was better than reported for the validated KeratinoSens™. These results indicate that the EndoSens assay could advance the predictivity of skin sensitization, thus making it a promising tool for in vitro skin sensitization testing.

Keywords Skin sensitization · Keratinocyte activation · Reporter cell line · CRISPR/Cas9

Introduction

Traditionally, hazard assessment of skin sensitizers relies mainly on animal testing, such as the Guinea pig maximization test and local lymph node assay (LLNA). With growing concerns for animal welfare and 3R principles, the EU has banned animal testing for cosmetic products and their ingredients since 2013 (EU 2009), a move subsequently followed by South Korea in 2018. Meanwhile, an increasing number of countries including the United States, Canada, Japan, Brazil, and China have been considering avoiding or minimizing animal testing, and promoting the adoption of defined alternative approaches for skin sensitization assessments (Casati et al. 2018; Daniel et al. 2018; Kleinstreuer et al. 2018).

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During the last decade, much progress has been made in developing in vitro test methods to replace current animal testing for assessment of skin sensitization. In 2012, the OECD described the adverse outcome pathway (AOP) for skin sensitization, comprising a number of molecular events leading to the final adverse effect (OECD 2012). Currently, mechanistically based in chemico and in vitro test methods addressing the first key event (Direct Peptide Reactivity Assay, TG 442C; OECD 2015a), the second key event (KeratinoSens™, TG 442D; OECD 2015b), and the third key event (h-CLAT, U-SENS™, IL-8 Luc assay, OECD TG 442E; OECD 2018) of the skin sensitisation AOP have been adopted for contributing to the evaluation of the skin sensitization hazard potential of chemicals. In addition, several other in vitro methods, such as the LuSens, the SENS-IS™, and the GARD assays have been included in the OECD TG work programme.

Many studies have indicated that HMOX1 is the most consistently observed genetic marker induced by skin sensitizers (Ade et al. 2009; Arkusz et al. 2010; Emter and Natsch 2015; Emter et al. 2013; Johansson et al. 2011; Natsch and Emter 2015; Neves et al. 2013; Van der Veen et al. 2013, 2014, 2015; Vandebriel et al. 2010). Sensitizers activate the Keap1-Nrf2 pathway to promote HMOX1 expression.

Moreover, HMOX1 can be upregulated by sensitizers through other pathways, such as heat shock factor, activator protein 1, and nuclear factor kappa B-related pathways (Alam and Cook 2007; Gozzelino et al. 2010). Hence, induction of HMOX1 expression could be a more sensitive assay to reflect the sensitization of keratinocytes.

Current reporter cell lines (KeratinoSens™ and LuSens) that mimic keratinocyte activation by conducting an antioxidant response element (ARE)-luciferase reporter plasmid (Emter et al. 2010; Ramirez et al. 2014) are considered to be an efficient method for sensitization testing (Bauch et al. 2012; Ramirez et al. 2016; Urbisch et al. 2015). However, plasmid-based exogenous reporter systems largely ignore the comprehensive aspects of gene expression regulation by complex factors such as epigenetic factors, transcription factors, and various *cis* elements (Basu et al. 2017; Soldner et al. 2016). Furthermore, the accuracy of regulatory function might also be affected by random integration of the ARE-luciferase reporter cassette (Lai et al. 2016; Uemura et al. 2016). Thus, it appears that precise insertion of a reporter gene into the endogenous gene frame would allow synchronous expression between the endogenous gene and reporter gene, and would closely mimic the state of cells (Lai et al. 2016).

With the development of CRISPR/Cas9 in recent years, reporter genes can be precisely inserted into endogenous gene loci by homologous recombination (He et al. 2016; Lai et al. 2016). Therefore, we developed a novel reporter HaCaT cell line (EndoSens) in which the luciferase reporter gene was precisely inserted into the *HMOX1* gene locus by CRISPR/Cas9 mediated knock-in (KI) technology. Thus, luciferase activity within the novel cell line actually reflected the expression of the endogenous *HMOX1* gene. The EndoSens assay was evaluated with 35 substances, including 20 coded test substances (OECD 2015a). Predictivity was assessed by comprehensive comparison with available human and LLNA data. All data from the EndoSens assay were compared with data available from Keratinosens™ and LuSens.

Materials and methods

Chemicals

All test chemicals were purchased from Aladdin (Shanghai, China), except oxazolone and methyldibromo glutaronitrile, which were obtained from Sigma-Aldrich (St. Louis, MO). All substances and their molecular weight, CAS number, physical state, purity, chemical class, EC3 (%) of LLNA data, human data, and respective literature references are listed in Table 1.

Plasmid construction

To insert the luciferase gene into the endogenous *HMOX1* locus and monitor its expression synchronously, the reporter luciferase gene was specifically knocked into the *HMOX1* locus by CRISPR/Cas9 and homologous recombination (HR). The endogenous *HMOX1* gene was linked with the luciferase gene by a 2A peptide, allowing co-translational "cleavage" of their protein products.

pCMV-Cas9 vector, which has a selectable neomycin marker, was obtained from Addgene (41,815). sgRNAs were designed near the stop codon of *HMOX1* by GN19NGG rule and constructed as previously described (Mali et al. 2013). In brief, oligonucleotides were annealed to form double-strand DNA and cloned into *BbsI* restriction enzyme-digested U6-sgRNA cloning vector. Sequences of oligonucleotides were as follows: sgRNA1, 5′-caccTTAACAGGTGGGCGTGCATC-3′ and 5′-aaacGATGCACGCCCACCTGTTAA-3′; sgRNA2, 5′-caccGGTCCTTACACTCAGCTTTC-3′ and 5′-aaacGAAAGCTGAGTGTAAGGACC-3′; sgRNA3, 5′-caccGCTTTATGCCATGTGAATGC-3′ and 5′-aaacGCATTCACATGGCATAAAGC-3′.

For the p2A-Luciferase (+HAs) donor, homologous arms were designed both upstream and downstream of the termination codon TGA site (Fig. 1a), and then amplified by genomic PCR and cloned into the pMD-18T vector (Takara, Tokyo, Japan). The entire coding sequence of luciferase was amplified from pGL4.10 (Promega Corporation, Madison, WI). Subsequently, the 2A sequence was ligated with the luciferase gene and inserted between the right and left arms. All constructs were confirmed by Sanger sequencing (BGI, Guangzhou, China). Plasmids were purified using an Endo-free Plasmid Mini kit (Omega Bio-Tek, Norcross, GA).

Cell culture and transfection

The human embryonic kidney 293T cell line was routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C with 5% CO_2 incubation.

293T cells were seeded onto 6-well plates (Corning, NY) 24 h before transfection. Cells were transfected using GenJet™ In Vitro DNA Transfection Reagent at 80–90% confluency (SignaGen, Rockville, MD) following the manufacturer's recommendations. For each well of a 6-well plate, a total of 1 µg of pCMV-Cas9 + pU6-sgRNA plasmid was used.

The human keratinocyte cell line HaCaT was cultured in Roswell Park Memorial Institute 1640 medium (RPMI

Table 1 Test substances used in the EndoSens assay

No.	Chemicals	Chemical information			LLNA data				Human data		
		Molecular weight	CAS#	Chemical class	EC3 (%)	Potency class	LLNA	Ref	Potency class	Human	Ref
1	Oxazolone	217.22	15646-46-5	Heterocyclic Compounds	0.003	Extreme	+	Urbisch et al. (2015)	n.a	+	Urbisch et al. (2015)
2	2,4-Dinitrochlorobenzene	202.55	97-00-7	Hydrocarbon, Halogenated; Nitro Compounds; Hydrocarbons, Cyclic	0.049	Extreme	+	Kleinstreuer et al. (2018)	1	+	Kleinstreuer et al. (2018)
3	4-Nitrobenzylbromide	216.03	100-11-8	Hydrocarbons, Cyclic; Nitro Compounds	0.05	Extreme	+	Urbisch et al. (2015)			No data
4	Para-phenylenediamine	108.14	106-50-3	Amines	0.16	Strong/extreme	+	Kleinstreuer et al. (2018)	1	+	Kleinstreuer et al. (2018)
5	4-Methylaminophenol sulfate	344.38	55-55-0	Amines, Phenols	0.8	Strong	+	Kleinstreuer et al. (2018)	3	+	Kleinstreuer et al. (2018)
6	Methyldibromo glutaronitrile	265.93	35691-65-7	Nitriles	0.9	Strong	+	Kleinstreuer et al. (2018)	2	+	Kleinstreuer et al. (2018)
7	Isoeugenol	164.2	97-54-1	Carboxylic Acids	1.5	Moderate	+	Kleinstreuer et al. (2018)	2	+	Kleinstreuer et al. (2018)
8	2-Mercaptobenzothiazole	167.25	149-30-4	Heterocyclic Compounds	9.7	Moderate	+	Kleinstreuer et al. (2018)	3	+	Kleinstreuer et al. (2018)
9	Citral	152.23	5392-40-5	Hydrocarbons, Other	13	Moderate	+	Kleinstreuer et al. (2018)	3	+	Kleinstreuer et al. (2018)
10	Eugenol	164.2	97-53-0	Carboxylic Acids	13	Weak	+	Kleinstreuer et al. (2018)	3	+	Kleinstreuer et al. (2018)
11	Phenyl benzoate	198.22	93-99-2	Carboxylic Acids	20	Weak	+	Kleinstreuer et al. (2018)	3	+	Kleinstreuer et al. (2018)
12	Ethylene glycol dimethacrylate	198.22	97-90-5	Carboxylic Acids	35	Weak	+	Kleinstreuer et al. (2018)	4	+	Kleinstreuer et al. (2018)
13	4-methoxy-acetophenone	150.17	100-06-1	Phenols	NC	n.a	-	Urbisch et al. (2015)	n.a	-	Urbisch et al. (2015)
14	Glycerol	92.09	56-81-5	Alcohols; Carbohydrates	NC	n.a	–	Kleinstreuer et al. (2018)	6	–	Kleinstreuer et al. (2018)
15	Isopropanol	60.1	67-63-0	Alcohols	NC	n.a	–	Kleinstreuer et al. (2018)	5	–	Kleinstreuer et al. (2018)
16	Salicylic acid	138.12	69-72-7	Phenols; Carboxylic Acids	NC	n.a	–	Kleinstreuer et al. (2018)	6	–	Kleinstreuer et al. (2018)
17	Chlorobenzene	112.56	108-90-7	Hydrocarbons, Cyclic Hydrocarbons, Halogenated	NC	n.a	–	Urbisch et al. (2015)			No data

Table 1 (continued)

No.	Chemicals	Chemical information			LLNA data				Human data		
		Molecular weight	CAS#	Chemical class	EC3 (%)	Potency class	LLNA	Ref	Potency class	Human	Ref
18	Lactic acid	90.08	50-21-5	Carboxylic Acids	NC	n.a	−	Kleinstreuer et al. (2018)	6	−	Kleinstreuer et al. (2018)
19	Methyl salicylate	152.15	119-36-8	Phenols; Carboxylic Acids	NC	n.a	−	Kleinstreuer et al. (2018)	5	−	Kleinstreuer et al. (2018)
20	Sulfanilamide	172.21	63-74-1	Amides; Sulfur Compounds; Amines	NC	n.a	−	Urbisch et al. (2015)	n.a	−	Urbisch et al. (2015)
21	Benzoquinone	108.1	106-51-4	Quinones	0.0099	Extreme	+	Urbisch et al. (2015)	n.a	+	Urbisch et al. (2015)
22	Cobalt chloride	58.9	7646-79-9	Elements; Metals	0.8	Strong	+	Urbisch et al. (2015)	n.a	+	Urbisch et al. (2015)
23	Benzisothiazolione	151.19	2634-33-5	Sulfur Compounds, Heterocyclic Compounds	2.3	Moderate	+	Kleinstreuer et al. (2018)	2	+	Kleinstreuer et al. (2018)
24	Resorcinol	110.11	108-46-3	Phenols	5.5	Moderate	+	Kleinstreuer et al. (2018)	4	+	Kleinstreuer et al. (2018)
25	3,4-Dihydrocoumarin	148.16	119-84-6	Heterocyclic Compounds	5.6	Moderate	+	Urbisch et al. (2015)	n.a	+	Urbisch et al. (2015)
26	Benzyl cinnamate	238.29	103-41-3	Hydrocarbons, Cyclic; Carboxylic Acids	18.4	Weak	+	Kleinstreuer et al. (2018)	4	+	Kleinstreuer et al. (2018)
27	2,4-Dichloronitrobenzene	192	611-06-3	NC	20	Weak	+	Emter et al. (2010)			No data
28	Cinnamic alcohol	134.18	104-54-1	Alcohols	21	Weak	+	Kleinstreuer et al. (2018)	3	+	Kleinstreuer et al. (2018)
29	Benzocaine	165.19	94-09-7	Carboxylic Acids	NC	n.a	−	Kleinstreuer et al. (2018)	4	+	Kleinstreuer et al. (2018)
30	Coumarin	146.15	91-64-5	Heterocyclic Compounds	NC	n.a	−	Kleinstreuer et al. (2018)	3	+	Kleinstreuer et al. (2018)
31	Propyl paraben	180.2	94-13-3	Phenols; Carboxylic Acids	NC	n.a	−	Kleinstreuer et al. (2018)	5	−	Kleinstreuer et al. (2018)
32	4-Hydroxybenzoic acid	138.12	99-96-7	Phenols; Carboxylic Acids	NC	n.a	−	Urbisch et al. (2015)	n.a	−	Urbisch et al. (2015)
33	Vanillin	152.15	121-33-5	Aldehydes	NC	n.a	−	Kleinstreuer et al. (2018)	5	−	Kleinstreuer et al. (2018)
34	6-Methylcoumarin	160.17	92-48-8	Heterocyclic Compounds	NC	n.a	−	Urbisch et al. (2015)	n.a	±	Urbisch et al. (2015)
35	Benzoic acid	212.2	65-85-0	Carboxylic Acids	NC	n.a	−	Urbisch et al. (2015)			No data

+ Indicates sensitizing, − indicates not sensitizing, *NC* indicates not calculated, *n.a.* indicates not applicable

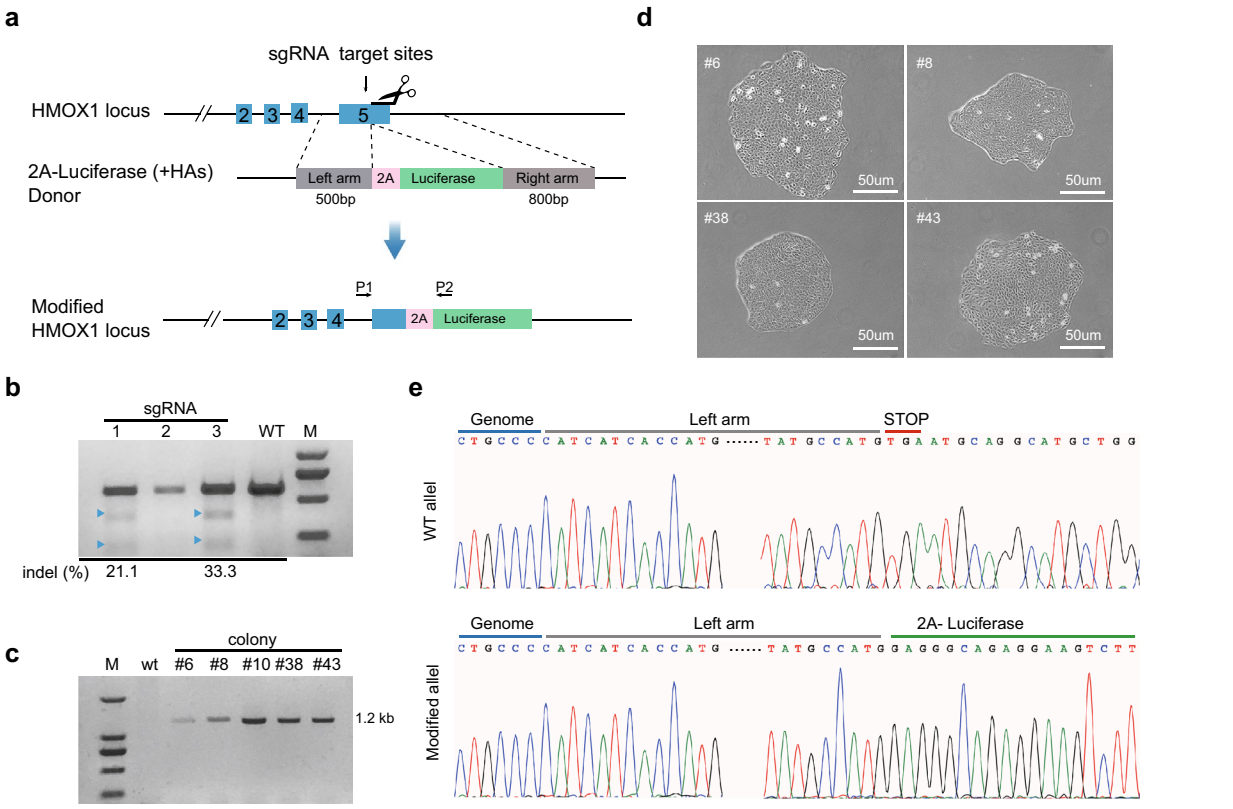

Fig. 1 Generation of endogenous *HMOX1* luciferase reporter. **a** Schematic of donor plasmids and targeting strategy. Transfection of sgRNAs targeting the stop codon (TGA) of exon 5. Donor vector design contains a 500-bp left arm encompassing part of intron 4 and exon 5 upstream from the stop codon, and a right arm of 800-bp downstream of the stop codon. **b** T7EI assay for sgRNAs of *HMOX1* in 293T cells. **c** PCR analysis of individual colonies. PCR amplification of the genome-plasmid junction showed DNA fragments of the expected size (1.2 kb), indicating correct insertion of luciferase via HR. **d** A portion of harvested single-cell colonies (**e**). Sequencing results of the WT allele and amplified fragments of genome-plasmid junctions in clone #38. Expected modifications were confirmed at the genome-plasmid junction, indicating the precise integration of 2A-Luciferase through HR-mediated repair

1640; Life Technologies, Waltham, MA) supplemented with 10% FBS (Life Technologies), 1% non-essential amino acids (Life Technologies), 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies). Cells were incubated at 37 °C in a humidified incubator containing 5% CO_2. HaCaT cells were transfected with 0.35 µg Cas9, 0.35 µg sgRNA3, and 0.3 µg linearized donor p2A-Luciferase (+HAs) plasmid using GenJet™ In Vitro DNA Transfection Reagent according to the manufacturer's protocol.

T7EI nuclease assay for genome modification

293T cells were collected 2–3 days after transfection, and genomic DNA was extracted using a DNA Mini Prep kit (Omega). Genomic regions containing sgRNA target sites were PCR amplified using primers (F: 5′-tgttttcacaatgtggcctgg-3′, R: 5′-ccattgcctggatgtgcttt-3′). For T7EI assay, 8.5 µl of PCR products were annealed in NEB Buffer 2 (New England Biolabs, Beverly, MA) in 10-µl total volume. The annealing procedure was 95 °C for 5 min, 95–85 °C at −2 °C s^{-1}, 85–25 °C at −0.1 °C s^{-1}, hold at 4 °C. Next, 10 µl of annealed products were digested by 5 UL^{-1} T7EI nuclease (New England Biolabs) at 37 °C for 30 min, followed by analysis on 2% agarose gels. Indel percentage was determined by the formula, $100 \times [1 - (1 - (b+c)/(a+b+c))^{1/2}]$, where a is the integrated intensity of the undigested PCR product, and b and c are the integrated intensities of each cleavage product (Zelensky et al. 2017).

Generation of stable 2A-Luciferase KI HaCaT cell lines

At 48 h post transfection, transfected HaCaT cells were seeded at low density (100 cells/10-cm dish) and cultured with selective medium (G418, 800 µg/ml). After 8–10 days of selection, cell colonies were isolated and further cultured on 24-well plates. When selected colonies grew to confluence, genomic DNA was extracted from individual wells by

incubation in lysis buffer containing Triton X-100 (0.45%, Sigma) and proteinase K (1 mg/ml, Merck, Germany) at 56 °C for 20 min, followed by 95 °C for 10 min. Plasmid-genome junctions were amplified via PCR with primers P1/P2 (Fig. 1a). Primers sequences were as follows: (P1) 5′-aaccagggatgggactgaac-3′, (P2) 5′-tcgcccaccagctacttaaa-3′. PCR products of colonies were sent for sequencing and BLAST searched against the human genome and donor plasmid to confirm 2A-Luciferase insertion. KI-positive colonies were expanded and frozen.

Testing of chemicals in stable KI HaCaT cell lines

Positive colonies were selected and tested for proficiency to activate HMOX1-Luciferase. Proficiency was evaluated by measuring the relative increase in luciferase activity following exposure to two weak sensitizers (cinnamic alcohol and ethylene glycol dimethacrylate) and one non-sensitizer (lactic acid). In brief, cells were seeded in white 96-well assay plates at a density of 1×10^4 cells/well and incubated for 24 h. After incubation, the culture medium was replaced with fresh medium containing specific concentrations of test chemicals (1000 µM, 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, 16.125 µM, 7.8125 µM, or 3.90625 µM) or vehicle control (DMSO). After 48-h treatment, cell culture medium was removed and cells were washed twice with 200 µl PBS. Subsequently, 100 µl PBS and 100 µl Bright-Glo™ Reagent (Promega) were added to each well. Plates were gently shaken for 2 min and luminescence was measured on an Infinite M200 plate reader (Tecan, Switzerland).

Standard testing of chemicals in stable KI HaCaT cell lines

For EndoSens assay, chemicals were first dissolved in DMSO or PBS at a concentration of 200 mM. Cells were seeded in 96-well white assay plates (Corning) at a density of 5×10^4 cells/ml (200 µl/well). After 24 h incubation, the culture medium was replaced with medium containing the test substance and a final concentration of 0.5% of DMSO. Each test substance was examined using a nine-point, two-fold serial dilution series ranging from 4 to 1000 µM. Each chemical was tested in triplicate and a parallel plate was prepared for cytotoxicity determination. All plates were covered with mineral oil (Sigma-Aldrich) to avoid evaporation and cross-contamination. After a 48-h treatment with test chemicals, the culture medium was removed and cells were washed once with 200 µl PBS. After adding 100 µl PBS to each well, cultured cells were equilibrated to room temperature. To each well, 100 µl of Bright-Glo™ Reagent was added and incubated for 2 min at room temperature to allow cell lysis. Plates were read by luminescence on an Infinite M200 plate reader, with an integration of luciferase activity for 0.5 s. In the parallel plate, cytotoxicity was determined by MTT assay. In brief, 27 µL of MTT (Merck Millipore) solution (5 mg/ml in PBS) was added to each well, and cells were incubated for another 4 h at 37 °C. After incubation for 4 h, the medium was removed and 200 µL of DMSO was added to each well. The optical density (O.D.) value at 570 nm was measured for each well.

Data analysis and statistical evaluation

All 35 test chemicals were validated using three replicates for each concentration. For each chemical tested, average maximal fold-induction of gene activity (I_{max}), average concentration inducing gene activity > 50% (EC1.5) and concentration causing 50% cytotoxicity (IC50) were calculated. In addition, EC2 and EC3 for 100 and 200% enhanced luciferase expression were also calculated, if possible. A test chemical was rated to positive, if it statistically significantly induced the luciferase activity more than 50% above control values at any of the tested concentrations and at which the cell viability was above 70%. A test compound was rated to negative if these effects were not observed.

To determine the predictive capacity of the EndoSens assay, Cooper statistical values were calculated as follows. Sensitivity: $(TP/[TP + FN] \times 100)$; specificity: $(TN/[TN + FP] \times 100)$; positive predictivity: $(TP/[TP + FP] \times 100)$; negative predictivity: $(TN/[TN + FN] \times 100)$; accuracy: $(TP + TN/[TN + TP + FP + FN] \times 100)$, with TP representing the number of true positives, FP the number of false positives, TN the number of true negatives, and FN the number of false negatives (Cooper et al. 1979).

Results

Generation of a stable HaCaT cell line with luciferase gene insertion into the *HMOX1* locus

The genome editing capacity of sgRNAs in 293T cells was tested, with 20–30% indel rates observed in sgRNA1 and sgRNA3 based on T7EI assays (Fig. 1a, b). To further establish a 2A-Luciferase KI cell line, HaCaT cells were cotransfected with pCMV-Cas9, pU6-sgRNA3, and the linearized donor plasmid p2A-Luciferase (+HAs) (Fig. 1a). After a 10-day G418 selection, a total of 70 colonies were picked and seeded onto 24-well plates. Sixty-one colonies grew to confluence (a portion of colonies are shown in Fig. 1d). Forty-four colonies were identified by PCR analysis. Five colonies (11.36%, 5/44) showed precise insertion of the 2A-Luciferase (Fig. 1c). Sanger sequencing further confirmed that 2A-Luciferase was correctly knocked-in at the *HMOX1* gene locus with no indel mutation in colony

#38 (Fig. 1e). The above results confirmed the successful establishment of a stable cell line with 2A-Luciferase gene insertion in the endogenous *HMOX1* gene.

Validation of luciferase induction by chemicals in the stable KI HaCaT cell line

To test whether the luciferase activity in colony #38 cells reflected expression of endogenous *HMOX1*, its induction by chemicals was compared with that of vehicle control. Luciferase activity increased significantly in a dose-dependent manner after 48 h of exposure to cinnamic alcohol and ethylene glycol dimethacrylate. In addition, the fold-induction of luciferase increased by 20-fold and 40-fold at a high concentration (1000 µM), respectively (Fig. 2). In contrast, lactic acid (non-sensitizer) showed no effect on luciferase induction in colony #38 cells. Collectively, these results indicated that the colony #38 cell line had a good basic output of luciferase induction and that luciferase activity could accurately reflect the expression of *HMOX1*. Furthermore, colony #38 showed a good dynamic range when treated with weak sensitizers (Fig. 2). Thus, we called this colony "EndoSens" and used it for further development of a standard operating procedure for testing chemicals.

Selection of chemicals for EndoSens assay

To meet the performance standard (PS) requirement (OECD 2015a), we initially selected 20 reference substances to validate the EndoSens assay, comprising 8 non-sensitizers and 12 sensitizers of various LLNA potencies (weak, moderate, strong and extreme) (No. 1–20). After validation, 15 additional substances (no. 21–35), some of which were consistently misclassified in vitro or in vivo (Table 1), were further tested by EndoSens assay (Emter et al. 2010; Natsch et al. 2013; Urbisch et al. 2015).

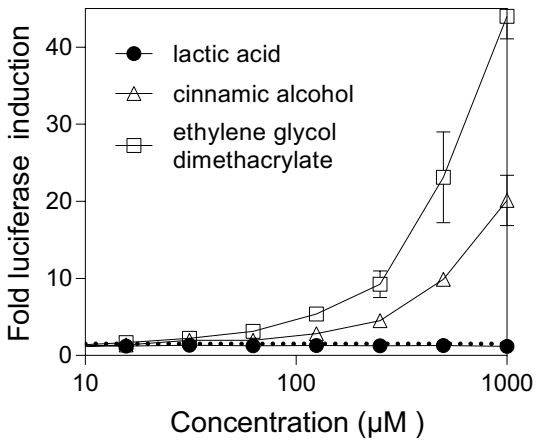

Fig. 2 Induction of luciferase activity in colony #38. After exposure to cinnamic alcohol (open triangles) and ethylene glycol dimethacrylate (open squares) an increase in luciferase expression above the 1.5-fold threshold was observed to be dose-dependent. No luciferase induction was observed above the 1.5-fold threshold when exposed to lactic acid (filled cycles)

Assay development

To validate the predictive capacity of the EndoSens assay, all 35 substances (Table 1) were tested in three independent experiments. For the 20 PS, all eight non-sensitizers elicited no luciferase induction effect, with the exception of 4-methoxy-acetophenone (Fig. 3a). Among the twelve sensitizers, eleven significantly induced luciferase activity above 1.5-fold, while methyldibromo glutaronitrile had no luciferase induction but increased cytotoxicity rapidly along with ascending concentration (Fig. 3b). For moderate/strong sensitizers, such as oxazolone and citral, luciferase induction was initiated at low concentrations without cytotoxicity (Fig. 3b). Dose–response curves of 15 additional test substances, presented in Fig. 4, demonstrate similar effects for dose–response curves.

The parameters of dose–response curves (I_{max}, EC1.5, and IC50) are summarized in Table 2. I_{max} ranged from 1.1-fold (4-hydroxybenzoic acid) to 133.1-fold (para-phenylenediamine), indicating a large dynamic range of luciferase induction. Most sensitizers induced over 1.5-fold luciferase activity, while particularly low EC1.5 values below 10 µM were recorded for most strong/extreme sensitizers; generally, over 70 µM was recorded for weak sensitizers. Moreover, cytotoxicity had only a modest effect on initial luciferase induction when comparing IC50 and EC1.5 values (Table 2).

Predictivity of EndoSens according to Cooper statistics

For the 20 PS, EndoSens showed accuracy of 90% (18/20), sensitivity of 91.7% (11/12), and specificity of 87.5% (7/8) (Table 4). All performance values of EndoSens were higher than 80%, thus exceeding the predictive capacity requirement (OECD 2015a). Indeed, compared with KeratinoSens™ (85%) and LuSens (85%), EndoSens showed higher accuracy (90%). Moreover, its sensitivity (91.7%) was higher than KeratinoSens™ (83.3%, 10/12) and equal to LuSens (91.7%, 11/12), and the specificity (87.5%) was higher than LuSens (75%, 6/8) and equal to KeratinoSens™ (7/8). Among the 20 PS test substances, one mis-predicted by both KeratinoSens™ and LuSens, phenyl benzoate, was correctly predicted by EndoSens. However, methyldibromo glutaronitrile showed high cytotoxicity and no luciferase induction, and, thus, was not properly identified as an FN in EndoSens; in addition, 4-methoxy-acetophenone was mis-predicted as positive, consistent with KeratinoSens™ and LuSens (Table 3).

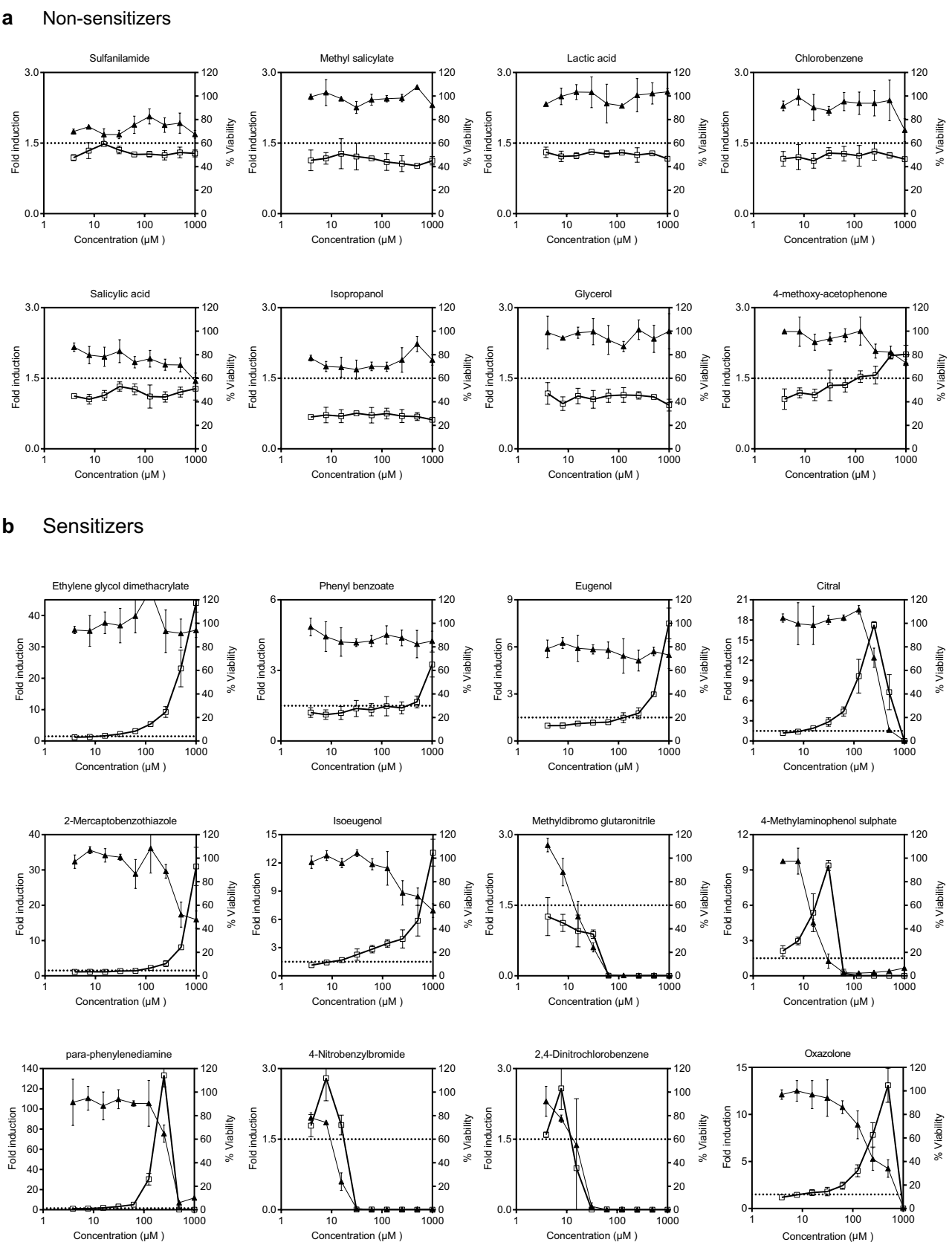

Fig. 3 Induction of luciferase activity (open squares) and cellular viability (closed triangles) for 20 PS in a full dose–response analysis. **a** Results of the eight non-sensitizers in a full dose–response analysis. **b** Results of the 12 sensitizers in a full dose–response analysis

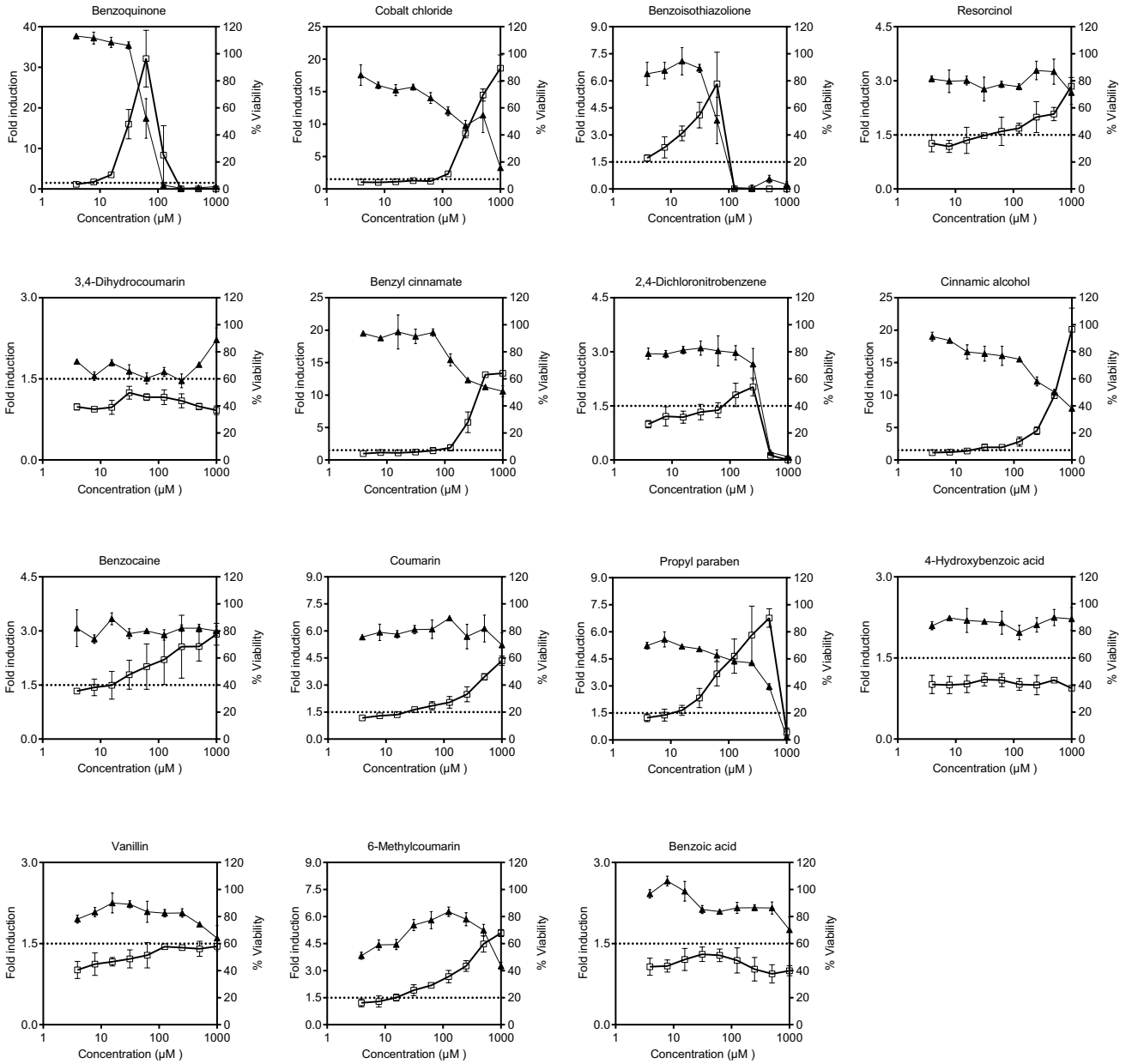

Fig. 4 Induction of luciferase activity (open squares) and cellular viability (closed triangles) for 15 additional test chemicals in a full dose–response analysis

For all 35 test substances, EndoSens showed an overall good accuracy of 90%, sensitivity of 94.7%, and specificity of 81.8% when compared with human data (Table 4). Among the 15 additional test substances, most prediction results were consistent with KeratinoSens™ (Table 3). Surprisingly, resorcinol, which has always been misclassified by KeratinoSens™ and Lusens, was predicted correctly by EndoSens. 6-Methylcoumarin was predicted as FP and 3, 4-Dihydrocoumarin was predicted as FN by EndoSens, consistent with the results of KeratinoSens™ (Fig. 4; Table 3).

Discussion

Given the marketing ban on testing cosmetics ingredients on animals in many parts of the world and the development of integrated approaches to testing and assessment (IATA), more feasible and robust in vitro skin sensitization test methods are urgently needed (Adler et al. 2011; Ezendam et al. 2016; Hoffmann et al. 2018; Kleinstreuer et al. 2018; Natsch and Emter 2015). In this study, a novel stable reporter gene assay based on keratinocytes, EndoSens, was developed and validated using 35 substances. EndoSens accurately reported

Table 2 Summary results of the EndoSens assay

No.	Test substances	Imax (fold)	EC1.5 (μM)	Repeat positive	EC2.0 (μM)	EC3.0 (μM)	IC50 (μM)	EndoSens prediction
1	Oxazolone	13.1	9.36	3/3	41.25	85.17	214.8	+
2	2,4-Dinitrochlorobenzene	2.58	3.28	3/3	5.5	n.i	13.37	+
3	4-Nitrobenzylbromide	2.8	2.48	3/3	4.73	n.i	9.3	+
4	Para-phenylenediamine	133.31	11.3	3/3	17.37	28.67	243.76	+
5	4-Methylaminophenol sulfate	9.38	1.75	3/3	3.49	7.93	17.62	+
6	Methyldibromo glutaronitrile	1.26	n.i	0/3	n.i	n.i	22.64	−
7	Isoeugenol	13.09	10.26	3/3	24.45	79.38	> 1000	+
8	2-Mercaptobenzothiazole	31.01	68.07	3/3	106.39	203.19	965.03	+
9	Citral	17.26	9.51	3/3	17.39	34.76	291.51	+
10	Eugenol	7.49	130.72	3/3	298.51	503.3	> 1000	+
11	Phenyl benzoate	3.25	341.78	3/3	606.15	920.69	> 1000	+
12	Ethylene glycol dimethacrylate	43.99	12.16	3/3	25.03	57.78	> 1000	+
13	4-methoxy-acetophenone	2.01	113.03	3/3	847.13	n.i	> 1000	+
14	Glycerol	1.17	n.i	0/3	n.i	n.i	> 1000	−
15	Isopropanol	1.11	n.i	0/3	n.i	n.i	> 1000	−
16	Salicylic acid	1.3	n.i	0/3	n.i	n.i	> 1000	−
17	Chlorobenzene	1.32	n.i	0/3	n.i	n.i	> 1000	−
18	Lactic acid	1.31	n.i	0/3	n.i	n.i	> 1000	−
19	Methyl salicylate	1.27	n.i	0/3	n.i	n.i	> 1000	−
20	Sulfanilamide	1.4	n.i	0/3	n.i	n.i	> 1000	−
21	Benzoquinone	32.15	6.17	3/3	8.96	13.45	55.58	+
22	Cobalt chloride	18.6	78.93	3/3	106.71	138.39	199.2	+
23	Benzisothiazolione	5.83	2.74	3/3	5.8	14.78	50.3	+
24	Resorcinol	2.85	36.97	3/3	267.57	n.i	> 1000	+
25	3,4-Dihydrocoumarin	1.24	n.i	0/3	n.i	n.i	> 1000	−
26	Benzyl cinnamate	13.36	71.78	3/3	128.57	160.43	827.98	+
27	2,4-Dichloronitrobenzene	2.02	79.84	3/3	237.44	n.i	167.26	+
28	Cinnamic alcohol	20.13	19.02	3/3	121.04	192.13	523.81	+
29	Benzocaine	2.9	16.07	3/3	61.13	n.i	> 1000	+
30	Coumarin	4.35	22.68	3/3	112.53	382.17	> 1000	+
31	Propyl paraben	6.76	11.35	3/3	23.78	46.91	113.06	+
32	4-Hydroxybenzoic acid	1.1	n.i	0/3	n.i	n.i	> 1000	−
33	Vanillin	1.45	n.i	0/3	n.i	n.i	> 1000	−
34	6-Methylcoumarin	5.08	15.45	3/3	41.63	195.31	> 1000	+
35	Benzoic acid	1.3	n.i	0/3	n.i	n.i	> 1000	−

+ Indicates positive/skin sensitizer prediction, − indicates negative/non-sensitizer prediction, *n.i.* indicates no significant induction above threshold

endogenous *HMOX1* expression synchronously induced by sensitizers, and closely mimicked the state of cells under various conditions of sensitization.

For the validation study, EndoSens assay was performed similar to the KeratinoSens™ assay (Emter et al. 2010) and adopted similar acceptance criteria. The results showed good ability to correctly identify skin sensitizers. For the 20 PS, the three values (accuracy, sensitivity, and specificity) of EndoSens were higher than 80%, indicating that the predictive capacity exceeded the performance standard requirement (OECD 2015a). These results also indicated a higher predictive capacity compared with KeratinoSens™ and LuSens for the 20 PS. For all 35 substances, the EndoSens still achieved higher predictivity than the validated KeratinoSens™. Thus, all the results indicated that the accuracy, sensitivity, and specificity of EndoSens were comparable or potentially better than KeratinoSens™ and LuSens assays.

Excitingly, some weak sensitizers (eugenol, resorcinol, and phenyl benzoate) misclassified by ARE induction-based KeratinoSens™ or LuSens (Emter et al. 2010;

Table 3 Comparison of prediction results among EndoSens, KeratinoSens™ and LuSens for the set of 35 test chemicals

No.	Test substances	LLNA	Human	EndoSens	KeratinoSens™	LuSens
1	Oxazolone	+	+	+	+	+
2	2,4-Dinitrochlorobenzene	+	+	+	+	+
3	4-Nitrobenzylbromide	+	No data	+	+	+
4	Para-phenylenediamine	+	+	+	+	+
5	4-Methylaminophenol sulfate	+	+	+	+	+
6	Methyldibromo glutaronitrile	+	+	−	+	+
7	Isoeugenol	+	+	+	+	+
8	2-Mercaptobenzothiazole	+	+	+	+	+
9	Citral	+	+	+	+	+
10	Eugenol	+	+	+	−	+
11	Phenyl benzoate	+	+	+	−	−
12	Ethylene glycol dimethacrylate	+	+	+	+	+
13	4-methoxy-acetophenone	−	−	+	+	+
14	Glycerol	−	−	−	−	−
15	Isopropanol	−	−	−	−	−
16	Salicylic acid	−	−	−	−	−
17	Chlorobenzene	−	No data	−	−	−
18	Lactic acid	−	−	−	−	−
19	Methyl salicylate	−	−	−	−	+
20	Sulfanilamide	−	−	−	−	−
21	Benzoquinone	+	+	+	+	+
22	Cobalt chloride	+	+	+	+	+
23	Benzisothiazolione	+	+	+	+	No data
24	Resorcinol	+	+	+	−	−
25	3,4-Dihydrocoumarin	+	+	−	−	No data
26	Benzyl cinnamate	+	+	+	+	No data
27	2,4-Dichloronitrobenzene	+	No data	+	+	+
28	Cinnamic alcohol	+	+	+	+	+
29	Benzocaine	−	+	+	+	No data
30	Coumarin	−	+	+	+	No data
31	Propyl paraben	−	−	+	+	+
32	4-Hydroxybenzoic acid	−	−	−	−	−
33	Vanillin	−	−	−	−	−
34	6-Methylcoumarin	−	+	+	+	+
35	Benzoic acid	−	No data	−	−	No data

+ Indicates positive/skin sensitizer prediction, - indicates negative/non-sensitizer prediction, *LLNA* and human results reference according to Kleinstreuer et al. (2018) and Urbisch et al. (2015); KeratinoSens™ prediction results reference according to Emter et al. (2010); LuSens prediction results reference according to Ramirez et al. (2014)

Ramirez et al. 2014) were correctly predicted by the *HMOX1*-induction-based EndoSens. Previous studies of KeratinoSens™ and Lusens claimed that these three substances could not activate the Keap1-Nrf2 pathway in keratinocytes as a result of deficient metabolism (eugenol, resorcinol) or inability to interact with the cysteine-rich Keap1 (phenyl benzoate), thus no ARE induction was observed (Natsch et al. 2013; Ramirez et al. 2016; Urbisch et al. 2015). However, the *HMOX1*-induction assay with EndoSens showed that eugenol and resorcinol could be metabolized in keratinocytes and significantly induce *HMOX1* expression. In addition, recent DPRA data demonstrated that phenyl benzoate had considerable cysteine-binding activity (Kleinstreuer et al. 2018). As such, the results from EndoSens assay and DPRA support the sufficiency of these substances to activate the Keap1-Nrf2 pathway. Therefore, the first explanation for the significant difference between *HMOX1* and *ARE* induction might be that some sensitizers activated not just the Keap1-Nrf2 pathway, but also many others. For example, the strong

Table 4 Comparison of predictivity among EndoSens, KeratinoSens™ and LuSens assay based on 35 test chemicals

	20 PS			35 Test chemicals	
	EndoSens	KeratinoSens™	LuSens	EndoSens	
	vs. LLNA	vs. LLNA	vs. LLNA	vs. LLNA	vs. Human
n	20	20	20	35	31
Sensitivity (%)	91.7	83.3	91.7	90.0	90.5
Specificity (%)	87.5	87.5	75.0	66.7	80.0
PPV (%)	91.7	90.9	84.6	78.3	90.5
NPV (%)	87.5	77.8	85.7	83.3	80.0
Accuracy (%)	90.0	85.0	85.0	80.0	87.1

sensitizer DNCB also activated Toll-like receptor signaling and upregulated *HMOX1* expression (van der Veen et al. 2014). Another explanation for the difference in induction might be that AREs of different genes have different affinities for Nrf2. For example, AREs from human *AKR1C1* (KeratinoSens™) and rat *NQO1* (LuSens) sometimes showed diverse induction for the same chemical (Emter et al. 2010; Ramirez et al. 2014). Furthermore, HMOX1 is considered the most common endpoint in many sensitization-related pathways. RT-PCR and gene-chip data also indicated that *HMOX1* was frequently induced by most sensitizers (Ade et al. 2009; Cottrez et al. 2016; Emter et al. 2013; van der Veen et al. 2013, 2015). Thus, *HMOX1* induction could reflect the sensitization characteristics of chemicals and might be a better endpoint for skin sensitization prediction.

However, the strong sensitizer methyldibromo glutaronitrile showed less induction of *HMOX1* expression and high cytotoxicity. This phenomenon, which was also reported for KeratinoSens™ and LuSens (Emter et al. 2010; Ramirez et al. 2014), is inconsistent with human in vivo data (Basketter et al. 2014). One explanation may be that chemicals with high cytotoxicity could increase oxidative stress and, thus, be prone to induce apoptosis instead of the Keap1-Nrf2 pathway (Magesh et al. 2012). Therefore, it might be inappropriate to evaluate the sensitization of chemicals with high cytotoxicity using the Keap1-Nrf2 pathway.

In conclusion, the predictive capacity of EndoSens for skin sensitization has been shown to exceed OECD requirements and performance of other ARE-based assays (namely, validated KeratinoSens™ or LuSens). Thus, the EndoSens assay may be a promising screening tool for in vitro skin sensitization testing and a good candidate for integrated strategies. Reproducibility within and between laboratories will further be assessed according to the OECD 2015 guideline (OECD 2015b).

Acknowledgements This work was funded by Guangzhou Science and Technology Major Project of Industry University Research Synergetic Innovation (201704020151), the National Training Program of Innovation and Entrepreneurship for Undergraduates (201710561169), the National Natural Science Foundation of China (31871292), and Fundamental Research Funds for the Central Universities (2014ZM0067) from South China University of Technology.

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