Received: 22 April 2020 | Revised: 2 September 2020 | Accepted: 2 September 2020

DOI: 10.1002/jat.4076

RESEARCH ARTICLE

Journal of Applied Toxicology WILEY

Characterization of dermal sensitization potential for industrial or agricultural chemicals with EpiSensA

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Abstract

The regulatory community is transitioning to the use of nonanimal methods for dermal sensitization assessments; however, some in vitro assays have limitations in their domain of applicability depending on the properties of chemicals being tested. This study explored the utility of epidermal sensitization assay (EpiSensA) to evaluate the sensitization potential of complex and/or "difficult to test" chemicals. Assay performance was evaluated by testing a set of 20 test chemicals including 10 methacrylate esters, 5 silicone-based compounds, 3 crop protection formulations, and 2 surfactant mixtures; each had prior in vivo data plus some *in silico* and in vitro data. Using the weight of evidence (WoE) assessments by REACH Lead Registrants, 14 of these chemicals were sensitizers and, six were nonsensitizers based on in vivo studies (local lymph node assay [LLNA] and/or guinea pig studies). The EpiSensA correctly predicted 16/20 materials with three test materials as false positive and one silane as false negative. This silane, classified as weak sensitizer via LLNA, also gave a "false negative" result in the KeratinoSens™ assay. Overall, consistent with prior evaluations, the EpiSensA demonstrated an accuracy level of 80% relative to available in vivo WoE assessments. In addition, potency classification based on the concentration showing positive marker gene expression of EpiSensA was performed. The EpiSensA correctly predicted the potency for all seven sensitizing methacrylates classified as weak potency via LLNA (EC3 ≥ 10%). In summary, EpiSensA could identify dermal sensitization potential of these test substances and mixtures, and continues to show promise as an in vitro alternative method for dermal sensitization.

KEYWORDS

DPRA, EpiSensA, h-CLAT, in vitro, KeratinoSens™, skin sensitization

1 | INTRODUCTION

Assessment of dermal sensitization potential is a requirement of the acute "6 pack" when assessing chemicals toxicity. Historically, dermal sensitization was evaluated in vivo using the guinea pig maximization test (GPMT), the Guinea Pig Buehler Test (Organisation for Economic Cooperation and Development [OECD] Test Guideline [TG] 406; OECD, 1992) or the murine local lymph node assay (LLNA) (OECD TG 429, OECD, 2010a; OECD TG 442A/B;, 2010b, 2018a; Kimber, Dearman, Basketter, Ryan, & Gerberick, 2002); however, there are some limitations with these assays, including subjective qualitative scoring (guinea pig-based tests), interanimal variability, chemistry incompatibility with the test system (e.g., long-chain fatty acids and surfactants in the LLNA), and difficulty distinguishing irritants from dermal sensitizers (LLNA and guinea pig studies; Basketter, Gerberick, & Kimber, 1998; Basketter & Kimber, 2010). Furthermore,

many regulatory programs are moving away from animal testing to the extent possible either through the acceptance of nonanimal alternative assays or through outright ban of animal-based tests (e.g., European Union Cosmetics Regulations).

The adverse outcome pathway (AOP) for dermal sensitization has provided a scientific framework for the development of in vitro assays that examine the molecular initiating event (MIE; i.e., covalent binding to skin proteins) and subsequent key events. These key events include activation of keratinocytes (i.e., inflammatory responses and altered gene expression including the antioxidant/electrophile response element [ARE]-dependent pathways), activation of dendritic cells (denoted by specific cell cytokines, chemokines, and surface markers), and last, T-cell activation and proliferation.

In recent years, the OECD has developed internationally accepted test guidelines for a number of nonanimal alternative methods (NAMs) to evaluate dermal sensitization potential, including assays that examine the MIE (covalent binding to proteins using the direct peptide reactivity assay [DPRA] or amino acid derivative reactivity assay [ADRA]; OECD TG 442C; OECD, 2019) and subsequent key events, including keratinocyte activation (KeratinoSens™ or LuSens tests; OECD TG 442D; OECD, 2018b), and activation of dendritic cells (human cell line activation test [h-CLAT], U937 cell line activation test [U-SENS™] or interleukin-8 reporter gene assay [IL-8 Luc assay]; OECD TG 442E; OECD, 2018c). These in vitro assays, when used as part of an Integrated Approach to Testing and Assessment (IATA), can distinguish sensitizers from nonsensitizers by detecting specific properties that make chemicals sensitizers (e.g., reactivity) as described in the Draft OECD Performance Based Test Guideline for Defined Approaches (DA) to Skin Sensitization (OECD, 2017, 2018d). The DA provides optimized approaches that use fixed data interpretation procedures to identify hazard potential and, if possible, provide information on potency (strong vs. weak sensitizer) to allow for Globally Harmonized System (GHS) classification and labeling (GHS, 2017).

Some classes of chemicals are difficult to test in these assays (e.g., lipophilic substances or substances requiring metabolic activation; Natsch et al., 2013; Takenouchi, Miyazawa, Saito, Ashikaga, & Sakaguchi, 2013); thus, the Epidermal Sensitization Assay (EpiSensA) was developed. This assay uses a 3D reconstructed human epidermis (RhE) model to measure four cell stress response genes related to critical keratinocyte responses during skin sensitization: (a) *Activating Transcription Factor 3* (*ATF3*), (b) *Glutamate-Cysteine Ligase Modifier subunit* (*GCLM*), (c) *DNAJ/HSP40 Homolog, Subfamily B, Member 4* (*DNAJB4*), and (d) *Interleukin-8 (IL-8)*. The up-regulation of these sensitive biomarkers in RhE after exposure to a test chemical indicates activation of the inflammatory response and the cytoprotective gene pathway in keratinocytes. These four marker genes have been demonstrated to detect positive control chemicals for skin sensitization (Saito et al., 2013). Previous studies with EpiSensA have demonstrated 90% accuracy from a diverse group of 72 chemicals, including both lipophilic and prehaptens/prohaptens (Saito, Takenouchi, Nukada, Miyazawa, & Sakaguchi, 2017).

This experimental work was undertaken to better evaluate the performance of the EpiSensA in a group of chemicals outside of the previous classes of tested chemicals, namely, methacrylates, silicone-based compounds, surfactant mixtures, and crop protection formulations. To test predictivity, the results were compared with in vivo dermal sensitization data, results from other in vitro dermal sensitization assays, and *in silico* predictions. When positive LLNA data were available, potency predictions from positive EpiSensA were compared with those derived from the effective concentration inducing a stimulation index of 3 (EC3) in the LLNA. To facilitate AOP-based weight of evidence (WoE) assessments, h-CLAT data also were generated for some chemicals.

2 | MATERIALS AND METHODS

2.1 | Test chemicals and available sensitization data

In the work described herein, 20 test chemicals containing 10 methacrylate esters, 5 silicone-based compounds, 3 crop protection formulations, and 2 surfactant mixtures were obtained from the Dow Chemical Company, Corteva Agriscience, Polysciences Inc., Evonik, or Sigma-Aldrich. Table 1 summarizes the information for the 10 methacrylate esters containing names, acronyms, CAS numbers, molecular weights, log Kow values (calculated by KOWWIN Version 1.69), and the previously reported results of in vivo skin sensitization tests. The dermal sensitization potential of these methacrylate esters has been reviewed recently, including in vivo (GPMT and LLNA), *in chemico* (DPRA), *in vitro* (KeratinoSens™/LuSens or U-SENS), and *in silico* (times metabolism simulator platform for predicting skin sensitization [TIMES-SS]) in Kimber (2019). The methacrylate esters are arranged in ascending order of molecular weight (Table 1). In addition, log Kow increased with increasing molecular weight, and six methacrylate esters are considered highly lipophilic (log Kow > 3.5). Based on the results of GPMT and LLNA for each methacrylate ester, WoE hazard and potency were summarized (e.g., European Chemicals Agency [ECHA] Registration Dossier study references, 1981, 1982, 1999, 2006, 2009, 2009, 2013, 2013, 2013, 2018). It should be noted that n-hexyl methacrylate (n-HMA) was determined to have sensitization potential in guinea pigs by two different reports, but the reliability of these tests may be insufficient to conclude as a true positive (ECHA Registration Dossier study references, 1982, 1999). In addition, 2-ethylhexyl methacrylate (EHMA) was reported as both positive and negative in the GPMT (Kimber & Pemberton, 2014). In the context of this assessment, the result of the guinea pig test for EHMA is described as "Positive/Negative," and the LLNA judged as negative with testing up to 100% concentration; thus, both compounds were identified as nonsensitizers in a recent review using a WoE approach (Kimber, 2019). However, from a regulatory standpoint, the REACH Lead Registrant (in the respective ECHA Registration Dossier; see ECHA information dissemination portal, 2020) concluded these chemicals have dermal sensitizing potential given the results of the GPMT; thus, this conclusion appears in the tables (Tables 1, 3, 4, and 5). In addition, a clear dose–response was observed when EHMA was tested by LLNA (the stimulation index values were 1.53, 2.66, and

TABLE 1 In vivo dermal sensitization results and weight of evidence (WoE) determinations for selected methacrylate used in the current evaluation of epidermal sensitization assay (EpiSensA)

					In vivo			
Test chemicals	Acronym	CAS no.	Mw (g/mol)	Log Kow KOWWIN v1.69[a]	Guinea pig	LLNA	WoE hazard	WoE potency
Methyl methacrylate	MMA	80-62-6	100.1	1.28	Positive[b]	Weak positive[c]	Sensitizer	Weak
Ethyl methacrylate	EMA	97-63-2	114.1	1.77	Positive[c]	Weak positive[c]	Sensitizer	Weak
n-Butyl methacrylate	n-BMA	97-88-1	142.2	2.75	Positive[d]	Weak positive[c]	Sensitizer	Weak
Isobutyl methacrylate	i-BMA	97-86-9	142.2	2.67	Negative[c]	Weak positive[c]	Sensitizer	Weak
n-Hexyl methacrylate	n-HMA	142-09-6	170.2	3.73	Positive[e, f]	Negative[b]	Sensitizer	Weak
2-Ethylhexyl methacrylate	EHMA	688-84-6	198.3	4.64	Positive/ Negative[c]	Borderline negative[b]	Sensitizer	Weak
n-Octyl methacrylate	n-OMA	2157-01-9	198.3	4.71	No data	Weak positive[g]	Sensitizer	Weak
Isodecyl methacrylate	IDMA	29964-84-9	226.4	5.62	No data	Negative[b]	NS	NS
Lauryl methacrylate	LMA	142-90-5	254.4	6.68	No data	Negative[b]	NS	NS
Tridecyl methacrylate	TDMA	2495-25-2	254.4-310.5	6.68-8.64	No data	Negative[b]	NS	NS

Abbreviations: LLNA, local lymph node assay; NS, nonsensitizer.
[a]Values may differ from those in the European Chemicals Agency (ECHA) registrations due to differing versions of KOWWIN; however, these differences are unlikely to be significant.
[b]Kimber, 2019.
[c]Kimber & Pemberton, 2014.
[d]ECHA Registration Dossier study reference, 2013.
[e]ECHA Registration Dossier study reference, 1982.
[f]ECHA Registration Dossier study reference, 1999.
[g]ECHA Registration Dossier study reference, 2018.

2.85 at 25%, 50%, and 100%, respectively; Kimber & Pemberton, 2014), and the result of the LLNA for EHMA is described as "Borderline negative."

Table 2 shows the information on five polyfunctional silicone-based compounds (PS-6: aminoethyl-aminoisobutyl methyl dimethoxysilane, PS-7: reaction products of vinyltriacetoxysilane and glycidoxypropyltrimethoxy silane, PS-8: methylamino siloxane with glycidyl trimethylammonium chloride, silane glycol, and silicone glycol), three crop protection formulations (CPF-1: emulsion concentrate of triclopyr butoxyethyl ester, CPS-2: soluble concentrate of aminopyralid triisopropanolammonium, and CPF-3: suspension concentrate of florasulam), and two surfactant mixtures (XUS-906 and XU-801), respectively. Petry et al. (2017, 2018) have already reported the results of testing that assessed the skin sensitization potential of a heterogeneous group of functionalized polysiloxanes and silanes in two previous articles. In order to compare these substances with the previous publications, the same substance codes as those used in the Petry et al. (2017, 2018) publications were used in this article (Table 2). An aminofunctional alkoxysilane (PS-6), a non-aminofunctional alkoxysilane (PS-7), and an aminofunctional siloxane (PS-8) were evaluated (Table 2). Two additional silicone-based compounds were tested with EpiSensA and h-CLAT: a polymer (silane glycol) and a silicone material (silicone glycol) (Table 2). Three crop protection formulations have been already evaluated by the KeratinoSens™ assay, and the results have been summarized with in vivo data in Settivari et al., 2015. The two other chemicals, two surfactant mixtures (XUS-906 and XU-801; Table 2), were assessed with EpiSensA and h-CLAT in this work.

The product classes mentioned in Table 2 followed the definitions written in OECD TG 442E, 2018c. Briefly, mixture was defined as a mixture or a solution composed of two or more substances in which they do not react. In addition, each substance was defined by its quantitative composition. If one main constituent is present to at least 80% (w/w), the substance was called a monoconstituent. Furthermore, if more than one main constituent is present in a concentration ≥10% (w/w) and <80% (w/w), the substance was defined as multiconstituent. The difference between mixture and multiconstituent substance is that a mixture is obtained by blending without chemical reaction. In contrast, multiconstituent substance is the result of a chemical reaction. The 10 tested chemicals (Table 2) were all

TABLE 2 In vivo dermal sensitization results and weight of evidence (WoE) determinations for selected silicone-based compounds, crop protection formulations, and surfactant mixtures used in the current evaluation of epidermal sensitization assay (EpiSensA)

Substance name/agrochemical active ingredient	Chemical nature/ formulation type	CAS no.	Molecular weight	Water solubility	Purity/Active ingredient concentration (%)	In vivo		
						Guinea pig, human	LLNA	WoE hazard
Silicone-based compounds								
PS-6 (aminoethyl aminoisobutyl methyl dimethoxysilane)	Aminofunctional alkoxysilane[a]	23410-40-4	220 g/mol	>1 g/L; hydrolysis upon H_2O contact	85–100	Positive[b] (GPMT)	Positive[b]	Sensitizer
PS-7 (reaction products of vinyltriacetoxysilane and glycidoxypropyltrimethoxy silane)	Alkoxysilane (non-aminofunctional)[c]	154518-41-9	Con[d]	>1 g/L; hydrolysis upon H_2O contact	90–100	Positive[b] (GPMT)	Positive[b]	Sensitizer
PS-8 (methylaminosiloxane with glycidyltrimethylammonium chloride)	Aminofunctional siloxane[c]	495403-02-6	Con[d]	Poorly soluble	70–90	Positive[b] (Buehler)	Positive[b]	Sensitizer
Silane glycol	Polymer[c]	Con[d]	Con[d]	No data	≥80.0 to ≤90.0	Negative[e] (GPMT)	No data	NS
Silicone glycol	Silicone material[a]	Con[d]	Con[d]	No data	≥70 to ≤80.0	Negative[f] (Human patch)	No data	NS
Crop protection formulations								
CPF-1 (triclopyr butoxyethyl ester)	Emulsion concentrate[g]	64700-56-7	356.6	Emulsifiable	61.6	Positive[h] (Buehler)	No data	Sensitizer
CPF-2 (aminopyralid triisopropanolammonium)	Soluble concentrate[g]	566191-89-7	398.3	Soluble	40.6	Negative[h] (Buehler)	No data	NS
CPF-3 (florasulam)	Suspension concentrate[g]	145701-23-1	359.3	No data	4.8	No data	Negative[h]	NS
Surfactant mixtures								
XUS-906 (sodium sulfate of secondary alcohol alkoxylates)	Surfactant[g]	Con[d]	Con[c]	Completely miscible	≥20.0 to ≤50.0	Positive[j] (GPMT)	No data	Sensitizer
XU-801 (sodium sulfate salt of branched alcohol alkoxylates)	Surfactant[g]	Con[d]	Con[c]	Completely miscible	≥20.0 to ≤50.0	Positive[j] (GPMT)	No data	Sensitizer

Abbreviations: CPF, crop protection formulation; GPMT, guinea pig maximization test; LLNA, local lymph node assay; NS, nonsensitizer; PS, polyfunctional silicone.

[a]Monoconstituent.

[b]Petry et al., 2017, 2018.

[c]Multiconstituent.

[d]Confidential.

[e]The Dow Chemical Company, 1999. Internal Technical Report.

[f]The Dow Chemical Company, 1998. Internal Technical Report.

[g]Mixture.

[h]Settivari et al., 2015.

[i]The Dow Chemical Company, 2015. Internal Technical Report.

[j]The Dow Chemical Company, 2013. Internal Technical Report.

TABLE 3 EpiSensA results, potency determinations, and comparison with weight of evidence (WoE) determinations for selected methacrylates

	EpiSensA								In vivo	
		I_{max}								
Test chemicals	IC20 (%)	ATF3	GCLM	DNAJB4	IL-8	Min EC (%)	Hazard	Potency	WoE hazard	WoE potency
MMA	11.3	1.8	**2.7**	1.8	1.4	2.1	Positive	Weak	Sensitizer	Weak
EMA	13.5	0.5	**3.6**	**2.3**	0.6	6.9	Positive	Weak	Sensitizer	Weak
n-BMA	13.4	**15.1**	**13.0**	**8.2**	**6.3**	1.0	Positive	Weak	Sensitizer	Weak
i-BMA	10.1	1.6	**8.4**	**5.9**	1.6	0.9	Positive	Weak	Sensitizer	Weak
n-HMA	14.2	**49.8**	**6.8**	**4.5**	**30.8**	2.8	Positive	Weak	Sensitizer	Weak
EHMA	80.4	4.8	**2.1**	**3.2**	**16.7**	12.5	Positive	Weak	Sensitizer	Weak
n-OMA	53.9	**147.3**	1.9	**3.8**	**240.4**	12.5	Positive	Weak	Sensitizer	Weak
IDMA	>100	8.6	**6.0**	**4.3**	**17.3**	10.7	Positive	Weak	NS	NS
LMA	>100	3.1	1.3	1.4	2.3	>100	Negative	NS	NS	NS
TDMA	>100	1.2	1.3	1.3	1.2	>100	Negative	NS	NS	NS

Note: I_{max}, maximum fold-induction of biomarker genes at concentrations with >80% cell viability. IC20 (%), inhibitory concentration resulting in a 20% decrease in cell viability. Min EC (%), minimum effective concentration producing the targeted increase in one or more biomarker genes resulting in a positive assay result.
Abbreviation: NS, nonsensitizer.

TABLE 4 h-CLAT results and comparison with weight of evidence (WoE) hazard determination for methacrylates

	h-CLAT						
		CD86		CD54			
Test chemicals	CV75 (μg/ml)	No. of positive	EC150 (μg/ml)	No. of positive	EC200 (μg/ml)	h-CLAT judgment	In vivo WoE hazard
MMA	>1,000	0/3	–	3/3	479.7	Positive	Sensitizer
EMA	>1,000	0/3	–	2/3	651.3	Positive	Sensitizer
n-BMA	253.4	1/3	–	2/3	282.5	Positive	Sensitizer
i-BMA	309.7	0/3	–	3/3	265.4	Positive	Sensitizer
n-HMA	89.6	0/3	–	3/3	54.8	Positive	Sensitizer
EHMA	38.8	0/3	–	3/3	24.2	Positive	Sensitizer
n-OMA	100.0	0/3	–	2/3	121.1	Positive	Sensitizer
IDMA	43.0	1/3	–	3/3	21.9	Positive	NS
LMA	80.2	0/3	–	3/3	43.2	Positive	NS
TDMA	123.4	0/3	–	3/3	75.7	Positive	NS

Abbreviations: h-CLAT, human cell line activation test; NS, nonsensitizer.

commercial grade raw materials provided by The Dow Chemical Company or Corteva Agriscience (formerly Dow AgroSciences). The activities of these 10 chemicals in in vivo tests for skin sensitization and WoE hazard are provided in Table 2.

2.2 | EpiSensA protocol

The EpiSensA is a RhE model-based assay and addresses the second key event in the AOP for skin sensitization (i.e., keratinocyte activation). The test protocol has been described in Saito et al. (2017) except for the potency prediction. An RhE model "LabCyte EPI-MODEL 24" (Japan Tissue Engineering Co. Ltd, Aichi, Japan) was pre-incubated overnight in culture medium. Each test chemical was dissolved in an appropriate vehicle, selected from either AOO (acetone:olive oil at 4:1, v/v), distilled water (DW), or 50 v/v% ethanol in DW (50% EtOH). As the vehicle selection criteria, a vehicle, which can dissolve a test chemical at the highest concentration, was selected. Two-fold serial dilutions were performed (basically from three to five concentrations), and 5 μl of working solutions were applied on tissue surfaces and incubated for 6 h. Cell viability was measured by lactate dehydrogenase (LDH) assay and the 20% inhibitory concentration (IC20) affecting a 20% reduction of cell viability was calculated by linear interpolation. After incubation, the tissues were rinsed with

TABLE 5 In vivo dermal sensitization results, weight of evidence (WoE) determinations, and results for EpiSensA and other *in silico*, *in chemico*, and in vitro sensitization assays for selected methacrylates

	In vivo		EpiSensA					
Test chemicals	WoE hazard	WoE potency	Hazard	Potency	h-CLAT	TIMES-SS[a]	DPRA[a]	LuSens (L)[a]/KeratinoSens™ (K)
MMA	Sensitizer	Weak	Positive	Weak	Positive	Negative	Positive	Positive (L)
EMA	Sensitizer	Weak	Positive	Weak	Positive	Negative	Positive	Positive (L)
n-BMA	Sensitizer	Weak	Positive	Weak	Positive	Negative	Positive	Positive (L)
i-BMA	Sensitizer	Weak	Positive	Weak	Positive	Weak positive	Positive	Positive (K)
n-HMA	Sensitizer	Weak	Positive	Weak	Positive	Weak positive	Negative	Negative (K)
EHMA	Sensitizer	Weak	Positive	Weak	Positive	Weak positive	Negative	Negative (K)
n-OMA	Sensitizer	Weak	Positive	Weak	Positive	Weak positive	Negative	Negative (K)
IDMA	NS	NS	Positive	Weak	Positive	Weak positive	Negative	Negative (K)
LMA	NS	NS	Negative	NS	Positive	Negative	Negative	Positive (K)
TDMA	NS	NS	Negative	NS	Positive	Negative	Negative	Positive (K)

Abbreviations: DPRA, direct peptide reactivity assay; EpiSensA, epidermal sensitization assay; h-CLAT, human cell line activation test; NS, nonsensitizer; TIME-SS, times metabolism simulator platform for predicting skin sensitization.
[a]Kimber, 2019.

Dulbecco's phosphate-buffered saline (DPBS) and collected. Total RNA was extracted, and reverse transcription of total RNA and quantitative reverse transcription–polymerase chain reaction (RT–PCR) was performed (QuantStudio 5 real-time PCR System, Thermo Fisher Scientific, Waltham, MA, USA). Cycle threshold (Ct) values of four skin sensitization marker genes (*ATF3*, *GCLM*, *DNAJB4*, and *IL-8*) and one endogenous control gene (*GAPDH*, encoding the housekeeping protein glyceraldehyde 3-phosphate dehydrogenase) were measured using RT–PCR. Relative gene expression levels normalized to *GAPDH* gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as fold-induction. Each chemical was tested in one run, and three tissues per tested concentration were used. The mean value (three tissues per group) of maximum fold-induction (I_{max}) was obtained using the data from the concentrations with more than 80% cell viability. When the I_{max} of at least one out of the four marker genes exceeded the respective cut-off value (*ATF3*, 15-fold; *GCLM*, 2-fold; *DNAJB4*, 2-fold; and *IL-8*, 4-fold), the chemical was judged as positive. For a potency prediction for positive chemicals in EpiSensA, the estimated concentration needed to reach respective cut-off values (EC) was calculated by linear interpolation for each marker gene and minimum EC value (Min EC) was determined. If the Min EC value was less than 0.098 w/v%, the test chemical was classified as strong sensitizer (i.e., equivalent to LLNA EC3 < 1%; European Centre for Ecotoxicology and Toxicology of Chemicals [ECETOC], 2008). On the other hand, if the Min EC value was more than or equal to 0.098 w/v%, the chemical was classified as weak sensitizer (i.e., equivalent to 1% < LLNA EC3 < 100%; ECETOC, 2008).

2.3 | h-CLAT protocol

The h-CLAT, which addresses the third key event in the AOP for skin sensitization (i.e., dendritic cell activation), was performed according to OECD TG 442E, 2018c. Briefly, the test chemical was dissolved or stably dispersed in saline or DMSO and diluted into culture medium. An initial cytotoxicity test was performed as a dose-finding study and CV75 (concentration yielding 75% cell viability after 24-h incubation) was determined from at least two independent runs. For the main study, THP-1 cells were treated at eight concentrations of 1.2-fold serial dilution-based on predetermined CV75 for 24 h. After exposure, the cells were stained with fluorescence-labeled antibodies for CD86 or CD54, and quantified by flow cytometry. At concentrations greater than or equal to 50% of control, the relative fluorescence intensity (RFI) to solvent control was calculated, and when CD86 RFI was more than or equal to 150% and/or CD54 RFI was more than or equal to 200%, the chemical was considered as positive. The test chemical was tested in three independent runs, and if at least one of the above conditions was met in at least two of three independent runs, the final judgment of h-CLAT was decided as positive; otherwise, the judgment was negative. The estimated concentrations inducing 150% of CD86 RFI and 200% of CD54 RFI (EC150 and EC200) were calculated by linear interpolation.

3 | RESULTS

3.1 | The results of 10 methacrylate esters

Table 3 presents the overview of results of EpiSensA. The methacrylate esters are arranged in ascending order of molecular weight. Focusing on IC20 values, five of the 10 methacrylate esters with lower molecular weight (MMA, EMA, n-BMA, i-BMA, and n-HMA) showed similar cytotoxicity in EpiSensA (10.1%–14.2%). In general, the IC20 values tended to increase (lower cytotoxicity) with increasing molecular weight across the methacrylate group, and no cytotoxicity was indicated in three methacrylate esters with higher molecular

weight (IDMA, LMA, and TDMA). A similar tendency generally was observed in Min EC values. Five methacrylate esters with lower molecular weight showed similar Min EC values (0.9%–6.9%), and the Min EC values tended to increase with the increasing molecular weight with the next three larger methacrylates showing similar values (EHMA: 12.5%, n-OMA: 12.5%, IDMA: 10.7%). Moreover, two methacrylate esters with higher molecular weight (LMA and TDMA) did not reach the threshold for a positive gene expression signature in EpiSensA. In conclusion, the sensitizing potential and potency prediction for eight methacrylate esters with lower molecular weight were judged as positive with weak potency, and two esters with higher molecular weight were judged as negative. Compared with in vivo WoE results, EpiSensA correctly predicted the hazard (sensitizing or nonsensitizing) and weak potency of diverse methacrylate esters with different molecular weights, except for IDMA (false positive).

Table 4 provides the results of h-CLAT for 10 methacrylate esters. Regarding cytotoxicity, CV75 values generally tended to decrease with the increasing molecular weight in contrast to EpiSensA. For example, MMA and EMA did not show cytotoxicity at concentrations up to 1,000 μg/ml, and n-BMA and i-BMA presented relatively high CV75 values (253.4 and 309.7 μg/ml, respectively). These results seemed counterintuitive as MMA and EMA are the strongest Michael acceptors. In contrast, n-HMA, EHMA, n-OMA, IDMA, and LMA had CV75 values that were less than or equal to 100 μg/ml. These cytotoxicity values may reflect a complex relationship between Kow and reactivity or be related to alkyl chain length (see Section 4).

In terms of CD86 and CD54 marker expression, none of the methacrylate esters tested showed a repeated positive increase in the CD86 marker. On the other hand, all methacrylate esters tested presented a positive response in CD54. In addition, the EC200 value typically decreased with the increasing molecular weight, similar to CV75, which again, may be related to a complex relationship between test material bioavailability and reactivity. Figure 1 illustrates the relationship between CV75 and EC200 for eight out of the 10 methacrylate esters (R^2 = 0.924). Note that MMA and EMA are absent from Figure 1 because CV75 could not be calculated. The positive response (i.e., CD54 response) for the remaining eight methacrylate esters appears to correlate with cytotoxicity, which in some cases, may be related to bioavailability (Kow). All methacrylate esters were judged as positive in the h-CLAT in this study, indicating that despite the absence of expected cytotoxicity by lower molecular weight methacrylates, there was sufficient bioavailability and activity in these materials to generate positive results. Three esters (IDMA, LMA, and TDMA) resulted in false-positive results compared with in vivo findings.

A compiled overview of the responses of the methacrylates in in vivo, *in chemico*, and *in silico* methods from the data described herein and in Kimber (2019) is presented in Table 5. The results of EpiSensA and h-CLAT have been mentioned above. For TIMES-SS, four methacrylate esters were correctly characterized for their hazard and weak potency (i-BMA, n-HMA, EHMA, and n-OMA), but three sensitizing methacrylate esters were incorrectly judged as negative (MMA, EMA, and n-BMA), and IDMA was incorrectly judged as positive. Regarding DPRA and LuSens/KeratinoSens™, four sensitizing methacrylate esters with lower molecular weight (MMA, EMA, n-BMA, and i-BMA) were correctly predicted for their sensitizing potential by both assays, but three sensitizing esters with higher molecular weight (n-HMA, EHMA, and n-OMA) were incorrectly called nonsensitizers. In addition, DPRA correctly predicted three nonsensitizing methacrylate esters, but KeratinoSens™ over-predicted the sensitizing potential of LMA and TDMA. Based on the overview in Table 5, EpiSensA demonstrated the best predictive performance among these five alternative methods for this class of chemistry.

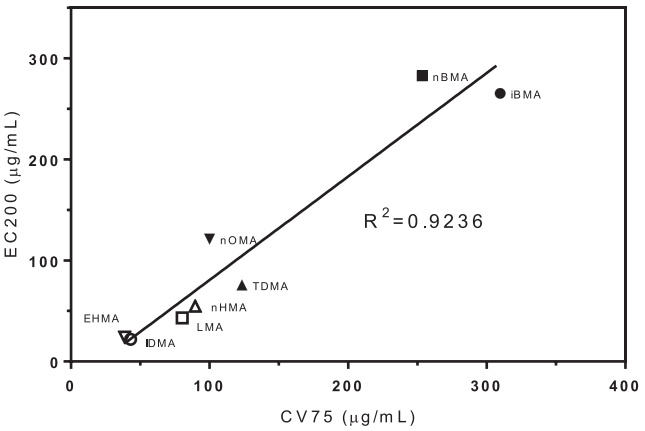

FIGURE 1 Relationship between cytotoxicity concentration yielding 75% cell viability (CV75) and the estimated concentration producing a 200% increase in CD54 relative fluorescent units (EC200) for selected methacrylates. There is a strong correlation between cytotoxic concentration and concentrations driving increased expression of CD54 (EC200), which may reflect a correlation between alkyl chain length and cytotoxicity or a complex relationship between intracellular bioavailability and reactivity. Note that MMA and EMA are not included in this graph as CV75 values could not be determined for these compounds

3.2 | The results of five silicone-based compounds, three crop protection formulations, and two surfactant mixtures

Table 6 provides the results of five silicone-based compounds (PS-6, PS-7, PS-8, silane glycol, and silicone glycol), three crop protection formulations (CPF-1, CPF-2, and CPF-3), and two polyether-derived surfactant mixtures (XUS-906 and XU-801). In addition, maximum tested concentrations of EpiSensA are also presented, and five chemicals (PS-8, silicone glycol, CPF-1, CPF-2, and CPF-3) were tested up to 100 w/v% (neat). On the other hand, four chemicals (PS-6, PS-7, XUS-906, and XU-801) were tested at a lower concentration due to cytotoxicity. The maximum tested concentration of silane glycol was 50 w/v% due to solubility limitations of the substance.

Regarding the silicone-based compounds, EpiSensA correctly predicted two of the three sensitizing silane/siloxanes as positive (PS-7 and PS-8). For PS-7 and PS-8, which have positive LLNA and EpiSensA data, in vivo and in vitro potency predictions can be

TABLE 6 In vivo dermal sensitization weight of evidence (WoE) determinations and results for EpiSensA and other *in chemico*/in vitro sensitization assays for selected silicone-based compounds, crop protection formulations, and surfactants

		EpiSensA				
Product name	In vivo WoE hazard	Max test conc. (w/v%)	Judgment	h-CLAT	DPRA	KeratinoSens™
PS-6	Sensitizer	6.25	Negative	Positive[a]	NC[a]	Negative[a]
PS-7	Sensitizer	6.25	Positive	Positive[a]	Positive[a]	Positive[a]
PS-8	Sensitizer	100 (neat)	Positive	Positive[b]	Negative[a]	Negative[a]
Silane glycol	NS	50	Positive	Positive[b]	No data	No data
Silicone glycol	NS	100 (neat)	Negative	Positive[b]	No data	No data
CPF-1	Sensitizer	100 (neat)	Positive	Positive[b]	Negative[c]	Positive[d]
CPF-2	NS	100 (neat)	Negative	Negative[b]	Negative[c]	Negative[d]
CPF-3	NS	100 (neat)	Positive	Positive[b]	Negative[c]	Negative[d]
XUS-906	Sensitizer	0.78	Positive	Positive[b]	Positive[e]	Positive[f]
XU-801	Sensitizer	1.56	Positive	Negative[b]	No data	No data

Abbreviations: CPF, crop protection formulation; DPRA, direct peptide reactivity assay; EpiSensA, epidermal sensitization assay; NC, not classifiable; NS, nonsensitizer; PS, polyfunctional silicone.
[a]Petry et al., 2017.
[b]Newly tested.
[c]Gehen et al., 2016.
[d]Settivari et al., 2015.
[e]The Dow Chemical Company, 2017. Internal Technical Report.
[f]The Dow Chemical Company, 2018. Internal Technical Report.

compared. PS-7 was estimated to be of weak-to-moderate potency using the LLNA EC3 (Petry et al., 2017), whereas the EpiSensA Min EC predicted PS-7 to be weak sensitizer (Min EC = 0.58 w/v%). PS-8 was predicted as a weak sensitizer using both approaches (Petry et al., 2017; Min EC = 10.9 w/v%). Across most in vitro and *in chemico* methods, PS-6 was incorrectly predicted (false negative); however, the h-CLAT accurately predicted PS-6 as positive for sensitization potential. Across these silicone-based compounds, DPRA correctly predicted PS-7 to be positive, whereas PS-6 could not be classified due to interference (Petry et al., 2017). KeratinoSens™ predicted two of three to be nonsensitizers despite all three compounds inducing sensitization in in vivo assays. For the two nonsensitizing silicone-based compounds, silicone glycol was correctly predicted for its nonsensitizing potential by EpiSensA, but silane glycol resulted in a false-positive finding. Contrary to the EpiSensA results, h-CLAT over-predicted the sensitization potential of both silane and silicone glycols. DPRA and KeratinoSens™ have not yet been conducted on the silane and silicone glycol compounds.

EpiSensA correctly predicted the sensitization potential of two out of three crop protection formulations (CPF-1 and CPF-2) but over-predicted CPF-3. Due to the absence of positive LLNA data, potency predictions for EpiSensA could not be compared with in vivo results. In comparison with other in vitro methods, h-CLAT also judged CPF-3 as positive, but DPRA and KeratinoSens™ correctly predicted it as negative. DPRA predictions were correct for two of three chemicals as this assay under-predicted CPF-1 as negative. KeratinoSens™ correctly predicted all three chemicals.

The two surfactant mixtures with sensitizing properties (XUS-906 and XU-801) were correctly predicted for their sensitization potential by EpiSensA. Again, the absence of LLNA data precludes an in vivo comparison with EpiSensA potency predictions. Contrary to the EpiSensA results, however, the h-CLAT assay falsely predicted the sensitization potential of one surfactant mixture (XU-801); thus, h-CLAT only predicted one of two chemicals correctly compared with EpiSensA and in vivo data. DPRA and KeratinoSens™ correctly predicted XUS-906 as a sensitizer, but these assays have not been conducted on XU-801.

4 | DISCUSSIONS

This study was designed to examine a broader chemical domain for the EpiSensA by examining its performance on some “difficult to test” chemicals. EpiSensA results were compared with in vivo dermal sensitization data for these chemicals. The overall concordance of the EpiSensA with in vivo dermal sensitization results for this limited set of chemicals is shown in Table 7. Relative to the in vivo conclusions on dermal sensitization, the sensitivity of the EpiSensA was 92%, the specificity was 57%, and the accuracy was 80%. Compared with a recent analysis with a larger data set (72 chemicals; Saito et al., 2017), the sensitivity of the EpiSensA for the current chemicals was similar; however, specificity and accuracy were slightly decreased compared with the larger data set (78% specificity and 90% accuracy; Saito et al., 2017).

Results in the EpiSensA also were compared with results of the KeratinoSens™ assay as both of these assays examine the same key event in the dermal sensitization AOP, activation of keratinocytes. In this comparison, the assay with the best predictive performance varied based on the chemicals being assessed. For methacrylates, the EpiSensA was a better predictor of dermal sensitization potential than

TABLE 7 Confusion matrix of EpiSensA performance with the selected test chemicals

		EpiSensA		
		Positive	Negative	
In vivo	Positive	12	1	13
	Negative	3	4	7
		15	5	20

Abbreviation: EpiSensA, epidermal sensitization assay.
Sensitivity = True Positives/Total positive = 12/13 = 0.92.
Specificity = True Negatives/Total negative = 4/7 = 0.57.
Accuracy = (True Positives + True Negatives)/Total = 16/20 = 0.80.

the KeratinoSens™ assay (relative to in vivo results). Similarly, the EpiSensA data more accurately predicted the dermal sensitization potential of the three silicone-based compounds (PS-6, PS-7, and PS-8) that had data in both assays, whereas the KeratinoSens™ assay better predicted the three agricultural chemicals included in the test set. EpiSensA and KeratinoSens™ both correctly predicted the one surfactant mixture (XUS-906) tested in both assays as a sensitizer. While these are limited data sets, there is some indication that, at least for some chemicals, the EpiSensA has better predictive capability for dermal sensitization potential than the KeratinoSens™ assay. Here, as noted in Section 2, n-HMA and EHMA were reported as both positive and negative in Guinea pig test and/or LLNA and interpreted as sensitizers in this study. Therefore, the result of the comparison might be subject to the interpretation. However, when the comparison between EpiSensA and KeratinoSens™ was performed based on nine methacrylates (n-HMA and EHMA removed), the EpiSensA was still a better predictor of dermal sensitization potential than the KeratinoSens™ assay.

In some cases, similarities to intact skin and in vivo study designs may favor EpiSensA sensitization predictions as more similar to in vivo results. Furthermore, the EpiSensA study design enables evaluation of test chemicals with direct application at higher concentrations, similar to in vivo methods for skin sensitization prediction, because the EpiSensA is not limited by aqueous-phase chemical exposures. For example, in this study, five chemicals were tested at 100 w/v% (neat). Furthermore, two surfactant mixtures were tested around 1 w/v%, a higher concentration than may be achieved in other assays as surfactants generally have highly cytotoxic properties in vitro. In addition, the metabolic competence of RhE models is generally more comparable to *ex vivo* human skin samples for detection of prehaptens/prohaptens, although relative activity differs for some enzymes (Eilstein et al., 2014; Götz et al., 2012, 2012; Luu-The et al., 2009; Oesch, Fabian, & Landsiedel, 2018). The 3D dermal structure also may better mimic the requirement for dermal flux that occurs with in vivo exposures.

4.1 | The results for 10 methacrylate esters

Based on the in vivo WoE results for the methacrylate esters evaluated, the three highest molecular weight substances (IDMA, LMA, and TDMA) are not sensitizers. In the LLNA, n-HMA, EHMA, IDMA, LMA, and TDMA were all negative. The rationale to explain LLNA negative results for these higher molecular weight substances was due to proposed size limitations in dermal flux (Gelbke, Ellis-Hutchings, Müllerschön, Murphy, & Pemberton, 2018; Kimber, 2019). The RhE model used in EpiSensA has a stratum corneum like human or animal skin. As molecular weight and log Kow increased across the methacrylates, cytotoxic concentration and Min EC values in EpiSensA tended to increase, indicating a tendency towards lower cytotoxicity and EpiSensA potency at higher molecular weights (Tables 1 and 3). Thus, similar to the LLNA, it appears that permeability of methacrylate esters in the RhE model affects EpiSensA reactivity. EpiSensA correctly predicted hazard and weak potency of nearly all evaluated methacrylate esters, including negative predictions for the highest molecular weight substances (LMA and TDMA). Contrary to the LLNA, the EpiSensA gave positive predictions for n-HMA, EHMA, and IDMA, suggesting that EpiSensA allows either greater dermal penetration than the LLNA and/or slower metabolism (inactivating hydrolysis) when penetration occurred.

As molecular weight increases across the methacrylate data set, IDMA is a transition compound for dermal sensitization. IDMA is considered negative via both the LLNA and REACH Lead Registrant interpretation. The false-positive EpiSensA result for IDMA may again suggest greater penetration and/or slower metabolism than occurs in vivo. This positive IDMA result is not related to protein reactivity as the DPRA assay was negative for all methacrylates greater in size than n-HMA. Hence, for methacrylates, EpiSensA may be able to predict skin sensitization potential and potency in the context of skin permeability of the test chemicals although there may be some qualitative differences in dermal flux relative to in vivo scenarios. This feature is important, not only for skin sensitization predictions, but also as a part of the EpiSensA application for category approach (i.e., read-across) when parameters related to molecular weight or skin permeability are considered.

Methacrylate esters also tended to show decreased reactivity in DPRA and KeratinoSens™ with increasing molecular weight (Kimber, 2019). For example, it was noted that the reactivity of LMA in KeratinoSens™ was borderline positive (e.g., EC1.5 = 912 μM for LMA; Dow Chemical/MPA, 2018). Moreover, DPRA and KeratinoSens™ methods did not detect hazard for three sensitizing methacrylate esters with intermediate molecular weight (n-HMA, EHMA, and n-OMA), but EpiSensA (and h-CLAT) correctly predicted their sensitization potential. The similarity in structure and experimental conditions between EpiSensA and animal tests (i.e., stratum corneum present in both models and similar direct exposure on the epidermis using a lipophilic vehicle) may have facilitated detection of the intermediate molecular weight methacrylates by EpiSensA. The negative DPRA assay suggests that parent n-HMA, EHMA, and n-OMA were not capable of covalent protein interactions in these in vitro systems.

Interestingly, h-CLAT over-predicted the sensitization potential for these methacrylates as described in Table 4. In addition, EC200 and CV75 were highly correlated with methacrylate esters (Figure 1). It has been reported that alkyl chain length can correlate with

cytotoxicity of chemicals based on data with benzalkonium chloride with various alkyl chain length (Groothuis et al., 2019). From this information, the positive response of h-CLAT in this study may be influenced by cytotoxicity derived from alkyl chain length of methacrylate esters. Alternatively, methacrylate in vitro cytotoxicity reportedly reflects a complex relationship between Kow and reactivity, where cytotoxicity is the result of both intracellular dose and reactivity (Fujisawa, Atsumi, & Kadoma, 2000; Fujisawa, Imai, Kojima, & Masuhara, 1978; Yoshii, 1997). In this case, optimum Kow values allow for greater intracellular bioavailability of some methacrylates with higher molecular weights (higher Kow values compared with more reactive MMA and EMA).

4.2 | The results of 5 silicone-based compounds, 3 crop protection formulations, and 2 surfactant mixtures

With respect to the remaining 10 chemicals, comparison of the EpiSensA results with the KeratinoSens™ assay can only be conducted for seven chemicals (three chemicals lack KeratinoSens™ data: Silane glycol, Silicone glycol, and XU-801). For these seven chemicals, the EpiSensA and KeratinoSens™ models performed similarly as both accurately predicted five of seven chemicals (71%).

The sensitizing aminofunctional alkoxysilane (PS-6) was interpreted as a false negative in EpiSensA, and KeratinoSens™, but was correctly predicted in the h-CLAT. It should be noted that PS-6 was judged as a weak sensitizer in the LLNA (10% < EC3 < 25%; Petry et al., 2017), but was also positive in the GPMT. In the DPRA assay, PS-6 could not be adequately categorized for protein reactivity due to analytical interference with the determination of lysine depletion (Petry et al., 2017). However, PS-6 was positive in two dendritic cell activation assays, the h-CLAT (here and in Petry et al., 2017), and the modified Myeloid U937 Skin Sensitization Test (mMUSST; Petry et al., 2017). Therefore, the sensitizing potential of PS-6 may be difficult to detect in some in vitro methods due to weak potency. Alternatively, these data may indicate that these in vitro assays are not well suited for use with certain challenging chemicals.

Similarly, the KeratinoSens™ assay mispredicted the sensitization potential of PS-8. Again, this may be due to the low water solubility of this substance, which limits dose administration for some in vitro assays. With the EpiSensA, 100% neat material was administered to the 3D tissue, which may have enabled a positive response.

CPF-3 was a false-positive in EpiSensA (and h-CLAT). Here, the active ingredient of CPF-3 (florasulam) was judged as negative in vivo (Magnusson and Kligman skin sensitization test) and in KeratinoSens™. In addition, CPF-3 formulation was also negative in KeratinoSens™ when it was tested based on its whole formulation at concentrations ranging from 0.4 to 800 ppm (Settivari et al., 2015). However, the CPF-3 formulation was a false positive in the KeratinoSens™ assay when tested at high concentrations based on the concentration of active ingredient (exposed at 18.3-fold higher concentration) (Settivari et al., 2015). In the EpiSensA, CPF-3 did not reach these concentrations; however, the positive result might be attributed to the co-formulant contributions to nonspecific cell stress and/or other nonspecific mechanisms that activate the Nrf2 pathway at high concentrations in this assay.

4.3 | Two out of three approach to identify dermal sensitization hazard

The two out of three approaches (Bauch et al., 2012; Urbisch et al., 2015) is one of the DA under consideration by OECD (OECD, 2017). In this approach, the available assay data must address two of three key events (if assay results agree) or three of three key events (if two of the assays yield disparate results). Thus, assay results are used in combination, representing Key Event 1 (covalent protein binding, DPRA), Key Event 2 (keratinocyte activation, LuSens/Keratinosens™/EpiSensA), and/or Key Event 3 (dendritic activation, h-CLAT, U-SENS™). In practical terms, if the first two assays predict the same result (positive or negative for sensitization potential), this dermal sensitization prediction is applied to the substance being tested, and no additional work is conducted. If the two assay results disagree, a third assay is conducted.

Using a two out of three approaches (Kleinstreuer et al., 2018), the combination of the DPRA + EpiSensA + h-CLAT outperformed the DPRA + Keratinosens™/LuSens + h-CLAT as the former assay combination correctly predicted 14 of 17 chemicals (82%), whereas the latter assay combination predicted 10 of 16 chemicals (63%) correctly (relative to in vivo WoE determinations). Notably three chemicals (XU-801, silane glycol, and silicone glycol) lack DPRA and KeratinoSens™ data; thus, predictions for these chemicals could only be performed for silane glycol where the two available in vitro assays (EpiSensA + h-CLAT) yielded consistent positive results (an incorrect result relative to the negative in vivo WoE determinations). In addition, the DPRA data for PS-6 was not classifiable, leaving inconsistent results between the h-CLAT data (positive) and the EpiSensA/KeratinoSens™ data (both negative) and therefore, failing to meet the "two out of three" criteria. Excluding the Silane glycol to allow equal data sets (16 chemicals each) improves the predictivity of the DPRA + EpiSensA + h-CLAT assays to 88% (14 of 16). When the comparison between the DPRA + EpiSensA + h-CLAT and the DPRA + KeratinoSens™ + h-CLAT was performed based on 11 test chemicals (n-HMA and EHMA removed), the predictivities were 82% (9 of 11) and 64% (7 of 11), respectively. DPRA + LuSens + h-CLAT predictions were accurate for the three positive methacrylates examined; thus, it performed equivalent to the EpiSensA in a two out of three approaches for the limited number of compounds evaluated.

5 | CONCLUSION

This study was intended to evaluate the performance of the EpiSensA on some difficult to test chemicals that have in vivo, in vitro, and *in silico* dermal sensitization data. When examining keratinocyte activation,

this study demonstrated that the EpiSensA, both alone and in the two out of three approaches, was a better predictor of dermal sensitization potential for methacrylates than the KeratinoSens™ assay. EpiSensA also accurately predicted the potency of active methacrylates as weak sensitizers. EpiSensA predicted the sensitization potential of a small subset of alkoxysilanes and siloxane better than KeratinoSens™, whereas the KeratinoSens™ assay better predicted the sensitization potential of a small subset of crop protection formulations. In addition, EpiSensA accurately identified two surfactant mixtures as sensitizers. Although only a limited set of chemicals was evaluated in the current study, these data suggest that the EpiSensA may be better at evaluating Key Event 2 (keratinocyte activation) of the skin sensitization AOP for some chemicals or perform equally well compared with the currently validated assays. The structural features (e.g., 3D epidermis model with intact stratum corneum) and experimental conditions (e.g., direct addition of test chemicals with permissible use of lipophilic vehicle and higher concentrations of test chemicals) may allow the EpiSensA to better mimic in vivo dermal sensitization studies. More work is needed to better understand the chemical properties that discern whether the EpiSensA or KeratinoSens™ assay should be used to predict sensitization potential; however, available data support the inclusion of EpiSensA as an in vitro model for contact sensitization assessment.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the test material information supplied by Dow toxicologists, Dr Robert Ellis-Hutchings, Dr Joanna Klapacz, and Dr Shawn Seidel, which was invaluable during the preparation of this manuscript. In addition, the work of the Dow Pharmacy (Mary Scherzer and Coreena Cheney) for test material procurement and shipping is greatly appreciated.

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How to cite this article: Mizumachi H, LeBaron MJ, Settivari RS, Miyazawa M, Marty MS, Sakaguchi H. Characterization of dermal sensitization potential for industrial or agricultural chemicals with EpiSensA. *J Appl Toxicol*. 2020; 1–13. https://doi.org/10.1002/jat.4076