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Application of Non-animal Test Methods and Defined Approaches to Skin Sensitization Assessment of Isothiazolinone Compounds

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National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences
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List of Acronyms and Abbreviations ¶

ANN	Artificial neural network
AOP	Adverse outcome pathway
Avg	Average
BBIT	1,2-Benzisothiazol-3(2h)-one, 2-butyl
BCF	Bioconcentration factor
BIT	1,2-Benzisothiazolin-3-one
BP	Boiling point
CASRN	Chemical Abstracts Service Registry Number
CD54	Cluster of Differentiation 54, a cell surface protein and intercellular adhesion molecule upregulated upon dendritic cell activation
CD86	Cluster of Differentiation 86, a cell surface glycoprotein and co-stimulatory molecule upregulated upon dendritic cell activation
CMIT	5-Chloro-2-methyl-4-isothiazolin-3-one
CMIT/MIT	Mixture of 5-Chloro-2-methyl-4-isothiazolin-3-one and 2-Methyl-4-isothiazolin-3-one
Cys	Cysteine
DAs	Defined approaches
DASS	Defined approaches for skin sensitization
DCs	Dendritic cells
DCOIT	4,5-Dichloro-2-octyl-3(2h)-isothiazolinone
DPRA	Direct Peptide Reactivity Assay
EC1.5 or 3	Effective concentration of a test substance that produces a stimulation index (SI) of 1.5-fold or 3-fold compared to the vehicle control
EC150	Effective concentration of a test substance that produces a 150% increase in the expression of the CD86 cell surface marker
EC200	Effective concentration of a test substance that produces a 200% increase in expression of the CD54 cell surface marker
EPA	U.S. Environmental Protection Agency
GHS	Globally Harmonized System of Classification and Labelling of Chemicals

GPMT	Guinea pig maximization test
h-CLAT	Human cell line activation test
IC50	Concentration of a test substance that produces 50% inhibition of the response compared to vehicle controls
Imax	Maximum induction
IT	Isothiazolinone
LLNA	Murine local lymph node assay
Log_{10} P	Base 10 logarithm of the oil:water partition coefficient
Log_{10} S	Base 10 logarithm of water solubility
Lys	Lysine
MIT	2-Methyl-4-isothiazolin-3-one
MP	Melting point
MW	Molecular weight
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NTP	National Toxicology Program
OECD	Organization for Economic Co-operation and Development
OIT	2-n-Octyl-4-isothiazolin-3-one
OPERA	Open Structure-activity/property Relationship App
QSAR	Quantitative structure-activity relationships
SI	Stimulation index
SMILES	Simplified molecular information line entry system
TG	Test guideline
UN	United Nations
VP	Vapor pressure

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*The U.S. Environmental Protection Agency (EPA) is one of the seven member agencies on the ICCVAM SSEG. However, EPA members were recused from this review given their separate evaluation of this information during the risk assessment process and therefore are not listed above.

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Executive Summary ¶

Integrated approaches to assessing skin sensitization potential and assigning potency category leverage the combination of multiple methods to overcome the limitations of individual tests. Approaches that use predetermined data sources with fixed data interpretation procedures to arrive at an outcome without the use of expert judgment are referred to as defined approaches (DAs). This report provides the performance of individual in chemico, in vitro, and in silico methods for predicting skin sensitization potency in comparison to murine local lymph node assay (LLNA) results, and evaluates two versions of the Shiseido Artificial Neural Network (ANN) DA for the prediction of skin sensitization potency. The ANNs use combinations of non-animal tests that align with multiple key events in the adverse outcome pathway for skin sensitization. The test substances for this case study were six isothiazolinone (IT) compounds:

- 4,5-Dichloro-2-octyl-3(2h)-isothiazolinone (DCOIT)
- 5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one (CMIT/MIT mixture)
- 2-n-Octyl-4-isothiazolin-3-one (OIT)
- 2-Methyl-4-isothiazolin-3-one (MIT)
- 1,2-Benzisothiazolin-3-one (BIT)
- 1,2-Benzisothiazol-3(2h)-one, 2-butyl (BBIT)

The IT compounds were tested using three non-animal skin sensitization tests described by internationally harmonized test guidelines issued by the Organisation for Economic Co-operation and Development (OECD): direct peptide reactivity assay (DPRA, TG442C), KeratinoSens ™ (TG442D), and human cell line activation test (h-CLAT, TG442E). Skin sensitization hazard was also predicted by in silico read-across algorithms in the OECD QSAR Toolbox. The LLNA data were curated based on a report submitted by Dow and a literature search performed by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, evaluated for study quality, and used to assign a representative in vivo potency value.

The skin sensitization hazard results showed that each of the individual non-animal test methods, as well as the in silico tool, classified all of the IT compounds as sensitizers, which is concordant with LLNA results. A potency evaluation using the individual in chemico and in vitro methods was also performed by ranking the substances using each test method (the in silico read-across results were used for hazard classification only and thus did not provide potency information). KeratinoSens and h-CLAT produced a similar ranking to that for the

LLNA. Peptide depletion values from DPRA were too similar to be useful for ranking the six IT compounds for skin sensitization potency.

The two versions of the Shiseido ANN DA (one relying on DPRA and h-CLAT only and the other relying on DPRA, h-CLAT, and KeratinoSens) provided quantitative values for the effective concentration at 3-fold induction (EC3) as outputs. The potency rankings based on the EC3 values predicted by the two ANN DAs were similar to one another and were also similar to those derived from the LLNA. The quantitative EC3 values predicted by the DAs were within 30-fold of the LLNA values for all IT compounds.

1.0 Introduction

Numerous non-animal alternatives for skin sensitization hazard assessment have been developed and are at various stages of evaluation (Ezendam et al. 2016, Mehling et al. 2012). Because skin sensitization is a complex process, it is unlikely that any individual alternative method will completely replace current animal tests. In fact, even the in vitro and in chemico methods that have been adopted as international test guidelines are not yet recommended as stand-alone replacements for animal test methods (OECD 2018a, b; 2019). Thus, a number of approaches to integrate the information from multiple non-animal methods as a way to overcome the limitations of individual tests and more accurately assess the potential for skin sensitization have been evaluated and compared to one another (Kleinstreuer et al. 2018). These approaches, which preclude the use of expert judgement by applying fixed data interpretation procedures to specific data streams, are referred to as "defined approaches" or "DAs." These DAs use combinations of non-animal tests that align with key events in the adverse outcome pathway for skin sensitization (OECD 2012).

1.1 Background

In partnership with the IT Task Force of the American Chemistry Council, the U.S. Environmental Protection Agency (EPA) nominated six isothiazolinone (IT) compounds (**Table 1**) to the National Toxicology Program (NTP) for testing in non-animal skin sensitization test methods. IT Task Force members donated the compounds for testing. The EPA will evaluate these data for use as a case study in ranking the potency of the six IT compounds and for performing quantitative risk assessment for these substances.

Table 1 Isothiazolinone Compounds Nominated for Testing

Common Name	Chemical Name	CASRN	Product Name	Donor	% Active Ingredient
DCOIT	4,5-Dichloro-2-octyl-3(2h)-isothiazolinone	64359-81-5	KATHON 287T Industrial Microbicide	Dow	99.3
CMIT/MIT	Mixture of 5-Chloro-2-methyl-4-isothiazolin-3-one and 2-Methyl-4-isothiazolin-3-one	55965-84-9	MERGAL MITZ	Troy Corporation	14.2
OIT	2-n-Octyl-4-isothiazolin-3-one	26530-20-1	ACTICIDE OIT	Thor	98.13
MIT	2-Methyl-4-isothiazolin-3-one	2682-20-4	KORDEK 573F Biocide	Dow	50.8
BIT	1,2-Benzisothiazolin-3-one	2634-33-5	MERGAL BIT Technical	Troy Corporation	85.2
BBIT	1,2-Benzisothiazol-3(2h)-one, 2-butyl	4299-07-4	VANQUISH 100	Lonza	98.4

Abbreviations: CMIT = 5-Chloro-2-methyl-4-isothiazolin-3-one

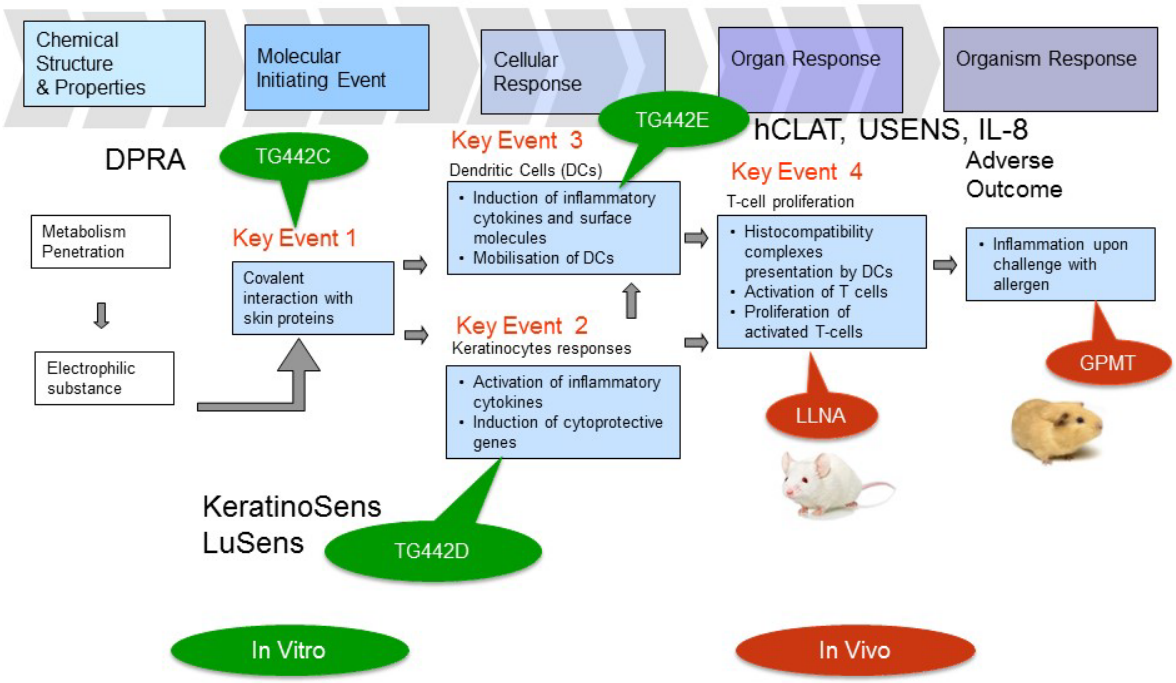
1.1.1 Adverse Outcome Pathway (AOP) for Skin Sensitization with Key Events as Targets of Alternative Method Development

An AOP is a conceptual framework constructed from existing knowledge that relates exposure of a type of toxic substance to subsequent molecular and cellular changes that in turn result in illness or injury to an individual or population (OECD 2012). The AOP for skin sensitization initiated by covalent binding to proteins (**Figure 1**) includes four key events with well-accepted biological significance: 1) binding of haptens to endogenous proteins in the skin, 2) keratinocyte activation, 3) dendritic cell activation, and 4) proliferation of antigen-specific T cells. The construction of the AOP for skin sensitization has prompted test method developers and users to align the available and conceivable methods with the key events of the AOP (Reisinger et al. 2015). Designers of defined approaches and integrated approaches to testing and assessment use the AOP as a framework to design strategies covering different multiple key events (OECD 2016). Assessment strategies using multiple methods are valuable for overcoming the limitations of the individual methods.

Figure 1 shows the association of the non-animal tests performed for this case study with the key events of the AOP. The non-animal tests include the in chemico direct peptide reactivity

assay (DPRA; OECD 2019), and the in vitro cell-based methods, KeratinoSens ™ (OECD 2018a) and human cell line activation test (h-CLAT; OECD 2018b). In silico read-across predictions (e.g. the QSAR Toolbox) cover the entire AOP because they are based on responses from in vivo methods, the murine local lymph node assay (LLNA) (OECD 2010) and guinea pig tests (OECD 1992).

Figure 1 Adverse Outcome Pathway for Skin Sensitization Caused by Covalent Binding to Proteins



Abbreviations: GPMT = Guinea Pig Maximization Test; TG = test guideline.

1.2 Objective

This report summarizes the in chemico, in vitro, in vivo, and in silico skin sensitization data and physicochemical properties for six isothiazolinone compounds and the integration of these data using defined approaches (DAs). This analysis is proposed as a case study in ranking the potency of these compounds and performing quantitative risk assessment. The report provides the performance of individual in chemico, in vitro, and in silico methods for predicting skin sensitization potency as determined by comparison to the murine local lymph node assay (LLNA). It also includes an evaluation of two DAs for the prediction of skin sensitization potency. The DAs evaluated include the Shiseido Artificial Neural Network Models "Model 1" and "Model 4" as published in Hirota et al. (2015), which rely on DPRA and h-CLAT or DPRA, h-CLAT, and KeratinoSens, and are referred to here as ANN D_hC and ANN D_hC_KS, respectively.

2.0 Methods

2.1 In Chemico and In Vitro Data Generated for This Project

Burleson Research Technologies, Inc., the NTP contract laboratory for immunotoxicity testing, tested the six isothiazolinone compounds using DPRA, KeratinoSens, and h-CLAT. Sections 2.1.1 to 2.1.3 review the tests performed by Burleson Research Technologies. The comprehensive test report, which includes detailed protocols for the methods and results, is provided in **Appendix A**.

2.1.1 DPRA

DPRA is an in chemico test that assesses the ability of a substance to form a hapten–protein complex (Gerberick et al. 2007; OECD 2019a), which is the molecular initiating event in the skin sensitization AOP (OECD 2012). Average cysteine and lysine depletion >6.38% indicates a sensitizer outcome. If the lysine peptide co-elutes with the test chemical, peptide reactivity can be assessed using cysteine depletion only. In that case, a sensitizer outcome is indicated when cysteine depletion is >13.89%. The measurement endpoints provided by the DPRA are: cysteine peptide depletion (Cys), lysine peptide depletion (Lys), average depletion of cysteine and lysine peptides (Avg.Lys.Cys), and sensitizer/nonsensitizer outcome. The DAs applied here, Shiseido ANN D_hC and ANN D_hC_KS, used the Avg.Lys.Cys values as inputs.

2.1.2 KeratinoSens

The KeratinoSens test method assesses the ability of a substance to activate cytokines and induce gene expression associated with specific cell signaling pathways in keratinocytes (Emter et al., 2010; OECD 2018a), the second key event in the skin sensitization AOP (OECD 2012). A sensitizer outcome is indicated when luciferase induction is statistically significant and at least 1.5-fold higher than control values at a concentration with cell viability >70%. The KeratinoSens assay provides the effective concentration at 1.5-fold luciferase induction (EC1.5), the effective concentration at 3-fold induction (EC3), the maximum induction (Imax) and the inhibitory concentration at 50% viability (IC50). The Imax was used in the DAs (Shiseido ANN D_hC_KS) applied here.

2.1.3 h-CLAT

h-CLAT assesses the ability of a substance to activate and mobilize dendritic cells in the skin (Ashikaga et al. 2016; OECD 2018b), the third key event of the skin sensitization AOP (OECD 2012). This test measures the induction of two cell surface markers, CD86 and CD54, which indicate dendritic cell activation. A cytotoxicity assay to determine 75% cell viability (CV75) is used to select the doses to be tested. The measurement endpoints for the

h-CLAT include the effective concentration at 150% induction for the CD86 marker (EC150) and the effective concentration at 200% induction for the CD54 marker (EC200). A sensitizer outcome is indicated when CD86 expression is at least 150% or CD54 expression is at least 200% with cell viability > 50%. All the DAs applied here used the minimum induction threshold from the CD86 and CD54 measurements. The minimum induction threshold is the lower value of these two measurements.

h-CLAT testing was also performed by the Institute for In Vitro Sciences and the data were used to assess consistency of the h-CLAT results (see **Section 3.1**); however, the defined approaches incorporated data generated at Burleson Research Technologies only. The Institute for In Vitro Sciences test report is provided as **Appendix B**. Data from the Institute for In Vitro Sciences in **Section 3** have been revised to report the effective concentrations of the active ingredients using the proportion of active ingredients in each product (**Table 1)**, but data in their report have not been revised.

2.2 Generation of In Silico Read-Across Hazard Predictions for Skin Sensitization Hazard: OECD QSAR Toolbox V4.3

QSAR Toolbox v4.3 (OECD 2019b), provided by the Organisation for Economic Co-operation and Development (OECD), was used to generate an in silico read-across hazard prediction (whether each substance was a sensitizer or nonsensitizer) based on in vivo LLNA and guinea pig data from structurally and mechanistically similar analogs. Inputs to the Toolbox were the SMILES chemical structure notation for each substance, obtained from the EPA Chemistry Dashboard (Williams et al. 2017). The automated workflow for skin sensitization was used to make the predictions. Because the automated workflow does not make predictions for substances that are not discrete chemicals, such as CMIT/MIT, predictions were made separately for CMIT and MIT. When the automated workflow could not make hazard predictions for single chemicals (e.g., due to an insufficient number of analogues), the "Skin Sensitization for DASS" profiler was implemented manually. This profiler assesses each substance, its auto-oxidation products, and skin metabolites for protein binding alerts for skin sensitization using the OASIS profiler. The results from this profiler indicate a sensitizer classification if any substance, its auto-oxidation products, or its skin metabolites are associated with a protein binding alert. The Skin Sensitization for DASS profiler will be automated in future versions of the Toolbox. The automated workflow failed to make hazard predictions for DCOIT, BIT, and CMIT. These predictions were performed by manually implementing the Skin Sensitization for DASS profiler.

2.3 Physicochemical Properties

The following physicochemical properties were collected for the IT compounds:

- Log_{10} P (octanol:water coefficient)
- Log_{10} S (water solubility) in M
- Log_{10} vapor pressure (VP) in mmHg
- Melting point (MP) and boiling point (BP), both in °C
- Molecular weight (MW) in g/mol
- Bioconcentration factor (BCF)

Experimental values for each physicochemical property were preferred, but when those were unavailable, predicted values were collected. Experimental log_{10} P values for each substance were provided by Andrew Byro, EPA. Means and ranges are shown for substances with multiple tests. All other values were obtained from OPERAv2.3, the Open Structure-activity/property Relationship App (https://github.com/NIEHS/OPERA). Only two experimental values, log_{10} VP and log_{10} S for OIT, were available. The remaining physicochemical properties for DCOIT, OIT, BIT, BBIT, and MIT were predicted. No physicochemical properties were available for CMIT/MIT because it is a mixture of two different structures. Physicochemical properties are shown in **Table 2**.

Table 2 Physicochemical Properties

Chemical	Log P	Log S (M)	BP (°C)	MP (°C)	Log VP (mmHg)	Log BCF	MW (g/mol)
DCOIT	4.4 (2.8-6.4)	-4.123	287	42.1	-3.983	1.942	281.04
CMIT/MIT	NA	NA	NA	NA	NA	NA	NA
OIT	3.4 (2.4-4.4)	-2.630	255	16.6	-4.434	1.148	213.12
MIT	-0.486	-0.435	154	131.3	0.349	0.309	115.01
BIT	1.35	-2.828	312	108.8	-4.845	0.651	151.01
BBIT	2.86	-4.002	310	87.7	-5.382	0.784	207.07

Abbreviations: NA = Not available.
All logarithms are base 10.

2.4 Evaluation of LLNA Reference Data

LLNA data were obtained from two major sources: a report submitted to EPA from Dow (Begolly 2019) (17 studies) and from publicly available scientific literature (15 studies). No LLNA studies were available for BBIT. Data from all LLNA studies are provided in **Appendix C.** With the exception of one study for MIT, all LLNA studies for all six IT substances yielded positive results. The negative MIT test, which was tested at a maximum concentration of 4.5% in water, was performed on the ultra-pure MIT product. The other four tests for MIT that yielded positive results used maximum concentrations of 0.985- 2.2% in acetone:olive oil or propylene glycol.

Using two different approaches, one from Dow and one from the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), the LLNA data were evaluated to determine a single representative EC3, defined in the context of the LLNA as the concentration inducing a stimulation index (SI) of 3. This representative EC3 was used to classify each substance according to the potency categories of the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS; UN 2019). Substances with EC3 ≤2% are 1A (strong) sensitizers, substances with EC3 >2% are 1B (other) sensitizers, and substances that do not produce a positive response in the LLNA are not classified.

The Dow report included two to four studies for each of five substances, totaling 17 LLNA studies. Dow determined a representative EC3 for each substance by selecting the tests that were performed using acetone or acetone:olive oil as the solvent. Their rationale was that they considered the best way to rank these substances for potency to be using tests with the same or similar solvents because it is well known that EC3 values can vary with solvent (Dumont et al. 2016; Anderson et al. 2011). Two representative tests with similar EC3 values, 0.20% and 0.25% were selected for OIT. Of the studies evaluated, the representative EC3 values were also the most potent values available for each substance (**Table 3**). The Dow approach classified all substances with LLNA results as GHS 1A sensitizers.

The NICEATM approach used the 17 studies provided by Dow and 15 studies from the scientific literature to determine a representative EC3 for each substance. Again, no studies were found for BBIT. A total of 32 studies were available with three to 13 studies for each of the other five substances. One MIT test with EC3 = 1.9% from Gerberick et al. (2005) was excluded because it was the same test reported by Basketter et al. (2003); it had the same stimulation index values with erroneous test concentrations and EC3 value (Roberts 2013). The remaining individual LLNA tests were evaluated for inclusion in determining a single representative mean EC3 using the approach designed by the OECD Expert Group for Defined Approaches for Skin Sensitization. To be included in the evaluation, studies were required to have these attributes:

- The test substance was applied topically to both ears of the mice.
- Lymphocyte proliferation was measured in the lymph nodes draining the site of test substance application.
- Lymphocyte proliferation was measured during the induction phase of skin sensitization.
- A vehicle control was included.
- Either individual or pooled animal data were collected.
- Concentrations tested and corresponding SI values were available.

- ^{3}H-methyl thymidine or other radiolabeled marker was administered in vivo rather than ex vivo
- Sodium lauryl sulfate was not applied to enhance the response.
- Extrapolated EC3 values passed the criteria from Ryan et al. (2007) as follows:
    - The lowest measured SI value was less than five.
    - The extrapolated EC3 was less than 10-fold of the closest tested concentration.
    - The slope ratio was less than two and non-negative. This value is the ratio of the slope from the high dose to the mid-dose to that from the mid-dose to the lowest dose.¶

The NICEATM evaluation rejected five studies because they did not meet the criteria for extrapolated EC3 values. Four studies were rejected because the lowest SI was greater than 5: these included three CMIT/MIT tests with EC3 = 0.021, 0.012, and 0.003% with lowest SI = 6.3, 10.43, and 8.1, respectively, and one DCOIT test (no EC3 calculated because lowest SI = 32.14). One CMIT/MIT test with EC3 = 0.002564% was rejected because the slope ratio was negative. Two to nine studies were then available for each of the five substances with LLNA studies. A representative EC3 for each substance was calculated by determining the mean EC3 for each substance (**Table 3**). The NICEATM approach classified all substances with LLNA results into as GHS 1A sensitizers, except for BIT, which was classified as a GHS 1B sensitizer.

Table 3 Representative LLNA EC3 Values

Chemical	Dow LLNA EC3 (%)	Dow GHS Classification	NICEATM LLNA EC3 (%)[a]	n for NICEATM LLNA EC3	NICEATM GHS Classification
DCOIT	0.004	1A	0.008 (0-0.053)	2	1A
CMIT/MIT	0.002	1A	0.018 (0.0011-0.034)	9	1A
OIT	0.2-0.25 (n=2)	1A	0.361 (0.029-0.69)	4	1A
MIT	0.863	1A	1.154 (0-3.476)	3[b]	1A
BIT	1.54	1A	10.57 (0-23.36)	7	1B
BBIT	NA	NA	NA	0	NA

Abbreviations: NA = not available (no LLNA data for BBIT)

[a] Numbers in parentheses are the 95% confidence intervals for the mean EC3

[b] NICEATM identified four acceptable LLNA studies for MIT, but one was negative and did not provide an EC3 value

2.5 Brief Description of Defined Approaches Used for This Project

The Shiseido artificial neural network (ANN) models are non-linear statistical models that combine multiple in vitro parameters covering various key events of the skin sensitization AOP and predict the LLNA EC3 as an output. The ANN models consist of an input layer (descriptors from in vitro results), a hidden layer, and an output layer (EC3 predictions). Two of the four Shiseido ANN models described in Hirota et al. (2015) were evaluated here, chosen based on availability of the input data and published performance of the models. The first model (ANN D_hC, "Model 1" in Hirota et al. 2015) used quantitative values from the DPRA (Avg.Lys.Cys) and the h-CLAT (minimum induction threshold) to predict the EC3 value that would be produced in the LLNA. The second model (ANN D_hC_KS, "Model 4" in Hirota et al. 2015) used the same structure with an additional value from the KeratinoSens (Imax) used as the third input. The ANN DAs were coded in R (available upon request), and in brief, logistic activation functions were used for the hidden and output layers, 10,000 iterations were used for training, and learning rate, scaling functions, and momentum parameters were inferred from Hirota et al. (2015). For each IT compound, each model was run 100 times and mean EC3 prediction and 95% confidence intervals were calculated. Additional details on DAs and performance-based validation on a set of 128 reference chemicals can be found in Kleinstreuer et al. (2018). All data used as information sources for the DAs, as well as the DA output predictions, are included in **Appendix D**.

2.6 Data Analyses

2.6.1 Comparison of Individual Non-Animal Methods Against LLNA

Concordance of the hazard classifications for in chemico and in vitro data amongst the non-animal tests was evaluated as well as concordance of the non-animal methods with the LLNA data. Concordance of potency was compared by ranking the IT compounds from most potent to least potent using both the LLNA EC3 values and the measured endpoints from the in chemico and in vitro methods. The in silico read-across predictions were not used for potency ranking because they are not quantitative.

2.6.2 Comparison of Defined Approaches Against LLNA

Concordance of the defined approaches with the LLNA data, with respect to hazard classifications and potency predictions, was evaluated. Concordance of potency was compared by ranking the IT compounds from most potent to least potent using both the measured LLNA EC3 values and the predicted EC3 values from the ANN DAs. Root mean square error (RMSE) and mean absolute error (MAE) were reported for the measured vs. predicted EC3 values.

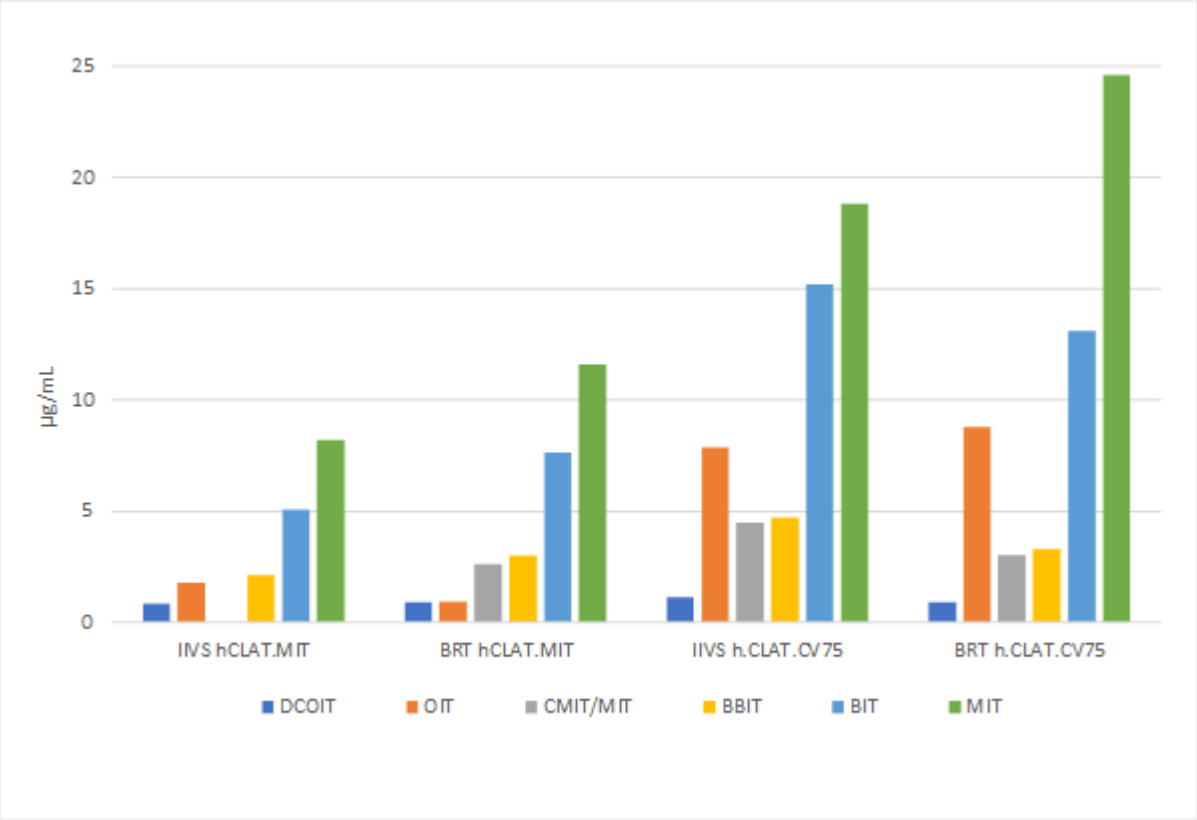
3.0 Results

3.1 Skin Sensitization Hazard Comparison of Individual Non-animal Methods with Respect to LLNA Results

The hazard classification results for each of the non-animal test methods, DPRA, KeratinoSens, h-CLAT, and for the in silico read-across, were the same for each of the six isothiazolinone compounds. All tests performed by Burleson Research Technologies classified all six compounds as sensitizers. With the exception of BBIT, which had no LLNA data, the hazard classification of the non-animal methods was concordant with that of the LLNA.

The h-CLAT results from Burleson Research Technologies and the Institute for In Vitro Sciences were comparable (**Figure 2**). With the exception of CMIT/MIT, which yielded negative results for cell surface marker expression at the Institute for In Vitro Sciences in 2/3 tests, the results from the two laboratories ranked the chemicals in the same order for both cell surface marker expression and cytotoxicity (CV75). h-CLAT data from the Institute for In Vitro Sciences are provided because they were available to show the consistency of h-CLAT data between laboratories. Reports of h-CLAT data hereafter are those from Burleson Research Technologies, which also performed the DPRA and KeratinoSens tests and is the official immunotoxicity testing contract facility for NTP.

Figure 2 Comparison of h-CLAT Results from Two Laboratories



Abbreviations: BRT = Burleson Research Technologies; IIVS = Institute for In Vitro Sciences

[a] All results have been corrected for % active ingredient

3.2 Skin Sensitization Potency Comparison of Individual Non-animal Methods with Respect to LLNA Results

Because the in chemico and in vitro methods are not to be used for potency classification (OECD 2018a, b; 2019), no GHS criteria for these methods have been proposed for classification of 1A and 1B sensitizers. However, the sensitization endpoint measurements from these methods (**Table 4**) were used as indicators of potency to rank the six IT compounds and compare with the LLNA EC3 rankings (**Table 5**).

Table 4 Skin Sensitization Measurement Endpoints for LLNA and Non-animal Methods

Chemical	Dow LLNA EC3 (%)	NICEATM EC3 (%)[a]	DPRA Mean Depletion (%)	Keratino-Sens EC1.5 (µM)[b]	Keratino-Sens Imax	h-CLAT Minimum Induction Threshold (µg/mL)[b]
DCOIT	0.004	0.008 (0-0.053)	55.2	1.32	4.37	0.92
CMIT/MIT	0.002	0.018 (0.0011-0.034)	55.3	3.41	5.61	2.63
OIT	0.2-0.25	0.361 (0.029-0.69)	50	2.19	3.70	0.95
MIT	0.863	1.154 (0-3.476)	50	9.54	15.84	11.6
BIT	1.54	10.57 (0-23.36)	NA	3.14	17.64	7.63
BBIT	NA	NA	50	3.84	19.61	3.01

Abbreviations: NA = not available

[a] Numbers in parentheses are the 95% confidence intervals for the mean EC3

[b] Results corrected for % active ingredient

Peptide depletion values from DPRA were not useful for ranking potency because all of the compounds reacted very strongly with the cysteine peptide and minimally, or not at all, with the lysine peptide (**Appendix A, Table 2**). The exception was BIT, which co-eluted with the lysine peptide. Based on a statistical comparison with the NICEATM LLNA ranking, KeratinoSens EC1.5 and h-CLAT yielded similar ranks (Wilcoxon signed rank test, p-values of 0.85 and 0.59, respectively). Both methods ranked DCOIT as the most potent and MIT as the least potent (**Table 5**).

The representative LLNA EC3 values used by Dow and NICEATM yielded the same ranks except for the positions of DCOIT and CMIT/MIT, which were 2 and 1 for the Dow approach and 1 and 2 for the NICEATM approach (**Table 5**). The ranks based on KeratinoSens EC1.5 and h-CLAT were roughly similar to that for the LLNA, which ranked DCOIT as very potent and BIT and MIT as least potent.

Table 5 Potency Rank by Test Method

Chemical	Dow LLNA	NICEATM LLNA	KeratinoSens	h-CLAT
DCOIT	2	1	1	1
CMIT/MIT	1	2	4	3
OIT	3	3	2	2
MIT	4	4	6	6
BIT	5	5	3	5
BBIT	NA	NA	5	4

NA = not available (no LLNA data for BBIT)

3.3 Comparison of Defined Approaches and LLNA Results for Hazard and Potency

The hazard classification result for each of the DAs was the same for each of the six isothiazolinone compounds, where all six compounds were classified as sensitizers. With the exception of BBIT, which had no LLNA data, the hazard classification of the DAs was concordant with that of the LLNA. The potency classification (**Table 6**) of 1A for all compounds was concordant across the DAs and with the LLNA data, with the exception of the NICEATM LLNA for BIT, which yielded a 1B classification, and BBIT, which had no LLNA data.

Table 6 Potency Classification Prediction for Isothiazolinones

Chemical	Dow LLNA	NICEATM LLNA	DA: ANN D_hC[a] Potency	DA: ANN D_hC_KS[b] Potency
DCOIT	1A	1A	1A	1A
CMIT/MIT	1A	1A	1A	1A
OIT	1A	1A	1A	1A
MIT	1A	1A	1A	1A
BIT	1A	1B	1A	1A
BBIT	NA	NA	1A	1A

[a] Model 1 from Hirota et al. 2015: DPRA + h-CLAT
[b] Model 4 from Hirota et al. 2015: DPRA + h-CLAT + KeratinoSens

3.4 Comparison of Predicted Potency to LLNA

The two ANN DAs provide quantitative EC3 predictions as outputs, shown below in comparison to the LLNA EC3 values from Dow or NICEATM (**Table 7**). When comparing the five IT compounds with in vivo data and quantitative DA predictions, the RMSE between the Dow LLNA EC3 values and the DA EC3 values was 0.49 for the model using only DPRA and h-CLAT (ANN D_hC) and 0.57 for the model using DPRA, h-CLAT, and

KeratinoSens (ANN D_hC_KS). The MAE between the Dow EC3s and the DA EC3s was 0.36 for ANN_D_hC and 0.38 for ANN D_hC_KS. The RMSE between the NICEATM LLNA EC3 values and the ANN DA EC3 values was 4.32 for the ANN D_hC model and 4.58 for the ANN D_hC_KS model, and the MAEs were 2.14 and 2.28, respectively. The differences in these comparative values were driven by the different representative LLNA EC3 values for BIT between the Dow data and the NICEATM data, where the DA EC3 predictions for BIT were more similar to the Dow data.

The quantitative EC3 predictions derived from the ANN DAs were similar to the NICEATM LLNA EC3 values, with overlapping 95% confidence intervals (CI) in most cases, with the exception of CMIT/MIT, where the upper bound of the in vivo CI was 3.5-fold less than the lower bound of the in silico CI (for the ANN D_hC DA). Because the in vivo EC3 values for CMIT/MIT were low in comparison to those for the most potent component, CMIT (EC3=0.009 and 0.01% from the NICEATM LLNA database [NICEATM 2013]), EC3 values weighted by the amount of each component were calculated (**Appendix E**). The weighted EC3 values of 0.21% (Dow approach) and 0.28% (NICEATM approach) were closer to the predicted values from the ANN DAs. While the in vivo and in silico CI for BIT did overlap, the average EC3 predictions derived from the DAs were closer to the in vivo estimate provided by Dow than that calculated by NICEATM. The largest discrepancy between the two ANN DAs was seen for the CMIT/MIT mixture, with a 4-fold difference between the average EC3 predictions.

Table 7 Quantitative EC3 Prediction for Isothiazolinones

Chemical	Dow LLNA EC3 (%)	NICEATM LLNA EC3 (%)[a]	DA: ANN D_hC[b] EC3 (%)[a]	DA: ANN D_hC_KS[c] EC3 (%)[a]
DCOIT	0.004	0.008 (0-0.053)	0.0566 (0.0555 – 0.0578)	0.023 (0.02 – 0.026)
CMIT/MIT	0.002[d]	0.018[e] (0.0011-0.034)	0.121 (0.119 – 0.123)	0.492 (0.4 – 0.605)
OIT	0.2-0.25	0.361 (0.029-0.69)	0.0569 (0.0559 – 0.058)	0.015 (0.013 – 0.017)
MIT	0.863	1.154 (0-3.476)	1.775 (1.732 – 1.818)	0.826 (0.759 – 0.9)
BIT	1.54	10.57 (0-23.36)	0.934 (0.909 – 0.959)	0.341 (0.317 – 0.367)
BBIT	NA	NA	0.148 (0.146 – 0.151)	0.061 (0.055 - 0.068)

[a] Numbers in parentheses are the 95% confidence intervals
[b] Model 1 from Hirota et al. 2015: DPRA + h-CLAT
[c] Model 4 from Hirota et al. 2015: DPRA + h-CLAT + KeratinoSens
[d] Weighted EC3 = 0.21% using CMIT data from NICEATM LLNA database that were selected using the same criteria used by Dow: vehicle was acetone or acetone:olive oil
[e] Weighted EC3 = 0.28% using the average of CMIT values from NICEATM LLNA database

The predicted EC3 values in **Table 7** from the ANN DAs were used to rank the six isothiazolinones by potency (**Table 8**) and compared to the potency rank derived from the LLNA studies based on the Dow submission or NICEATM literature review (also from **Table 7**). The DAs ranked DCOIT and OIT as the most potent IT compounds in the class, followed by CMIT/MIT, BBIT, and BIT (with differing ranks for this middle group between the two DAs) and lastly MIT. With the exception of BBIT, which had no LLNA data, the ranks for ANN D_hC and D_hC_KS were similar to in vivo results, based on a statistical comparison with the NICEATM LLNA rank (Wilcoxon signed rank test, p-values of 0.59 in each case).

Table 8 Potency Rank Comparison

Chemical	Dow LLNA	NICEATM LLNA	DA: ANN D_hC[a]	DA: ANN D_hC_KS[b]
DCOIT	2	1	1	2
CMIT/MIT	1	2	3	5
OIT	3	3	2	1
MIT	4	4	6	6
BIT	5	5	5	4
BBIT	NA	NA	4	3

[a] Model 1 from Hirota et al. 2015: DPRA + h-CLAT
[b] Model 4 from Hirota et al. 2015: DPRA + h-CLAT + KeratinoSens

3.5 Consideration of Uncertainties for the In Vivo, In Chemico, and In Vitro Data, and for the Defined Approaches

3.5.1 Uncertainties Related to the In Vivo Data

The LLNA is a standardized test method described in an internationally harmonized OECD test guideline for skin sensitization assessment. This method has been validated as relevant and reproducible for skin sensitization hazard and potency. It is applicable for testing most substances unless there are properties associated with a substance that may interfere with the accuracy of the LLNA (e.g., certain metals and surfactants).

The in vivo nature of the test incorporates the absorption, distribution, metabolism, excretion, and pharmacodynamic elements of the adverse outcome pathway between chemical exposure and key event 4, T-cell proliferation. The inherent reproducibility of the LLNA has been shown by multiple analyses (e.g. Hoffman et al. 2018, Dumont et al. 2016) to be in the range of 70-80% for hazard prediction and 60-70% for potency prediction, depending on the summary statistic used for comparison (e.g., median, mean, etc.). The NICEATM EC3 values reported in **Table 7** for the IT compounds represent the means of EC3 values from tests that meet criteria designed to identify the most reliable EC3 values (**Section 2.4**). Presenting the 95% confidence intervals around the mean EC3 provides a quantitative measure of uncertainty in the results. Dow EC3 values were derived to limit EC3 values to those in the same or similar solvents and were the most potent EC3 values available for each substance. Qualitative uncertainties regarding the LLNA data include:

- The LLNA incorporates all four key events of the AOP, but not the adverse outcome of skin sensitization.
- Mice, the experimental model used in the LLNA, are not humans, the species of interest.

3.5.2 Uncertainties Related to the In Chemico and In Vitro Data

The DPRA, KeratinoSens, and h-CLAT assays are standardized test methods described in internationally harmonized OECD test guidelines. These test methods have been validated as relevant and reproducible for regulatory use when used with other information (i.e., they are not intended to be used as stand-alone tests). The reproducibility of these tests and the accuracy, sensitivity, and specificity with respect to LLNA hazard classifications are provided in the OECD test guidelines:

- DPRA: reproducibility was approximately 85% within laboratories and 80% between laboratories; accuracy = 80% (126/157), sensitivity = 80% (88/109), and specificity = 77% (37/48) (OECD 2019).
- KeratinoSens: reproducibility was approximately 85% within and between laboratories; accuracy = 77% (155/201), sensitivity = 78% (71/91), and specificity = 76% (84/110) (OECD 2018a).
- h-CLAT: reproducibility was approximately 80% within and between laboratories; accuracy = 85% (121/142), sensitivity = 93% (94/101), and specificity = 66% (27/41) (OECD 2018b).

These in chemico and in vitro tests use human cellular and molecular targets to provide information on the activation of a key event by a test substance without the potential interference of upstream effects. The results of the DPRA were not helpful for distinguishing potencies of the IT compounds; all produced similar results. Confidence in the KeratinoSens and h-CLAT results is increased because they provided similar potency ranks for the IT chemicals and they have higher reproducibility than the in vivo results (Kleinstreuer et al. 2018). The qualitative uncertainties for DPRA, h-CLAT, and KeratinoSens results include the following:

- These methods assess the first three key events of the skin sensitization AOP, but not the fourth key event, T-cell proliferation, or the adverse outcome.
- The in chemico and in vitro tests do not mimic the absorption, distribution, metabolism, and excretion of a test substance that occur in vivo.

3.5.3 Uncertainties Related to the ANN

The ANN DAs incorporate information from two to three of the in chemico or in vitro tests. Model 1 uses DPRA and h-CLAT data and Model 4 uses DPRA, h-CLAT, and KeratinoSens data. Because DPRA was not effective in ranking the IT compounds for potency, and Model 4 includes both KeratinoSens and h-CLAT data, which ranked the IT compounds similarly for potency, confidence in Model 4 results is higher than that for Model 1. Model 4 also covers three key events of the AOP, rather than two. The reported ANN EC3 values are means

resulting from 100 runs of each model. The 95% confidence intervals around the mean ANN EC3 values provide a quantitative measure of uncertainty in the results based on the variation inherent in the machine learning algorithm. The variability of the in vitro methods is not explicitly incorporated, but during the OECD validation studies all methods were shown to have ≥80% within- and between-lab reproducibility. The performance of the ANN DAs with respect to predicting LLNA potency classification (strong, weak, and nonsensitizing) for a diverse group of 126 chemicals were provided in Kleinstreuer et al. (2018):

- Model 1 (ANN D_hC): accuracy = 65.1% (82/126), over-predicted = 21.4% (27/126), and under-predicted = 13.5% (17/126)
- Model 4 (ANN D_hC_KS): accuracy = 69.8% (88/126), over-predicted = 23.0% (29/126), and under-predicted = 7.1% (9/126)

Kleinstreuer et al. (2018) also provides performance of the ANN DAs for predicting human potency classification (strong, weak, and nonsensitizing):

- Model 1 (ANN D_hC): accuracy = 61.1% (77/126), over-predicted = 22.2% (28/126), and under-predicted = 16.7% (21/126)
- Model 4 (ANN D_hC_KS): accuracy = 62.7% (79/126), over-predicted = 25.4% (32/126), and under-predicted = 11.9% (15/126)
- In comparison, the LLNA performance against this set was: accuracy = 59.4% (76/128), over-predicted = 19.5% (25/128), and under-predicted = 21.1% (27/128)

The qualitative uncertainties for ANN Model 4 include the following:

- The ANN models were trained to predict T-cell proliferation results in mice (EC3 values), and not the adverse outcome in humans, the species of interest.

4.0 Conclusions

All of the non-animal methods, DPRA, KeratinoSens, h-CLAT, and the in silico read-across OECD QSAR Toolbox, were concordant with the LLNA in yielding a sensitizer hazard classification for each of the six isothiazolinone compounds. Peptide depletion values from DPRA were not useful for ranking the six IT compounds for skin sensitization potency because they were too similar to one another. KeratinoSens and h-CLAT produced a similar ranking to that based on the LLNA. The quantitative EC3 values generated from the DAs were comparable to those derived from the LLNA data. The DAs ranked DCOIT and OIT as the most potent IT compounds in the class, followed by BBIT, CMIT/MIT, BIT and MIT.

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