



Application of the KeratinoSens™ assay for assessing the skin sensitization potential of agrochemical active ingredients and formulations



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ABSTRACT

Assessment of skin sensitization potential is an important component of the safety evaluation process for agrochemical products. Recently, non-animal approaches including the KeratinoSens™ assay have been developed for predicting skin sensitization potential. Assessing the utility of the KeratinoSens™ assay for use with multi-component mixtures such as agrochemical formulations has not been previously evaluated and is a significant need. This study was undertaken to evaluate the KeratinoSens™ assay prediction potential for agrochemical formulations. The assay was conducted for 8 agrochemical active ingredients (AIs) including 3 sensitizers (acetochlor, meptyldinocap, triclopyr), 5 non-sensitizers (aminopyralid, clopyralid, florasulam, methoxyfenozide, oxyfluorfen) and 10 formulations for which *in vivo* sensitization data were available. The KeratinoSens™ correctly predicted the sensitization potential of all the AIs. For agrochemical formulations it was necessary to modify the standard assay procedure whereby the formulation was assumed to have a common molecular weight. The resultant approach correctly predicted the sensitization potential for 3 of 4 sensitizing formulations and all 6 non-sensitizing formulations when compared to *in vivo* data. Only the meptyldinocap-containing formulation was misclassified, as a result of high cytotoxicity. These results demonstrate the promising utility of the KeratinoSens™ assay for evaluating the skin sensitization potential of agrochemical AIs and formulations.

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1. Introduction

Assessment of skin sensitization potential serves as an important component of chemical safety evaluation programs across multiple chemical industry sectors (i.e., agrochemicals, personal care, industrial chemicals). In the agrochemical sector, assessment of skin sensitization potential is a requirement for global registration of active ingredients (AIs) and their corresponding formulated products (EPA, 2007; SANCO, 2010). This endpoint serves specifically to establish product classification and labeling with the ultimate goal of advising on the safe handling and use practices (i.e. PPE requirements, hazard statements) to ensure protection of agricultural workers and operators who may come into contact with the product.

Historically, animal tests including Buehler, Magnusson & Kligman guinea pig (M&K) test and Local Lymph Node Assay

(LLNA) have served as the accepted methods for assessing sensitization potential of chemicals (Basketter et al., 1996; Basketter and Kimber, 2010; Boverhof et al., 2008; Gerberick et al., 2007; Kimber et al., 1999; Woolhiser et al., 2000). Today, advances in the understanding of the key events involved in the skin sensitization response have led to development of *in vitro* strategies for assessment of skin sensitization leading to possible future elimination of animal use for this purpose. The KeratinoSens™ assay is one *in vitro* test method that has demonstrated utility in predicting sensitization potential with good correlation to *in vivo* data (Emter et al., 2010; Emter et al., 2013; Natsch et al., 2011; Natsch et al., 2013). The assay functions through a luciferase reporter gene system which detects activation of the Keap-1/Nrf2 signaling pathway following covalent binding of a potential sensitizer with endogenous skin peptide (Natsch et al., 2011). The KeratinoSens™ assay has recently completed the validation trials for prediction of sensitization potential at the European Centre for the Validation of Alternative Methods (ECVAM) and a draft proposal for a new test guideline has been developed at the

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Organization for Economic Co-operation and Development (OECD, 2014).

While the utility of the KeratinoSens™ assay has been demonstrated with testing single chemicals, there is a need to expand the applicability to multi-component mixtures. Testing of multiple component mixtures such as formulations in these systems may present significant challenges since each component may influence the overall sensitization response as well as absorption or uptake of components by the test system. While challenging, successful application of *in vitro* sensitization methods for the assessment of the sensitization potential of agrochemical formulations could have a substantial impact on reducing animal use because each AI may be present in numerous formulations, each of which would otherwise need to be individually assessed for sensitization potential to meet regulatory testing requirements. This approach offers an additional opportunity in designing sustainable formulations, through its implementation in early formulation development stage.

The purpose of the current study was to confirm the utility of the KeratinoSens™ assay for a set of agrochemical AIs and extend the applicability to corresponding multi-component formulations. In this study, the performance of the KeratinoSens™ assay was investigated using eight agrochemical active ingredients and ten formulations for which *in vivo* sensitization data were available. Based on the diverse set of test materials included in this study, the results support the applicability of the KeratinoSens™ for assessing sensitization potential of agrochemical active ingredients and formulations.

2. Materials and methods

2.1. Test materials

As part of initial experiments to demonstrate technical proficiency of the conducting laboratory (i.e., The Dow Chemical Company), the proficiency test substances in the OECD KeratinoSens™ draft test guideline were evaluated for skin sensitization potential and the in-house results were compared to existing *in vivo* as well as published KeratinoSens™ assay results (Table 1). All proficiency compounds were purchased from Sigma Aldrich (St. Louis, MO).

In the main study, eight agrochemical AIs and ten corresponding multi-component formulations were assessed for skin sensitization potential using the KeratinoSens™ assay (Tables 2a and 2b). All samples were of commercial quality, obtained from Dow AgroSciences, Indianapolis and had prior *in vivo* Buehler, M&K or LLNA results. Three different types of formulations were tested in the present study: soluble concentrate (SL), emulsion concentrate (EC) and suspension concentrate (SC). In an SL type formulation, the AI is presented as a water soluble salt. In an EC type formulation, the AI is solubilized in an organic solvent to be applied as an emulsion after dilution in water. In an SC type formulation a low water soluble and high melting point AI is suspended in water.

2.2. Test procedure of the KeratinoSens™ assay

The KeratinoSens™ cell line was kindly provided by Dr. Andreas Natsch (Givaudan Schweiz AG, Switzerland). The cell-line is derived from human keratinocyte cells, HaCaT (Boukamp et al., 1988) and contains a stable insertion of a luciferase gene under the control of the antioxidant response element (ARE) of the AKR1C2 gene (Nrf2-Keap1-ARE dependent gene). Generation of the stable transgenic cell line and optimization of the test procedure have been previously described in detail (Emter et al., 2010). The KeratinoSens™ assay was performed following standard protocols, as described by Emter et al. (2010). Briefly, KeratinoSens™ cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM) culture medium with GlutaMAX™-I (Life technologies, Carlsbad, CA) supplemented with 9.1% fetal bovine serum (FBS) and 0.55 mg/mL Geneticin® (G-418; Life technologies, Carlsbad, CA) in humidified atmosphere at 37 °C and 5% CO₂. The cells were sub-cultured every 3–4 days at 85–90% confluency, for a maximum of 25 passages.

For chemical exposure, the KeratinoSens™ cells were seeded at a density of 10,000 cells per well, in 96-well plates (in medium containing 9.1% FBS). Following 24-h of culture (on the day of treatment), cell culture medium in each well was replaced with fresh medium containing 1% FBS. Stock solutions were prepared by dissolving each test substance (proficiency compounds, agrochemical AIs and agrochemical formulations) in dimethyl sulfoxide (DMSO) at a concentration of 200 mM and further serially diluted (2-fold) in DMSO to obtain 12 doses of the stock solutions (0.1–200 mM; 100× stock). These stock solutions were further diluted

Table 1

OECD recommended substances for demonstrating technical proficiency with the KeratinoSens™ test method.

Proficiency substances	CAS#	Physical form	<i>In vivo</i> classification ^a	KeratinoSens™ classification ^a	In house KeratinoSens™ results		
					EC1.5 (μM)	Cell viability ^b (%)	Classification
Isopropanol	67-63-0	Liquid	Non-sensitizer	Negative	NA	>70%	Non-sensitizer
Salicylic acid	69-72-7	Solid	Non-sensitizer	Negative	NA	>70%	Non-sensitizer
Lactic acid	50-21-5	Liquid	Non-sensitizer	Negative	NA	>70%	Non-sensitizer
Glycerol	56-81-5	Liquid	Non-sensitizer	Negative	NA	>70%	Non-sensitizer
Cinnamyl alcohol	104-54-1	Solid	Sensitizer (weak)	Positive	261.3	>70%	Sensitizer
Ethylene glycol dimethacrylate	97-90-5	Liquid	Sensitizer (weak)	Positive	59.4	>70%	Sensitizer
2-Mercaptobenzothiazole	149-30-4	Solid	Sensitizer (moderate)	Positive	93.0	>70%	Sensitizer
Methyldibromo glutaronitrile	35691-65-7	Solid	Sensitizer (strong)	Positive	15.6	>70%	Sensitizer
4-Methylaminophenol sulfate	55-55-0	Solid	Sensitizer (strong)	Positive	3.9	>70%	Sensitizer
2,4-Dinitro-chlorobenzene	97-00-7	Solid	Sensitizer (extreme)	Positive	3.8	>70%	Sensitizer

^a OECD draft proposal for testing chemicals using the KeratinoSens™ assay.

^b Cell viability at the EC1.5 determining concentration.

Table 2a

Agrochemical active ingredients tested in the current study using the KeratinoSens™ assay.

Active ingredients (AI)					
Common name (type)	CAS#	Chemical class	MW	AI conc., %	Chemical structure
Acetochlor (Herbicide)	34256-82-1	Chloroacetanilide	269.8	95.7	
Meptyldinocap (Fungicide)	131-72-6	Dinitrophenol	364.4	91.5	
Triclopyr butoxyethyl ester (Herbicide)	64700-56-7	Pyridinecarboxylic acid	356.6	95.5	
Aminopyralid triisopropanolammonium (Herbicide)	566191-89-7	Pyridinecarboxylic acid	398.3	94.5	
Clopyralid monoethanolammonium (Herbicide)	57754-85-5	Pyridinecarboxylic acid	253.1	96.2	
Florasulam (Herbicide)	145701-23-1	Triazolopyrimidine	359.3	99.2	
Methoxyfenozide (Insecticide)	161050-58-4	Diacylhydrazine	368.5	98.5	

Table 2a (continued)

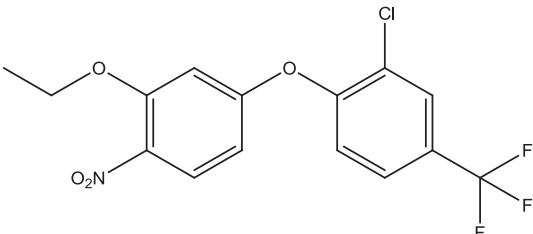
Active ingredients (AI)					
Common name (type)	CAS#	Chemical class	MW	AI conc., %	Chemical structure
Oxyfluorfen (Herbicide)	42874-03-3	Diphenyl ether	361.7	97.9	

Table 2b

Agrochemical formulations tested in the current study using the KeratinoSens™ assay.

Formulations			Application
Formulation	Formulation type	AI conc., %	
Acetochlor-EC	Emulsion concentrate (EC)	81.8	Herbicide
Meptyldinocap-EC	Emulsion concentrate (EC)	34.4	Fungicide
Triclopyr choline-SL	Soluble concentrate (SL)	54.6	Herbicide
Triclopyr butotyl-EC	Emulsion concentrate (EC)	61.8	Herbicide
Aminopyralid triisopropanolammonium-SL	Soluble concentrate (SL)	40.1	Herbicide
Clopyralid monoethanolammonium-SL	Soluble concentrate (SL)	43.2	Herbicide
Florasulam-SC	Suspension concentrate (SC)	4.9	Herbicide
Methoxyfenozide-SC	Suspension concentrate (SC)	22.8	Insecticide
Oxyfluorfen-EC-1	Emulsion concentrate (EC)	22.3	Herbicide
Oxyfluorfen-EC-2	Emulsion concentrate (EC)	22.6	Herbicide

AI: active ingredient.

25-fold into the culture medium containing 1% FBS (4× stock; working solutions). These working solutions were used for treatment with a further 4-fold dilution factor. Final testing concentrations (1×) of each compound ranged from 1 to 2000 μM. Each 96-well testing plate consisted of up to 7 test chemicals, the positive control (Cinnamic aldehyde) at five doses (4–64 μM), negative control (media with 1% DMSO; 6 wells) and one well serving as a no-cell blank. During the treatment period, all plates were covered with air-tight sealing foil (Sealing tape SI, Nunc) to avoid test substance evaporation and also to prevent cross-well contamination. Test substance treatments were performed under standard cell culture conditions for 48 h. At the end of treatment period, chemical-mediated induction in luciferase activity (SteadyGlo™; Promega, USA) and cytotoxicity (3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction test) were determined using the BMG LABTECH spectrophotometer. For each test chemical, (a) the average maximal induction of gene activity ($I_{\max}$) and (b) the average concentration inducing gene activity >50% (1.5-fold) above control values (EC1.5) were determined (as follows):

$$EC1.5 = (C_b - C_a) \times \left(\frac{1.5 - 1_a}{1_b - 1_a} \right) + C_a$$

where

C_a is the lowest concentration (μM) with >1.5-fold induction,
 C_b is the highest concentration (μM) with <1.5-fold induction,
 1_a is the average fold-induction measured at the lowest concentration with >1.5-fold induction,
 1_b is the average fold-induction at the highest concentration with < 1.5-fold induction.

2.3. Test acceptance criteria and data interpretation

All proficiency and prototypical compounds as well as agrochemical AIs were tested in at least duplicate wells for each replicate and each experiment was repeated on at least two separate days (independent replicates). Each replicate was considered acceptable when the following criteria were met: (a) the positive control induced a dose-dependent increase in luciferase activity, with EC1.5 between 7 and 30 μM and maximum luciferase induction at 64 μM was between 2- and 8-fold (b) average coefficient of variability of the luminescence reading for the solvent-control wells was below 20%. A test substance was considered positive for skin sensitization potential within the KeratinoSens™ assay when the following conditions were met: (a) the $I_{\max}$ was higher than 1.5-fold as compared to the solvent control, (b) the cell viability was higher than 70% at the lowest concentration with gene induction above 1.5-fold, (c) the EC1.5 was less than 1000 μM, (d) apparent overall dose-response for luciferase induction. When these criteria were not met, the response was considered to be negative.

2.4. Study design for testing agrochemical formulations

Unlike the approach for single substances, testing at a single specific molar concentration is not possible in the case of formulations due to their multi-component nature. In the present study, two approaches were evaluated for dose selection for testing of the agrochemical formulations.

2.4.1. Approach 1: Based on AI concentration

In Approach 1, the dose selection was based on the molecular weight (MW) and concentration of AI (ranged from 4.9% to

81.8%) in the formulation without regard to the concentrations of the co-formulants. This approach resulted in testing the AI at the KeratinoSens™ assay SOP-prescribed (for single substances) dose concentration; however, resulted in testing the co-formulants at higher concentrations. As some co-formulants may possess inherent cytotoxicity potential, testing at higher concentrations may contribute to non-specific stress responses and false predictions for the formulation, as well as pose test substance compatibility issues. Consistently, earlier studies suggested poor performance of the KeratinoSens™ assay when tested at higher concentrations due to non-specific induction of the stress response leading to false positives (Emter et al., 2010). For these reasons a second approach was implemented in parallel.

2.4.2. Approach 2: Based on whole formulation

In Approach 2, doses were selected based on the total formulation (considered formulation as single entity) rather than concentrations of individual components. In this approach, a common MW of 400 was assumed, consistent with approximate MW of the agrochemical AIs. Thus, in Approach 2, each formulation was tested in the range from 0.4 to 800 ppm (whole formulation) with no consideration for AI concentration, consequently avoiding testing the co-formulants at higher concentrations. This approach permitted testing formulations within the KeratinoSens™ SOP suggested dose concentration range, potentially below a range where non-specific stress responses might be expected.

To determine the optimal testing approach for future formulations testing, all ten formulations were evaluated using Approaches 1 and 2 and the results were compared to existing *in vivo* data. The KeratinoSens™ results were also compared to human skin sensitization data, when available.

3. Results

3.1. Laboratory proficiency with the KeratinoSens™ assay

To demonstrate laboratory technical proficiency and reproducibility with the KeratinoSens™ assay, the ten proficiency substances listed in OECD draft test guidelines were evaluated (Table 1). The OECD list consists of six skin sensitizers and four non-sensitizers. The in-house results for all the proficiency list of compounds were consistent with the results reported in OECD test guidelines and published literature (Table 1). All the tested sensitizers exhibited dose-dependent increases in luciferase activity above the threshold of 1.5-fold at concentrations below 1000 µM and had IC30 (concentration leading to 30% cytotoxicity) values

greater than the EC1.5 response. The non-sensitizers either did not induce luciferase activity or induced the response only at cytotoxic concentrations (cell viability <70%). These results demonstrated technical proficiency of the laboratory to efficiently conduct the KeratinoSens™ assay.

3.2. Evaluation of agrochemical AIs in the KeratinoSens™ assay

To evaluate the performance of the KeratinoSens™ assay, eight agrochemical AIs were assessed for skin sensitization potential (Table 3). Amongst the selected AIs, three were *in vivo* sensitizers (acetochlor, meptyldinocap and triclopyr) and five were non-sensitizers (aminopyralid, clopyralid, florasulam, methoxyfenozide, and oxyfluorfen).

For the agrochemical AIs, the KeratinoSens™ assay correctly predicted all the tested sensitizers and non-sensitizers, when compared to *in vivo* data (Fig. 1). The EC1.5 values for the tested sensitizers, acetochlor, meptyldinocap and triclopyr butoxyethyl ester were, 1.43, 1.79 and 71.25 µM, respectively (i.e. less than 1000 µM) whereas the EC1.5 values were >1000 µM for the non-sensitizers (Table 3). Oxyfluorfen was borderline positive in one replicate ($I_{\max}$: 1.51-fold; EC1.5: 810 µM) and borderline negative in a second replicate ($I_{\max}$: 1.49-fold; EC1.5: NA). In general, such a response would trigger additional testing; however, in both replicates oxyfluorfen did not exhibit a prototypical dose response for luciferase induction (Fig. 1h), as observed for typical skin sensitizers. Therefore, based on assay interpretation criteria and expert judgment, oxyfluorfen was considered as a non-sensitizer in the KeratinoSens™ assay.

3.3. Evaluation of agrochemical formulations in the KeratinoSens™ assay

The applicability domain and prediction performance of the KeratinoSens™ assay was next evaluated for agrochemical formulations with AIs corresponding to those assessed in the earlier study. Ten formulations were evaluated (Table 4) using Approaches 1 and 2, as described in Section 2.

Using Approach 1, the KeratinoSens™ assay correctly predicted skin sensitization potential of two of the four sensitizers and three of the four non-sensitizers compared to *in vivo* data (Table 4). Amongst the tested sensitizers, the KeratinoSens™ assay under-predicted meptyldinocap-EC and triclopyr choline-SL as potential non-sensitizers. Although both meptyldinocap-EC and triclopyr choline-SL exhibited a dose-dependent increase in luciferase activity above the threshold of 1.5-fold (consistent with response for a prototypical sensitizer), they exhibited a steep dose

Table 3
KeratinoSens™ assay results for agrochemical active ingredients.

Test material (AI)	<i>In vivo</i> data classification	KeratinoSens™ assay results		
		EC1.5 (µM)	Cell viability (%) ^a	Classification
Acetochlor	Sensitizer (M&K ^d)	1.43	>70%	Sensitizer
Meptyldinocap	Sensitizer (LLNA ^e)	1.79	>70%	Sensitizer
Triclopyr butoxyethyl ester	Sensitizer (Buehler ^f)	71.25	>70%	Sensitizer
Aminopyralid triisopropanolammonium	Non-sensitizer (M&K)	NA ^b	>70%	Non-sensitizer
Clopyralid monoethanolammonium	Non-sensitizer (M&K)	NA	>70%	Non-sensitizer
Florasulam	Non-sensitizer (M&K)	NA	>70%	Non-sensitizer
Methoxyfenozide	Non-sensitizer (M&K)	NA	>70%	Non-sensitizer
Oxyfluorfen	Non-sensitizer ^c (LLNA)	810	>70%	Non-sensitizer

^a Cell viability at the EC1.5 determining concentration.

^b Not applicable.

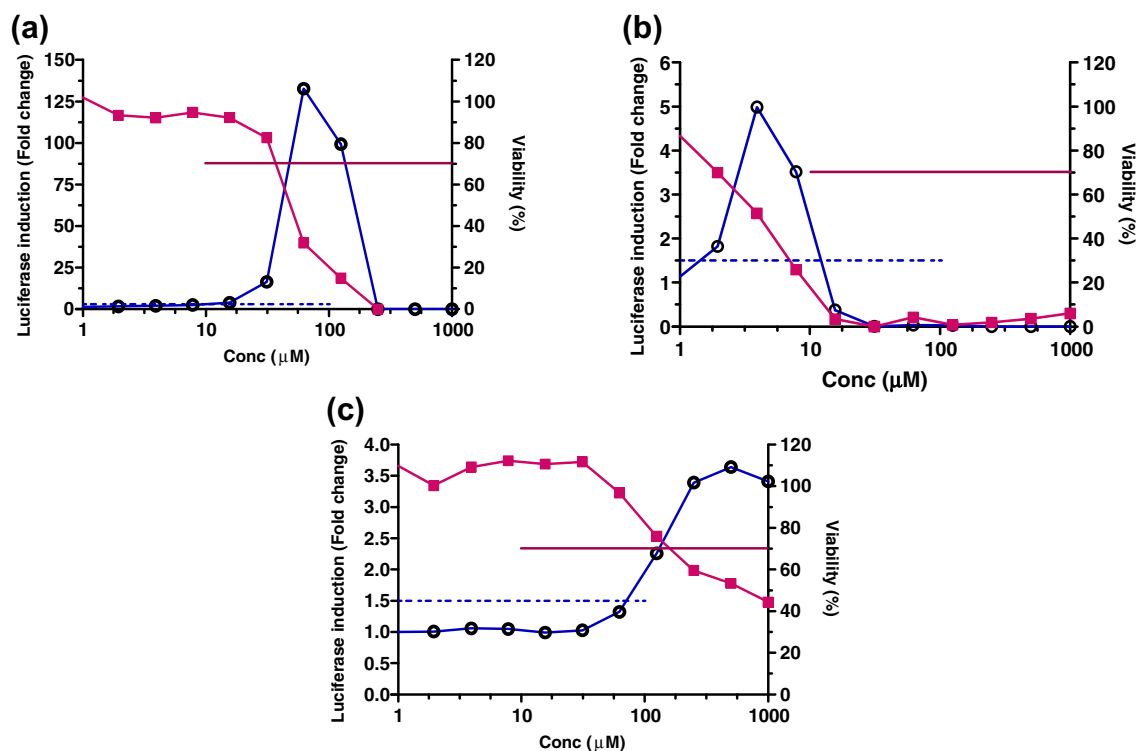
^c Borderline response.

^d Magnusson and Kligman skin sensitization test.

^e Local lymph node assay.

^f Buehler skin sensitization test.

Sensitizers



Non-sensitizers

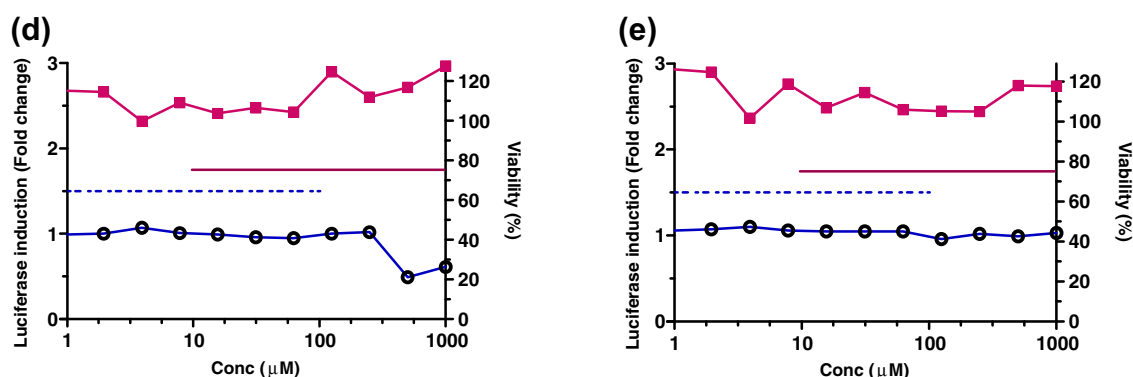


Fig. 1. Luciferase induction (open circles) and cellular viability (closed squares) for agrochemical active ingredients: (a) acetochlor, (b) meptyldinocap, (c) triclopyr butoxyethyl ether, (d) aminopyralid, (e) clopyralid, (f) florasulam, (g) methoxyfenozide and (h) oxyfluorfen are shown.

response for cytotoxicity such that at EC1.5, cell viability was less than 70%. As a result, meptyldinocap-EC and triclopyr choline-SL were misclassified as a potential non-sensitizers. Conversely, florasulam-SC, which was a non-sensitizer *in vivo*, was classified as a sensitizer in Approach 1 as it exhibited a dose-dependent increase in luciferase activity and cell viability at EC1.5 was greater than 70% (Table 4). Likewise, oxyfluorfen-EC-1 and oxyfluorfen-EC-2 formulations, which had equivocal response in *in vivo* studies, were identified as potential sensitizers in Approach 1. As the respective AIs for meptyldinocap-EC, triclopyr choline-SL, florasulam-SC and oxyfluorfen-ECs were correctly classified in the present study, it is reasonable to assume that the co-formulants contributed to the false predictions of the formulations, when tested at higher concentrations.

Using Approach 2, the KeratinoSens™ assay correctly predicted three of the four sensitizers and all non-sensitizers, consistent with *in vivo* results (Fig. 2). Similar to the observations in Approach 1,

meptyldinocap-EC exhibited a dose dependent increase in luciferase activity above the threshold of 1.5-fold; however, a steep cytotoxic response (cell viability <70% at EC1.5) was also observed (Fig. 2c). Based on these results, meptyldinocap-EC would be misclassified as a potential non-sensitizer. The KeratinoSens™ assay classified oxyfluorfen-containing formulations, oxyfluorfen-EC-1, and oxyfluorfen-EC-2 as non-sensitizers, consistent with the classification for the respective AI, oxyfluorfen.

Overall, these results demonstrated that the KeratinoSens™ assay can be implemented to test agrochemical formulations and Approach 2 provided better prediction when compared to *in vivo* results. Of the tested compounds, reliable human skin sensitization data was available only for an emulsified product containing acetochlor (Dong et al., 2014), which caused skin sensitization. This is consistent with the KeratinoSens™ study results noted in the present study. No reports of sensitization were observed for meptyldinocap, however, dinocap, which is a mixed isomer product

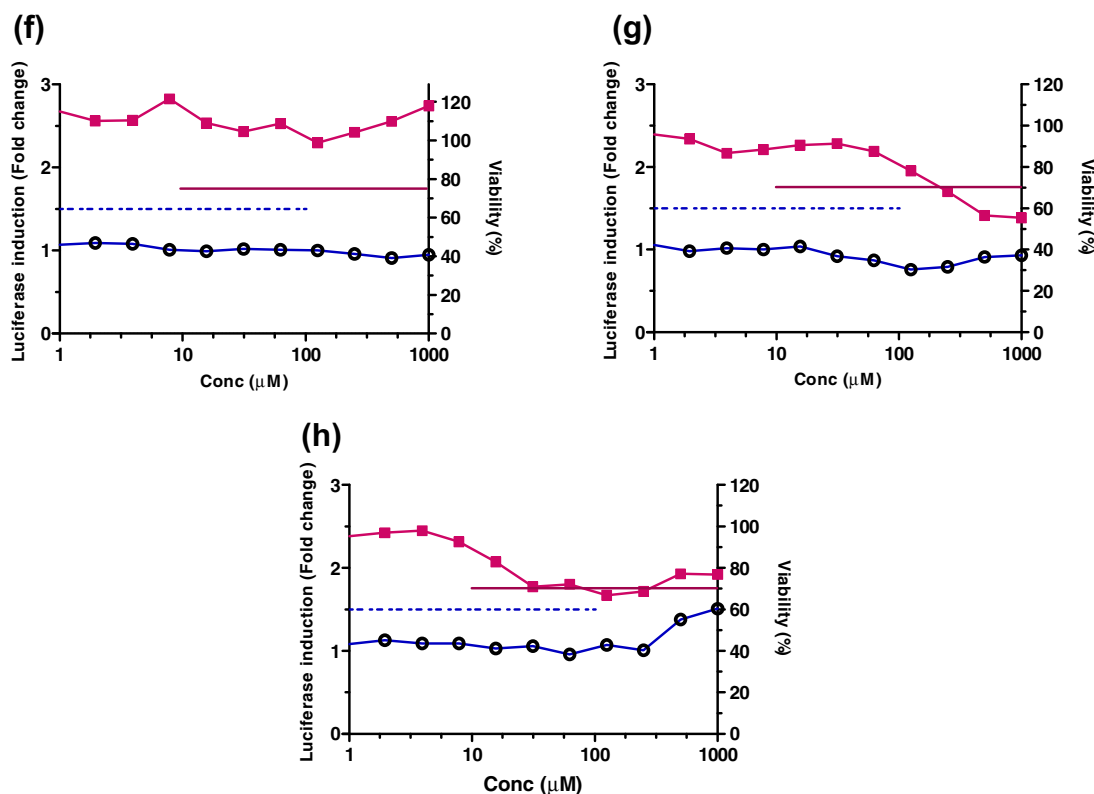


Fig. 1 (continued)

Table 4
KeratinoSens™ assay results for agrochemical formulations.

Test material (formulations)	<i>In vivo</i> data (formulations)	KeratinoSens™ assay data for AI	KeratinoSens™ assay data (Approach 1)	KeratinoSens™ assay data (Approach 2)
Acetochlor-EC	Sensitizer (Buehler ^a)	Sensitizer	Sensitizer	Sensitizer
Meptyldinocap-EC	Sensitizer (LLNA ^b)	Sensitizer	Non-sensitizer	Non-sensitizer
Triclopyr choline-SL	Sensitizer (LLNA)	Sensitizer	Non-sensitizer	Sensitizer
Triclopyr butotyl-EC	Sensitizer (Buehler)	Sensitizer	Sensitizer	Sensitizer
Aminopyralid triisopropanolammonium-SL	Non-sensitizer (M&K ^c)	Non-sensitizer	Non-sensitizer	Non-sensitizer
Clopyralid monoethanolammonium-SL	Non-sensitizer (LLNA)	Non-sensitizer	Non-sensitizer	Non-sensitizer
Florasulam-SC	Non-sensitizer (LLNA)	Non-sensitizer	Sensitizer	Non-sensitizer
Methoxyfenozide-SC	Non-sensitizer (M&K)	Non-sensitizer	Non-sensitizer	Non-sensitizer
Oxyfluorfen-EC-1	Equivocal (LLNA)	Non-sensitizer	Sensitizer	Non-sensitizer
Oxyfluorfen-EC-2	Equivocal (M&K)	Non-sensitizer	Sensitizer	Non-sensitizer

^a Buehler skin sensitization test.

^b Local lymph node assay.

^c Magnusson and Kligman skin sensitization test.

that contains meptyldinocap was positive for skin sensitization in humans (Hayes and Laws, 1991). These observations are consistent with the KeratinoSens™ study results noted in the present study.

4. Discussion

This study was conducted to assess the performance of the *in vitro* KeratinoSens™ assay for the prediction of skin sensitization potential of agrochemical active ingredients and corresponding formulations. The KeratinoSens™ assay, as well as other existing *in vitro* assays, has been characterized using prototypical single chemicals, but their application for testing agrochemical formulations (i.e. mixtures) has not been previously addressed. Unlike

single chemicals, formulations are complex multi-component mixtures which consist of AIs and co-formulants (e.g. surfactants, dry fillers/carriers, solvents, thickeners, biocides, anti-freezers, odorants, stabilizers, dyes, etc.), each of which may possess potential for sensitization and/or cytotoxicity. The present study was designed to validate and extend applicability of the KeratinoSens™ assay for predicting skin sensitization potential of complex agrochemical formulations.

Prior to testing the agrochemical samples, 10 prototypical sensitizers and non-sensitizers (OECD proficiency compounds) were evaluated to confirm proper conduct of the KeratinoSens™ assay in-house. The KeratinoSens™ assay was next utilized to investigate the sensitization potential of agrochemical AIs. In this assessment, all tested sensitizing and non-sensitizing AIs were correctly

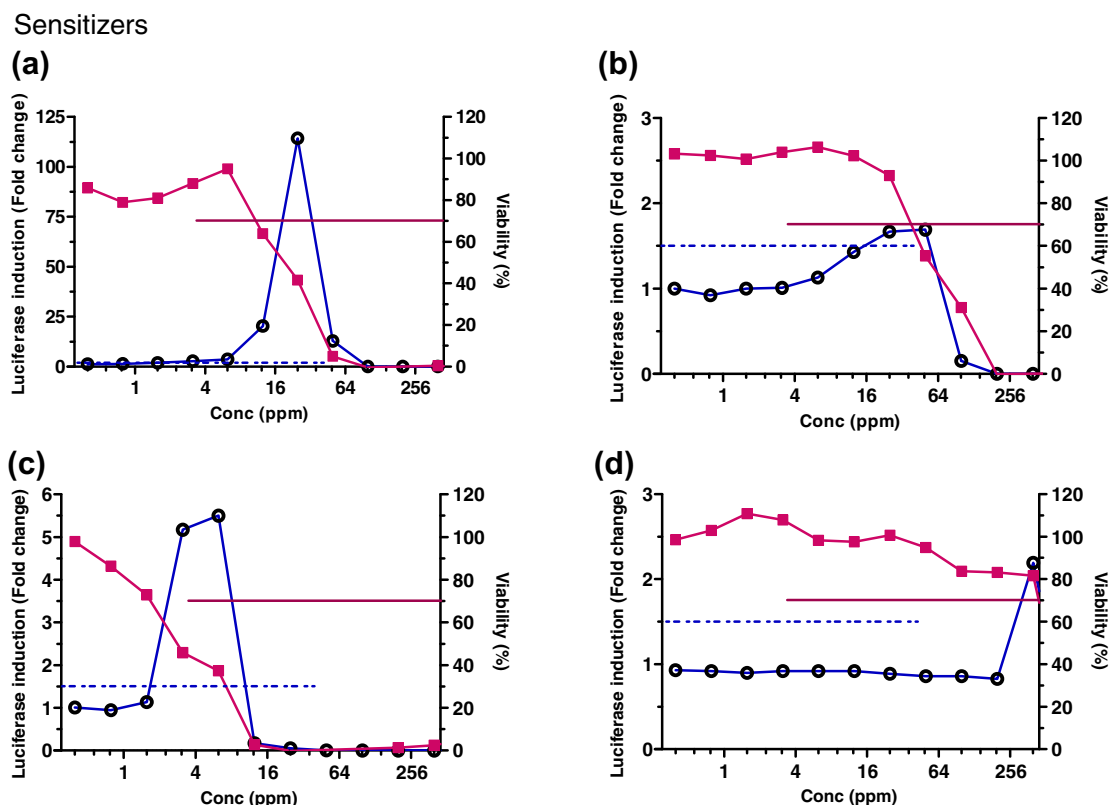


Fig. 2. Luciferase induction (open circles) and cellular viability (closed squares) for agrochemical formulations testing using Approach 2: (a) acetochlor-EC, (b) triclopyr butotyl-EC, (c) meptyldinocap-EC, (d) triclopyr choline-SL, (e) aminopyralid triisopropanolammonium-SL, (f) clopyralid monoethanolammonium-SL, (g) florasulam-SC, (h) methoxyfenozide-SC, (i) oxyfluorfen-EC-1 and (j) oxyfluorfen-EC-2 are shown.

predicted compared to existing *in vivo* results (Table 3). Of note, in previous *in vivo* studies oxyfluorfen was a borderline negative in M&K (26% were positive versus the 30% required for a positive classification) and had inconsistent responses with borderline results in the LLNA interlaboratory evaluation (SI values ranging from 2.3 to 5.4) (Boverhof et al., 2008). Based on a weight of evidence assessment, previous expert judgment was applied to conclude that oxyfluorfen is negative for sensitization potential. In the KeratinoSens™ assay, oxyfluorfen did not exhibit a dose response for luciferase induction as noted for prototypical sensitizers. Oxyfluorfen exhibited a borderline positive response in one replicate and a borderline negative response in the second replicate. Therefore, based on the assay interpretation criteria and expert judgment, oxyfluorfen was interpreted as a non-sensitizer in the KeratinoSens™ assay. These results demonstrate good performance of the KeratinoSens™ assay for predicting agrochemical AIs and highlight that in the case of both *in vivo* and *in vitro* studies, application of expert judgment and weight of evidence approaches will continue to play an important role in chemical evaluations.

A novel extension of the study was to explore performance of the KeratinoSens™ assay for agrochemical formulations. In Approach 1, it was assumed that the sensitization potential of the formulation is primarily driven by the AI. Therefore, in this approach, the dose selection for each formulation was based solely on the concentration of AI in the formulation (4.9–81.8%) without regards to the concentrations of the co-formulants. As a result, each AI within the formulation was tested at the SOP-suggested dose concentrations; however, in this approach the doses of the co-formulants were higher than in Approach 2. This approach yielded more false predictions than Approach 2. Two of the four sensitizers (meptyldinocap-EC and triclopyr choline-SL) were identified as false negatives and three of the non-sensitizers

(florasulam-SC, oxyfluorfen-EC-1 and oxyfluorfen-EC-2) were identified as false positives. These false predictions were likely due to the inherent cytotoxicity or non-specific stress response resulting from the co-formulants, which when tested at higher concentrations, led to misclassification of the formulation. These conclusions were apparent when the results of AI and formulations tested in Approach 1 were compared (Table 4). Furthermore, Fig. 3 illustrates the KeratinoSens™ assay results for florasulam-SC when tested using Approach 1 (identified as a sensitizer) and 2 (identified as a non-sensitizer). Interestingly using Approach 1, false positive results for florasulam-SC were observed at non-cytotoxic concentrations perhaps indicating other non-specific mechanisms may operate to activate Nrf2 pathway at higher concentrations. These findings are consistent with previous publications which suggested poor prediction performance for the KeratinoSens™ assay when the chemicals (for single compounds) are tested at higher concentrations than that prescribed in the assay SOP (Emter et al., 2010) and further warrant caution while selecting testing concentrations of formulations.

In Approach 2, it was assumed that the co-formulants may possess inherent sensitization and/or cytotoxic potential, and therefore, each formulation was considered as a single entity and was tested within assay prescribed dose concentrations. This approach ensured that neither the AI nor the co-formulants were tested at higher than SOP-recommended concentrations. Results from this approach were consistent with *in vivo* classifications, with the exception of meptyldinocap-EC, which was a false negative in the KeratinoSens™ assay due to excess cytotoxicity potentially resulting from the co-formulants. Meptyldinocap-EC exhibited a dose-dependent increase in luciferase activity >1.5-fold; however, a steep decline in the cellular viability was also observed leading to the false-negative prediction. For such formulations containing

Non-sensitizers

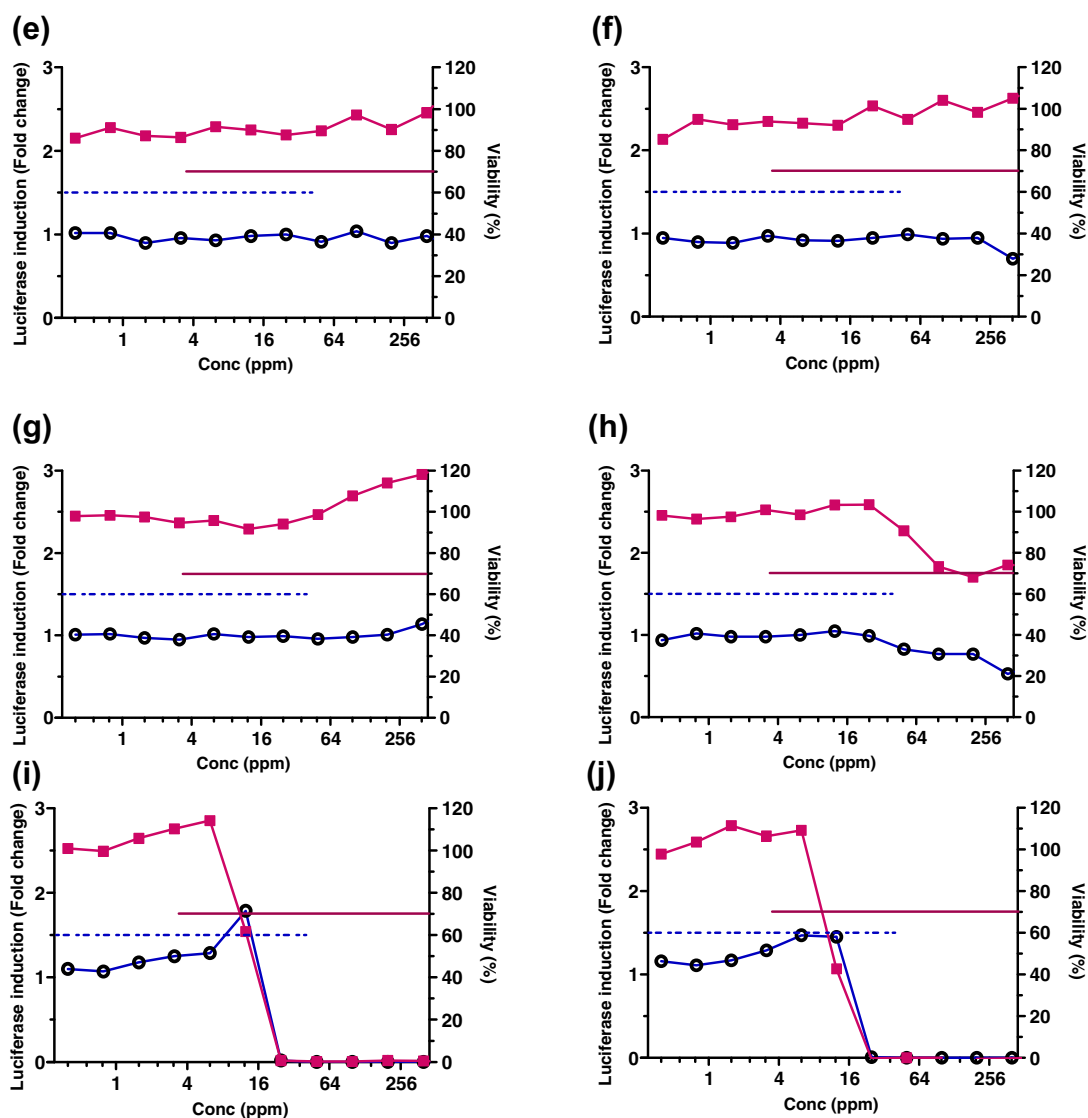


Fig. 2 (continued)

highly cytotoxic co-formulants, earlier reports recommended to perform fractionation of the mixtures (column chromatography, distillation of volatile compounds etc) followed by testing individual fractions using the KeratinoSens™ assay (Andres et al., 2013; Ball et al., 2011). The draft OECD test guideline for the KeratinoSens™ assay suggests implementing a narrow dose concentration range (1.33 or 1.41-fold) for such compounds to delineate luciferase activity at non-cytotoxic concentrations. Alternatively, testing such compounds in other *in vitro* test procedures may support a weight of evidence determination.

Overall, these results suggested that the KeratinoSens™ assay provided good predictions for testing complex agrochemical formulations and Approach 2 provided better prediction capabilities compared to *in vivo* data. In fact, Approach 2 is consistent with recently released draft OECD test guideline, (2014) for the KeratinoSens™ assay to test chemicals which have no defined MW. Per the draft guideline, the maximal test concentration (100× stock) is prepared to a default concentration of 40 mg/ml (4% w/v or 40000 ppm) (Andres et al., 2013) without considering AI concentration within the formulation. This dose selection is based on an assumed MW of 200, which is an approximate average

MW for cosmetic ingredients and plant extracts. In the present study, a similar approach was applied with the exception that a common MW of 400 was assumed, consistent with approximate MW of the agrochemical AIs. The KeratinoSens™ assay demonstrated good predictions for all three different types of formulations (SL, EC and SC) evaluated in the present study.

While the KeratinoSens™ assay demonstrated utility for the agrochemicals assessed in the present study, it is generally agreed that a single test method may not always recapitulate complex *in vivo* systems (Jaworska et al., 2011; MacKay et al., 2013; Pirone et al., 2014). The KeratinoSens™ assay assesses one of the earliest key events of sensitization, i.e. modification of the skin proteins by the sensitizing ingredient (Andres et al., 2013; Delaine et al., 2011; Emter et al., 2013; Natsch et al., 2011). Additional *in vitro* test methods such as direct peptide reactivity assay (DPRA), Myeloid U937 Skin Sensitization Test (MUSST), and human cell line activation test (h-CLAT) assays are being developed to assess the other sequential chain of key events of the sensitization adverse outcome pathway (AOP) (Ashikaga et al., 2006; Gerberick et al., 2004; Natsch and Gfeller, 2008; Nukada et al., 2012; Python et al., 2007; Sakaguchi et al., 2006; Sakaguchi et al.,

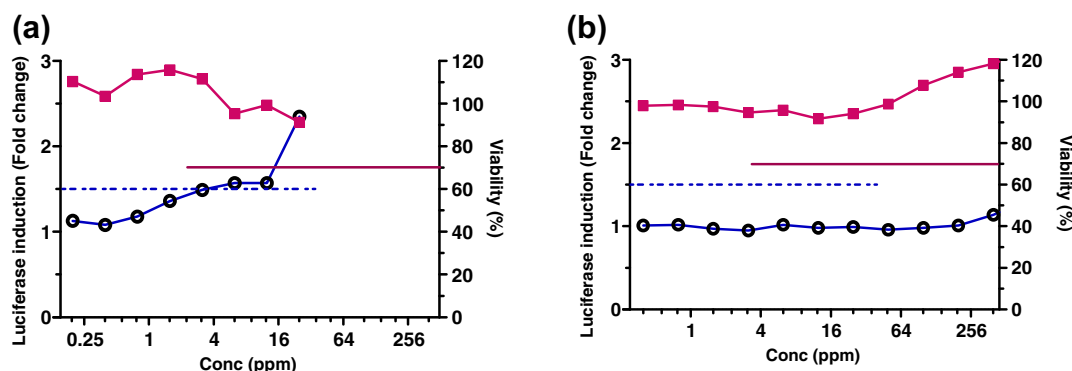


Fig. 3. Comparison of KeratinoSens™ assay results for florasulam-SC between Approach-1 and -2. Florasulam-SC induced luciferase activity (open circles) at higher concentrations in Approach-1; however, did not induce in Approach-2. Cellular viability (closed squares) in both approaches was >70% at all concentrations tested.

2010). Each of these test methods has prediction performances of around 80% to distinguish sensitizers from non-sensitizers; however, the methods are not adequate for sensitization potency characterization. While expert-based 'weight of evidence' approaches are routinely used to qualitatively integrate and interpret sensitization data, promising quantitative approaches such as the Bayesian network models are emerging, which enable integration of data from multiple sources (chemical's structure, physicochemical properties, and *in vitro* test results) to predict skin sensitization potency, consistent with murine LLNA (Jaworska et al., 2013; Jaworska et al., 2011; Patlewicz et al., 2014; Pirone et al., 2014; Tollefsen et al., 2014). In instances of incomplete or uncertain data, Bayesian networks provide a logical probabilistic framework for guiding decisions on the classification of a substance or the need for additional testing (Pirone et al., 2014). Recently, a toxicokinetic and toxicodynamic (TKTD) model has been proposed which provides the relationship between exposure (concentration at the site of the Molecular Initiating Event (MIE)) and likelihood and/or severity of the adverse outcome to determine safe exposure to a chemical (MacKay et al., 2013). While these models are still under development for predicting single compounds, extending their applications to formulations may present exciting opportunities.

5. Conclusions

This proof-of-concept study indicates promising application of the KeratinoSens™ assay for predicting the skin sensitization potential of the selected agrochemical AIs and their formulations. Two different approaches were evaluated for the assessment of agrochemical formulations in the KeratinoSens™ assay. The Approach 2 which assumed a common MW provided better prediction performance compared to *in vivo* data. The high concordance observed for AIs and their formulations with *in vivo* data (100 and 90%, respectively) strongly supports utilization of the KeratinoSens™ test as a key component in developing an animal-free testing approach for assessment of the skin sensitization potential of agrochemical formulations. This study does not cover all types of agricultural formulations and therefore future work needs to be done to evaluation a broader range of formulation types in the KeratinoSens™ assay.

Conflict of interest

The authors of this article are employed by The Dow Chemical Company or Dow AgroSciences, LLC, which produce the active ingredients and formulations tested and also funded this work.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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