Case study 1: The AOP-based “two out of three” skin sensitization ITS for hazard identification

A. Mehling, D. Urbisch, N. Honarvar, T. Ramirez, S.N. Kolle, B. van Ravenzwaay, R. Landsiedel

Alternatives for skin sensitization testing and assessment: A joint Cefic-LRI / Cosmetics Europe / EPAA workshop April 23, 2015

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Overview:

- Title and purpose (e.g. screening/hazard identification/potency prediction)
- Rationale underlying the construction of the approach including coverage of the AOP
- Description of the information sources used and readouts used (e.g. DPRA, prediction obtained with the proposed prediction model or % Cys depletion or % unreacted Cys)
- Chemical used to develop (train) and test the approach (number, selection, reference in vivo data, others)
- Process applied to derive the prediction/assessment (i.e. tiered testing strategy, support vector machine, Bayesian, Neural networks, others possibly illustrated with a diagram/workflow)
- Predictive capacity of the approach
- Limitations in the application of the approach:

  a) because of technical limitations and

  b) because of wrong predictions
- Conclusions

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Title and purpose (e.g. screening/hazard identification/potency prediction)

Title:

The AOP-based “two out of three” skin sensitization integrated testing strategy (ITS) for hazard identification

Purpose:

Skin sensitization **hazard** identification and classification for regulatory purposes such as:

- Globally Harmonized System of Classification, Labelling and Packaging of Chemicals **(GHS)**
- Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals **(REACH)**
- Regulation (EC) No 1223/2009 of the European Parliament and of the Council on Cosmetics **(EU Cosmetics Regulation)**

Without the need to use animal-based test methods!

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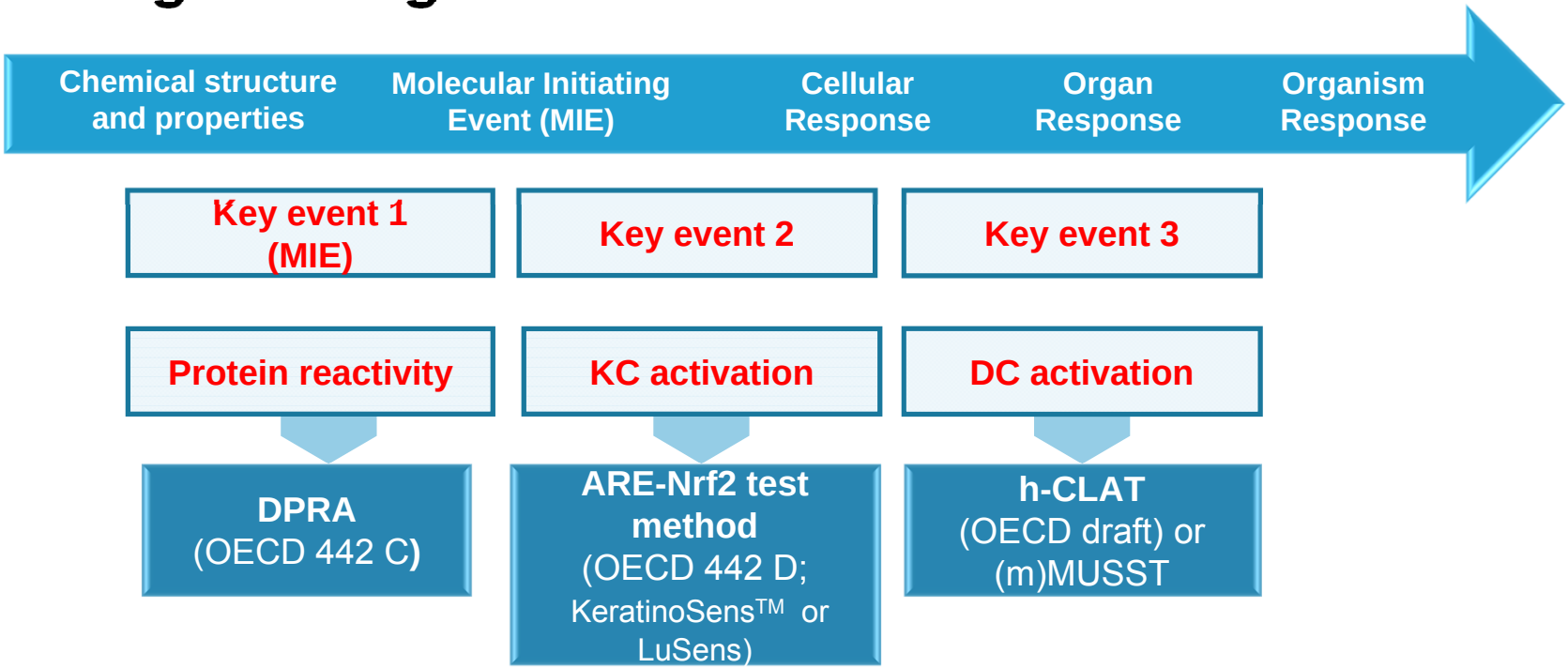
Rationale underlying the construction of the approach including coverage of the AOP



- This strategy covers key events 1, 2 and 3 of the AOP
- All methods are published in peer-reviewed journals, 2 are OECD TGs and 1 is an OECD TG draft
- A broad range of chemicals have been assessed using the individual methods
- High quality human and/or LLNA data was available for these chemicals

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Description of the information sources used and readouts used

The readouts/prediction models of the individual methods are those previously described and published (not all readouts listed)

- **Key event 1:** Direct peptide reactivity assay (**OECD TG 442 C** peptide depletion due to Cys and Lys adducts; peptide depletion of over 6.38% indicates a sensitizer
- **Key event 2:** ARE-Nrf2 luciferase test method (**OECD TG 442 D**): A luciferase reporter gene is used to assess keratinocyte activation. An increase ≥ 1.5 indicates a sensitizer (cell-viability ≥ 70%); KeratinoSens (!); LuSens (Ramirez et al., 2014)
- **Key event 3:** 1) Human cell line activation test (h-CLAT; **OECD TG draft**): Upregulation of CD54 (EC 200)/CD86 (EC150) on THP-1 cells (cell-viability≥50%); 2) (modified) Myeloid U937 skin sensitization test [(m)MUSST]: Upregulation CD86 (EC150) on U937 cells (Ade et al., Bauch et al., 2011; Urbisch et al., 2015)
- **OECD Toolbox:** in silico; to define possible peptide reactivity mechanisms

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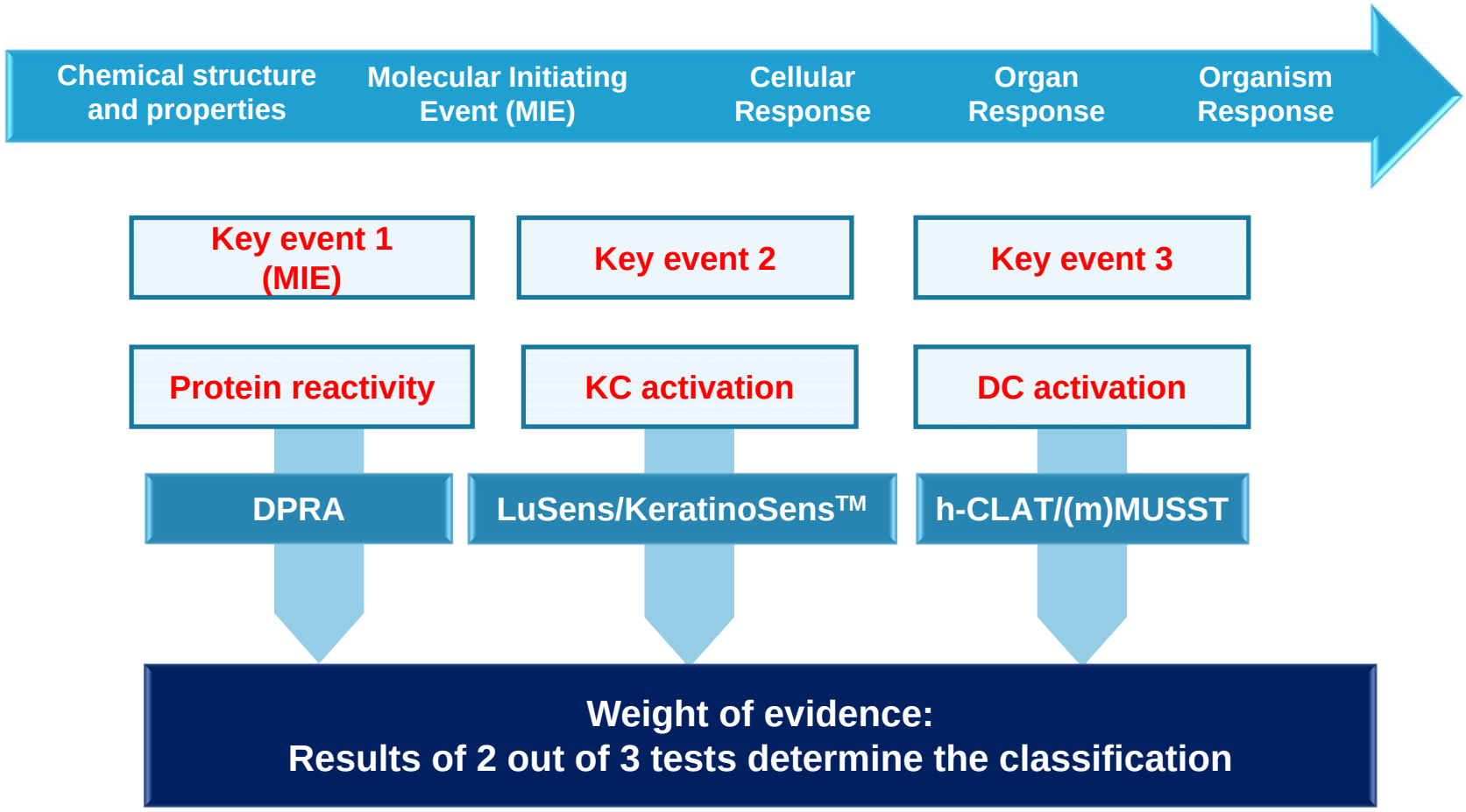
Process applied to derive the prediction/ assessment



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Data Set 1:
Chemical used to develop and test the approach

No predefined chemical training set was used – the prediction model was developed after analyzing the results:

- 54 substances: Additives/ stabilizers/ detergents (30%); fragrances (24%), cosmetic preservatives (22%), cosmetic solvents (11%), cosmetic dyes (7%)
- LLNA and human skin sensitization information available (references in Bauch et al., 2012)
- The chemicals selected included the performance standards for OECD TG 429 (LLNA)

Compared to human data		Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy
In vivo standard	LLNA	96%	81%	87%	94%	90%
Prediction model	DPRA, LuSens and mMUSST	93%	95%	96%	91%	94%

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Data set 2:
Predictive capacity: ‘real life’ substances


In vitro skin sensitization testing strategy – in-house post-validation with “real-life” compounds

Katharina Guth[1], Susanne N. Kolle[1], Naveed Honarvar[1], Britta Wareing[1], Annette Mehling[2], Bennard van Ravenzwaay[1], Robert Landsiedel[1]

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Guth et al., WC9, 2014; Poster-ID: II-1-103

	in-house post-validation					
	WoE I	WoE II	WoE I w/o PEI, AF	WoE II w/o PEI, AF	WoE I w/o PEI, AF, PE	WoE II w/o PEI, AF, PE
n	38	35	24	21	24	21
vs.	LLNA/ GPMT	LLNA/ GPMT	LLNA/ GPMT	LLNA/ GPMT	LLNA/ GPMT	LLNA/ GPMT
Sensitivity [%]	71	75	88	94	93	93
Specificity [%]	86	73	85	70	90	86
Accuracy [%]	76	74	87	85	92	90

WoE I: DPRA, LuSens, mMUSST; **WoE II:** DPRA, LuSens, h-CLAT; AF: agrochemical formulation; PEI: polyethylene imine; PE: plant extract

- The protocols for the test methods are intended for defined substances - not mixtures, plant extracts, etc. (use of molar equivalents)
- Formulations and polyethylene imine polymers were not well predicted indicating a need to adapt the methods (e.g. further optimize the gravimetric approach used)

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Data set 3:
Predictive capacity: additional substances


Research Article

Journal of Applied Toxicology

A dataset on 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation

Andreas Natsch[a*], Cindy A. Ryan[b], Leslie Foertsch[b], Roger Emter[a], Joanna Jaworska[c], Frank Gerberick[b] and Petra Kern[c]

Cooper statistics compared to LLNA and for WoE 'positive if 2 of 3 tests positive'

Compared to LLNA data	U937-CD86 Test	DPRA	KeratinoSens™	WoE (2 of 3 tests)
Sensitivity [%]	71	82	79	82
Specificity [%]	70	74	72	77
Accuracy [%]	71	80	77	81
n	141	145	145	145

2 out of 3	Accuracy 54 chemicals (Bauch et al., 2012) compared to human data	Accuracy 54 chemicals (Bauch et al., 2012) compared to LLNA data	Accuracy 145 chemicals (Natsch et al., 2013) compared to LLNA data
DPRA, ARE-based assay and U937/CD86 Test	**94%**	**83%**	**81%**

- 43 non-sensitizers according to the LLNA, 33 weak, 39 moderate, 19 strong and 11 extreme sensitizers
- cLogP: majority ranged between 0 and 4
- Molecular weight: majority ranged between 100 and 200 Da

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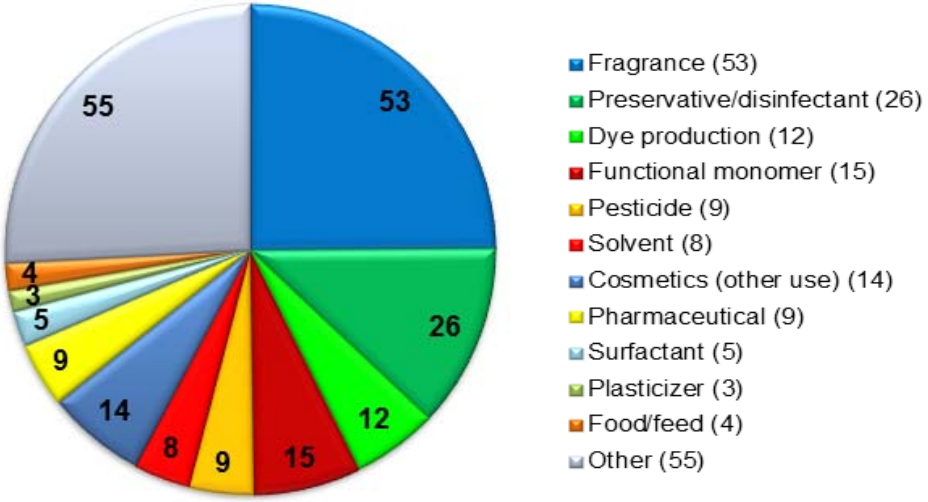
Data set 4:
Predictive capacity of the approach




Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Assessing skin sensitization hazard in mice and men using non-animal test methods

Daniel Urbisch [a], Annette Mehling [b], Katharina Guth [a], Tzutzuy Ramirez [a], Naveed Honarvar [a], Susanne Kolle [a], Robert Landsiedel [a,*], Joanna Jaworska [c], Petra S. Kern [d], Frank Gerberick [e], Andreas Natsch [f], Roger Emter [f], Takao Ashikaga [g], Masaaki Miyazawa [h], Hitoshi Sakaguchi [h]

Compared to HUMAN/LLNA data	n	Sensitivity [%]	Specificity [%]	Positive predictive value [%]	negative predictive value [%]	Accuracy [%]
DPRA + KeratinoSens + h-CLAT	**101/180**	90/82	90/72	96/89	79/59	**90/79**
DPRA + KeratinoSens + (m)MUSST	**95/171**	84/79	100/77	100/90	70/59	**88/78**
DPRA + LuSens + h-CLAT	**90/133**	90/83	89/78	95/91	80/64	**90/82**
DPRA + LuSens + (m)MUSST	**75/126**	87/84	100/84	100/93	75/69	**91/84**

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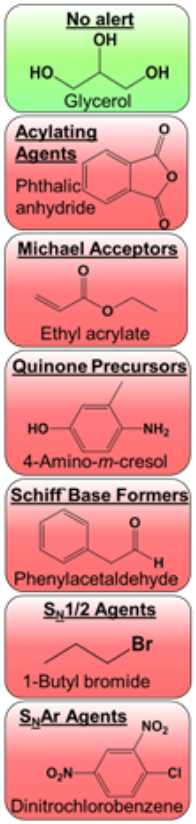
Mechanistic domains
Predictive capacity of the approach



Accuracies [%]	DPRA	Keratino-Sens	LuSens	h-CLAT	(m)MUSST	'2 out of 3' ITS
no alerts						
vs. human data	76	82	80	59	82	80
vs. LLNA data	68	64	64	69	71	70
n	34	33	25	29	28	30
Acylating agents						
vs. human data	82	58	50	83	56	83
vs. LLNA data	100	42	33	83	67	83
n	11	12	6	12	9	12
Michael acceptors						
vs. human data	86	100	100	90	80	95
vs. LLNA data	86	90	100	100	87	95
n	22	21	10	20	15	22
Quinone precursors						
vs. human data	91	90	71	91	80	91
vs. LLNA data	91	90	71	91	80	91
n	11	10	7	11	10	11
Schiff'base formers						
vs. human data	77	79	86	93	75	92
vs. LLNA data	85	71	86	86	67	85
n	13	14	7	14	12	13
S_N1/2 agents						
vs. human data	100	83	100	92	78	100
vs. LLNA data	82	100	100	75	78	83
n	11	12	5	12	9	12
S_NAr agents						
vs. human data	Only 2 chemicals with human data in this domain					
vs. LLNA data	100	83	100	100	75	100
n	5	6	2	4	4	6

Protein binding mechanisms: defined via OECD toolbox vers. 3.2 and scientific literature

Highest accuracies:
- Michael acceptors
- Nucleophilic substitutions (S_N)
- Quinone precursors

Lowest accuracy:
Acylating agents
(→ no/little Cys binding capacities)

Assessing protein binding mechanisms offers a way to obtain a more accurate estimate of the predictive performance

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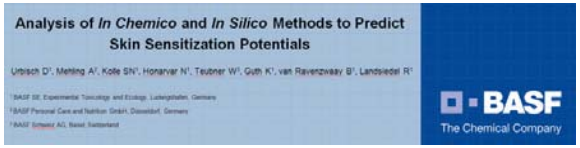
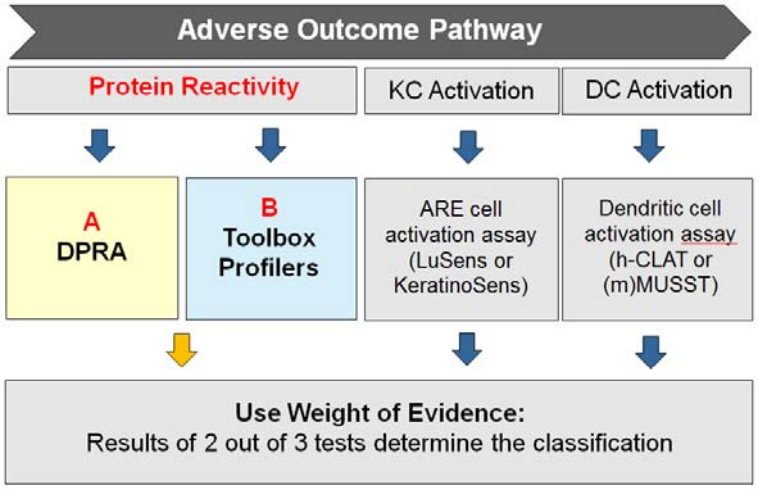
Using an *in silico* + *in vitro* combination
Predictive capacity of the approach



Urbisch et al., WC9, 2014; Poster-ID: II-1-103

Reference: LLNA data; Results in [%]	*in chemico* Approach	*in silico* Approach (QSAR Toolbox v3.2)		
	DPRA	OASIS Profiler	OECD Profiler	Overall *in silico* Result[2]
Sensitivity	82	67	65	66
Specificity	72	86	86	90
Accuracy	79	73	71	73

Ref.: LLNA & Human data; Results in [%]	'2 of 3 WoE' = KeratinoSens, (m)MUSST + …			
	A + DPRA		B + Toolbox Profilers	
Sensitivity	80	86	79	82
Specificity	80	96	85	96
Accuracy	80	89	81	86



- 45 non-sensitizers and 113 sensitizers
- The OECD toolbox 3.2 offers a tool for protein reactivity
- When used alone, the overall accuracy **is moderate**
- When combined with other in vitro methods with an AOP based rationale (2 out of 3) **high accuracies can be achieved**
- **Note:** Skin metabolism and autoxidation simulator not considered

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Limitations in the application of the approach:
because of

a) technical limitations

- Physical state, e.g. gases, highly lipophilic substances (cell culture)
- Stability under test conditions, e.g. DPRA/high pH Lys
- Interference with the detection system (bubbles formed by surfactants can interfere with flow cytometric detection in some cytometers, depletion of peptides not due to adduct formation, pigments could interfere with viability readouts)
- Substances with high cytotoxicity cannot always be tested to a sufficiently high concentration
- Complex mixture, e.g. plant extracts or formulations are difficult to evaluate as molecular weights or molar equivalents are used in most tests

b) wrong predictions

- Pre- and prohaptens are not always reliably predicted (e.g. due to the limited metabolic capacities)
- Depending on the protein binding mechanisms, the individual assays may have varying predictivities

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Conclusions

- Based on the extensive comparative studies conducted (currently n = 180), the ‘2 out of 3’ weight of evidence approach affords high predictivity for skin sensitization hazard identification (even better than the LLNA)
- A large data set has been made available, including human data (Urbisch et al., 2015; open-access)
- Identification of the proposed mechanistic peptide reactivity can help to build even more confidence in the predictions
- Pre- or prohaptens, highly lipophilic, cytotoxic substances, mixtures, etc. substances are challenging
- Potency assessments remain a challenge
- No toxicological test is perfect (not even the LLNA) – it is important to know their strengths and limitations

- Non-animal approaches can now be used for hazard identification with a sufficient degree of confidence for many chemicals (both for non-sensitizers and sensitizers)

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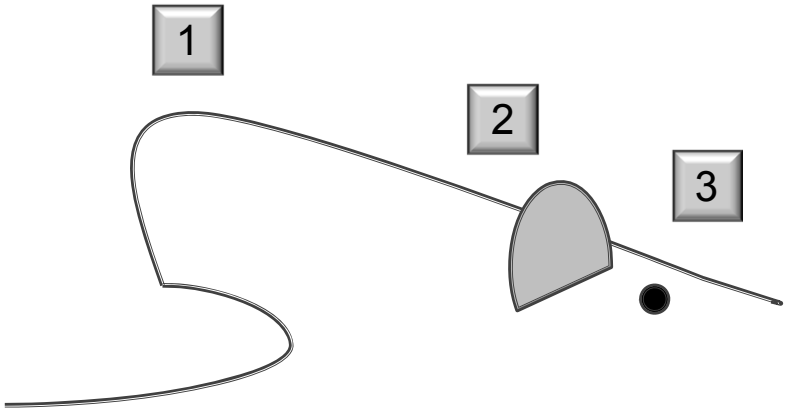
The beauty (?!) of simplicity



3 Elements:

Very simple - but we‘re getting the picture!

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Thank you for your attention!

Acknowledgements

Many thanks to all the companies and their employees that developed the methods, published results (both human, animal and nonanimal) and that made the various publications possible!

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