Weight of individual parameters measured in NAMs: meta-analysis and how it can be applied to NESIL determination – including case studies

IDEA meeting on NAM

10.12.2019, Andreas Natsch

Agenda

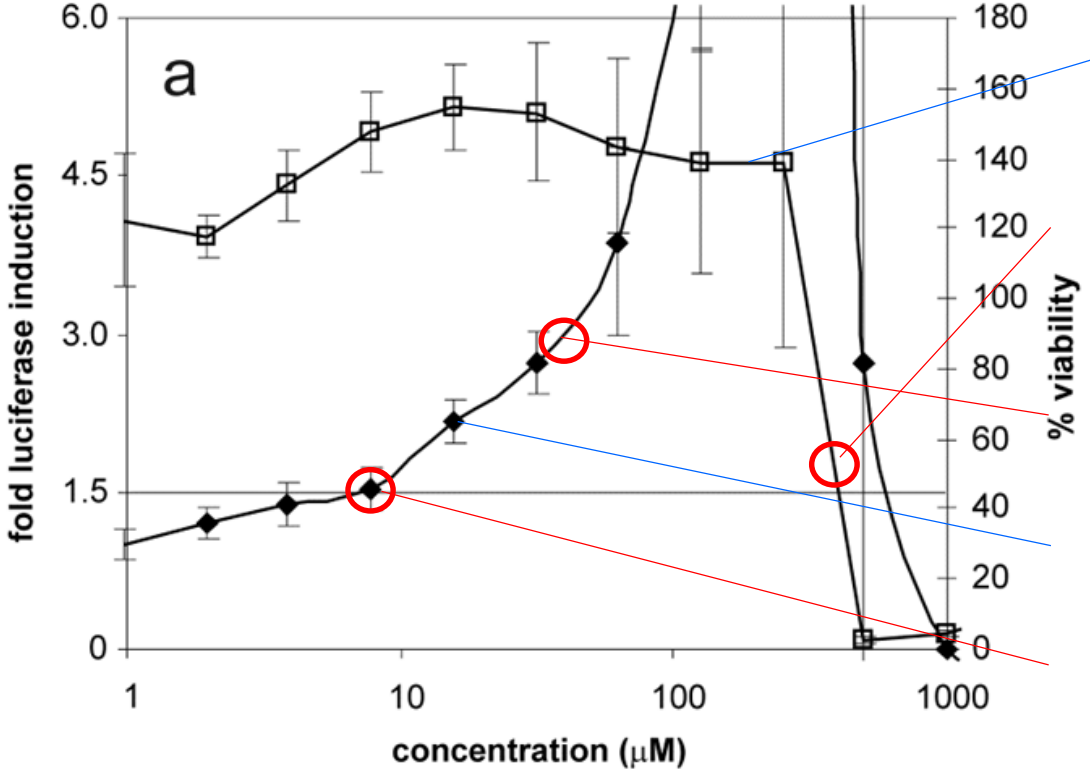
1. Quantitative parameters in validated *in vitro* assays
2. Correlation of individual parameters to LLNA potency
3. Combinations of parameters for models
4. Givaudan approach for deriving a NESIL
5. Case studies on existing molecules
6. Case studies on new materials

Quantitative contribution to potency assessment of individual tests

- Next to binary prediction model, the individual tests contain quantitative (*dose-response*) information – quantitative information not part of validation
- **KeratinoSens**
  - EC1.5, EC3 –Dose for 1.5 / 3-fold Luciferase induction
  - IC50 for 50% reduction in cell viability
- **hClat**
  - EC150 – dose for 1.5-fold induction of CD86
  - EC200 – dose for 2-fold induction of CD54
  - MIT minimum of EC150 and EC200
  - CV75 for 50% reduction in cell viability
- **DPRA**
  - % depletion for Cys and Lys peptide
- **Kinetic DPRA – new modified DPRA**
  - Kinetic rate for peptide depletion

In vitro tests used: **KeratinoSens® - Typical dose-response curve**

- In each test, chemicals are tested at 12 different concentrations
- **EC1.5, EC3 and IC50 are recorded in μM**



% cell viability

IC50, Concentration for 50% reduction in cell viability

EC 3, Concentration for 3-fold induction

fold luciferase induction

EC 1.5, Concentration for 1.5-fold induction

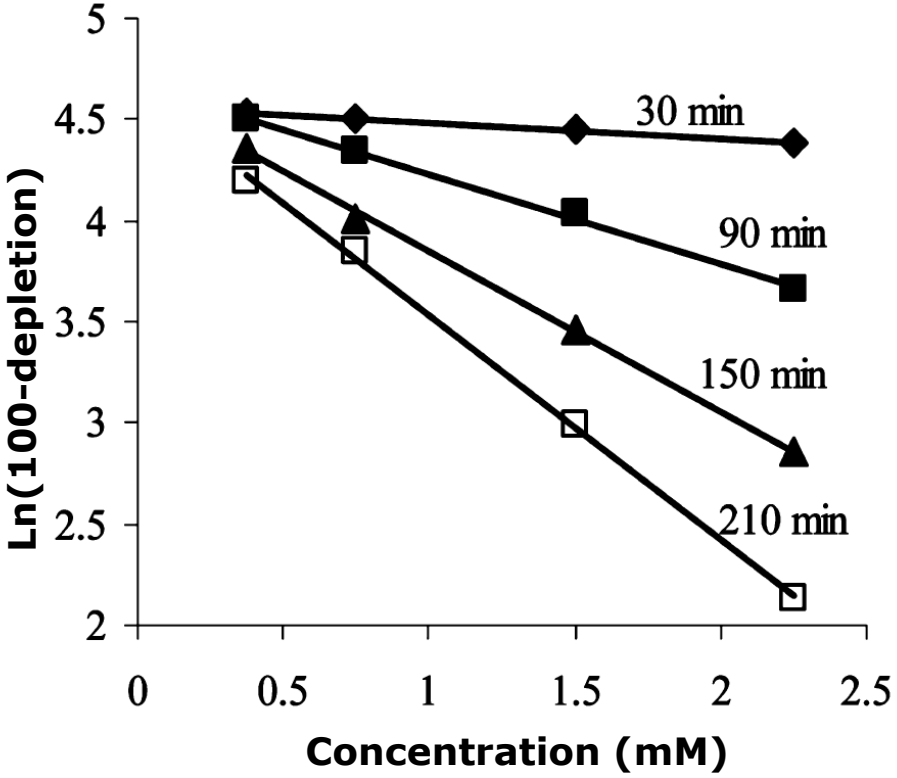
Example for the hair dye component *p*-phenylendiamine (strong sensitizer)

A. Natsch, R. Emter, *Arch Toxicol* **2015,** *89*.

In vitro tests used: Kinetic rate constants with peptides

- Idea: Kinetic rate (velocity) of the reaction between peptide and sensitizer **indicates how much allergenic protein modifications are made**
- Kinetic peptide reactivity assay measures this rate
- Same assay as with HPLC-UV (DPRA) or LC-MS peptide reactivity assay:
  - Incubate peptide and sensitizer – monitor reaction

- Multiple doses and multiple time points – high throughput assay in microtiter plates
- Peptide depletion mesured by fluorescent test
- Ln(100-depletion) is ploted vs. time or vs. concentration
- ⇒ **rate constant K_{max}**
- = kDPRA – Validation study currently under peer-review



D. Roberts, A. Natsch, *Chem Res. Toxicol.* **2009,** *22,592-603*.

Quantitative contribution to potency assessment of individual tests

- All parameters correlate to potency
- Shown for all chemicals (sensitizers and non-sensitizers)
  - This analysis 'includes' the ability of the tests for hazard ID
  - Strongest for the quantitative peptide reactivity

Table 1: R^2 coefficient for linear regression of logarithmic *in vitro* parameters vs. pEC3

		Set I: With KeratinoSens (n = 173)	Set II: with KeratinoSens and h-CLAT and DPRA (n = 154)
kDPRA	k_{max}	0.51	0.45
KeratinoSens	EC1.5	0.29	0.27
	EC3	0.35	0.35
	IC50	0.34	0.34
h-CLAT	EC150		0.28
	EC200		0.16
	MIT[1)]		0.36
	CV75		0.43
DPRA	kCys		0.33
	kLys		0.16

Quantitative contribution to potency assessment of individual tests

- Shown here **excluding the non-sensitizers** (EC3 < 30%):
  - Correlation is weaker as for all chemicals (hazard ID no longer included)
  - Strongest for the quantitative peptide reactivity
  - MIE may be key rate limiting step most strongly correlating to potency

R^2 coefficient for linear regression of logarithmic *in vitro* parameters vs. pEC3

		Set I: With KeratinoSens, EC3 <30% (n = 121)	Set II: with KeratinoSens and h-CLAT and DPRA, EC3 <30% (n = 107)
kDPRA	k_{max}	0.40	0.32
KeratinoSens	EC1.5	0.13	0.11
	EC3	0.17	0.16
	IC50	0.14	0.14
h-CLAT	EC150		0.17
	EC200		0.04
	MIT[1)]		0.20
	CV75		0.21
DPRA	kCys		0.19
	kLys		0.17

Combining datasources: Improved predictivity and data redundancy

- Combining data-inputs with multiple regression improves predictivity
- Combining peptide reactivity with one cellular test most predictive – beyond there is data redundancy

R^2 coefficient for linear multiple regression of logarithmic in vitro parameters vs pEC3

	All chemicals (n = 154)	**Clear sensitizers, EC3 <30% (n = 107)**
k_{max}	0.45	0.32
KS+k_{max}	0.57	0.38
h-CLAT+k_{max}	0.59	0.40
h-CLAT +KS+k_{max}	0.60	0.41
h-CLAT +KS+DPRA	0.54	0.27
h-CLAT +KS	0.51	0.27

Cases study Givaudan: Deriving NESIL without animal testing

- All the input data are Log-transformed and normalized (set to zero if molecule is inactive)
- Multiple regression models used to predict pEC3
  - Logarithmic molar EC3 value
- This predicts a **Likely LLNA EC3** as point of departure (PoD)

Global model:

$$pEC3 = 0.04 + 0.38 \times \textbf{Log } K_{norm} + 0.25 \times \text{Log } \textbf{EC1.5}_{norm} + 0.25 \times \text{Log } \textbf{IC50}_{norm} - 0.19 \times \text{Log } VP_{norm}$$

Peptide reactivity **KeratinoSens** **Volatility**

Natsch, A., Emter, R., Gfeller, H., Haupt, T., and Ellis, G. (2015). *Toxicol. Sci.* **143**(2), 319-32.

Published also as OECD case study Nr. 7 in ENV/JM/MONO(2016)29/ANN1

Domain and global assessments

- Idea: Closely related chemicals acting by the same chemical reaction mechanism (=mechanistic domains) will behave similarly
  - a) In *in vitro* tests
  - *b)* In *in vivo* tests
- Thus a local / domain model ist trained with chemicals from one mechanistic domain
- Chemicals are ideally predicted with a local / domain model
- **Chemicals which do not fall into a domain model will be predicted with a global model**

Uncertainty assessment

- Search for closely related molecules **with existing *in* vivo data** in database with **similar substructure** for the putative reactive part of the molecule
- Perform same assessment (DA / DIP /IATA)
- **Compare outcome to *in vivo* situation**
  - This helps to assess uncertainty for the very specific subdomain of chemicals
  - Based on the uncertainty assessment, NESIL may be adjusted

- If uncertainty is low ⇒ Adjustment factor = 2
  - Note: NESIL is defined as a NOEL
  - LLNA is extrapolated between NOEL and LOEL – 3-fold proliferation is already an 'effect'

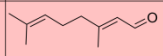
- If uncertainty is high – adjust based on uncertainty assessment

Application to derive NESIL: Case study Citral

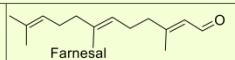
- One infocard covers all steps for each molecule; same info card generated for each molecule to be assessed

Case Study on Citral

a) Data, assessment with DIP and additional mechanistic tests

Name:	**Citral**	**DPRA:**	Cys-depletion: 85.7 % Lys-depletion : 16.9 % Positive in high category
Structure:		**KeratinoSens:**	EC 1.5: 23 µM IC 50: 183 µM Positive
TIMES parent:	Strong sensitizer, Di-substituted αβ-unsaturated aldehydes	**Prediction global model:**	EC3 5.2 %
TIMES metabolite:	Weak sensitizer, hydroperoxide	**Prediction Local model:**	EC3 6.8 %
LC-MS:	Cor1C420 depletion: 27.2 % Adduct: direct Michael Acceptor (MA) adduct 8.1%; Peptide oxidation predominant	**Additional mechanistic tests:**	Reactivity with amine groups to test for Schiff Base MoA
Domain attribution:	Michael acceptor	**Results mechanistic tests:**	Low amine reactivity, local model with BA-test indicates lower Sensitization potential (EC3 = 11.6%); MA MoA confers stronger sensitization potential, assess with MA model.

b) Analysis of close analogues for uncertainty assessment

Close analogue:		
Rationale for selecting close analogue:	β-alkyl-substituted αβ-unsaturated aldehydes	Di-substituted αβ-unsaturated aldehydes
Prediction close analogue global model:	EC3 2.3%	EC3 1.7%
Prediction close analogue local model (MA):	EC3 6.9 %	EC3 3.4 %
In vivo results close analogue:	EC3 11.7 %	EC3 7.5 %
Prediction accuracy analogues:	Local model predicts within 2-fold error; on conservative side	

c) IATA assessment and discussion

Weight of evidence assessment: Directly reactive Michael acceptor based on LC-MS, aldehyde MoA of lower potency. Take EC3 = 6.8% from local MA model, moderate sensitizer, PoD: 1700 µg/cm^2
Uncertainty assessment based on close analogues: Predictions with local model for close analogues indicate high certainty, predictions on conservative side. Adjustment factor to derive NESIL = 2.
In vivo results: LLNA EC3 5.7% (1425 µg/cm^2, weighted average 11 studies[16]), 9.3% (Median 6 studies[31]), PoD LLNA and human: 1400 µg/cm^2, LOEL human 3870 µg/cm^2
Discussion: *In vitro* prediction vs. *in vivo* data: PoD derived from *in vitro* tests close to LLNA and human PoD, below human LOEL.

Prediction by regression model

IATA: additional weight of evidence

Uncertainty analysis: Close analogues with DA / DIP results and in vivo data

WoE and conclusions

Case study Citral: Prediction by DA and IATA

- Local Michael acceptor model predicts EC3 of 6.8%
- Close to global model (EC3 = 5.2%)

Prediction by local model

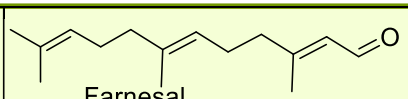
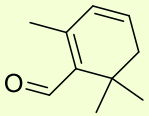
TIMES indicates MA acceptor, which is verified by LC-MS based protein binding test

Name:			Cys-depletion: 85.7 % Lys-depletion : 16.9 % Positive in high category
Structure:		**s:**	EC 1.3: 23 µM IC 50: 183 µM Positive
TIMES parent:	Strong sensitizer, Di-substituted αβ-unsaturated aldehydes	**Prediction global model:**	**EC3 5.2 %**
TIMES metabolite:	Weak sensitizer, hydroperoxide	**Prediction Local model:**	**EC3 6.8 %**
LC-MS:	Cor1C420 depletion: 27.2 % Adduct: direct Michael Acceptor (MA) adduct 8.1%; Peptide oxidation predominant	**Additional mechanistic tests:**	Reactivity with amine groups to test for Schiff Base MoA
Domain attribution:	Michael acceptor	**Results mechanistic tests:**	Low amine reactivity, lo with BA-test indicates lov tization potential (EC3 = 11.6%); MA MoA confers stronger sensitization potential, assess with MA model.

Additional testing for specific molecular classes

Case study Citral: Uncertainty assessment

- Related β-branched, αβ-unsaturated aldehydes assessed
- Local MA models predicts EC3 within 2-fold error, on conservative side
- **Indicates high certainty of the prediction for Citral**

Close analogue:	 Farnesal	 Safranal
Rationale for selecting close analogue:	β-alkyl-substituted αβ-unsaturated aldehydes	Di-substituted αβ-unsaturated aldehydes
Prediction close analogue global model:	**EC3 2.3%**	**EC3 1.7%**
Prediction close analogue local model (MA):	**EC3 6.9 %**	**EC3 3.4 %**
In vivo results close analogue:	**EC3 11.7 %**	**EC3 7.5 %**
Prediction accuracy analogues:	**Local model predicts within 2-fold error; on conservative side**	

Case study Citral: Conclusions

Weight of evidence assessment:

- Directly reactive Michael acceptor based on LC-MS
- **EC3 = 6.8% from local Michael Acceptor model**, moderate sensitizer, **PoD: 1700 µg/cm²**

Uncertainty assessment based on close analogues: Predictions with for close analogues indicate high certainty, predictions on conservative side. Use adjustment factor of 2

In vivo results:

- **LLNA EC3 5.7%** (weighted average 11 studies) = **1400 µg/cm²**
- **Human: NOEL 1400 µg/cm²**, LOEL human 3870 µg/cm²

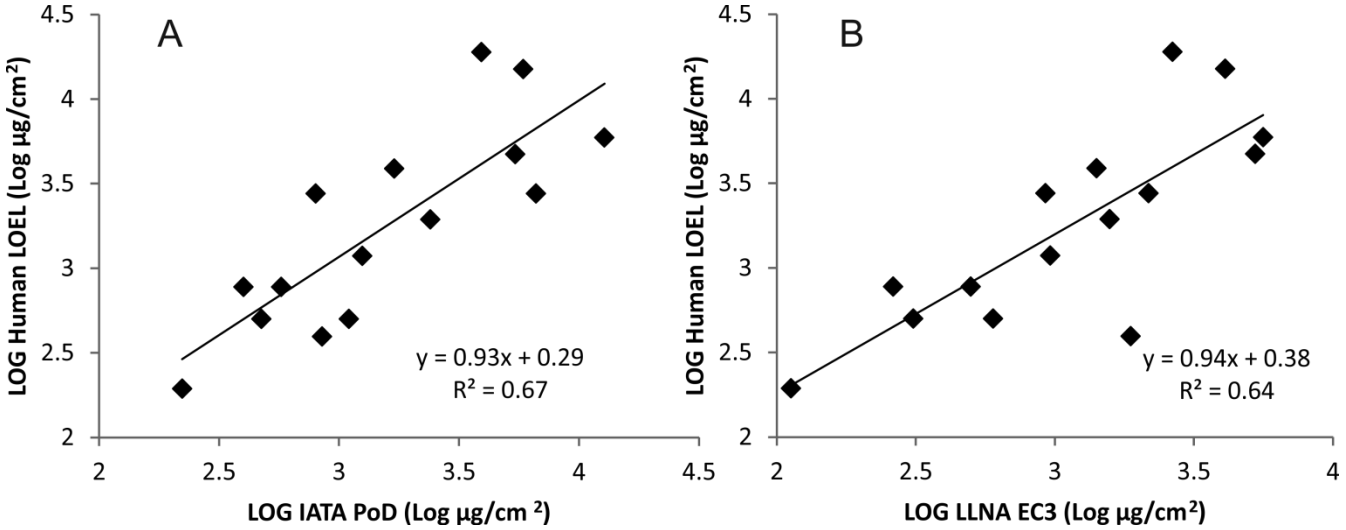
(NOEL = No observed effect level, LOEL lowest observed effect level)

Discussion: **PoD derived from *in vitro* tests close to LLNA and human PoD, below human LOEL**

With adjustment factor of 2: In vitro derived NESIL is 850 µg/cm²

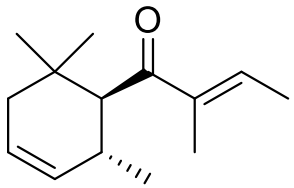
Case studies: Molecules with high quality LLNA and human data

- Same assessment done on 15 fragrance molecules with human NOEL, LOEL and LLNA EC3
- The PoD (= predicted LLNA EC3) is compared to LLNA and human data
  - Overall good correlation of *in vitro* - drived PoD with Human LOEL, PoD 0.29 Log units (=2-fold) below LOEL
  - Similar correlation between LLNA EC 3 and human LOEL



Case studies on new molecules: α-methyldamascone

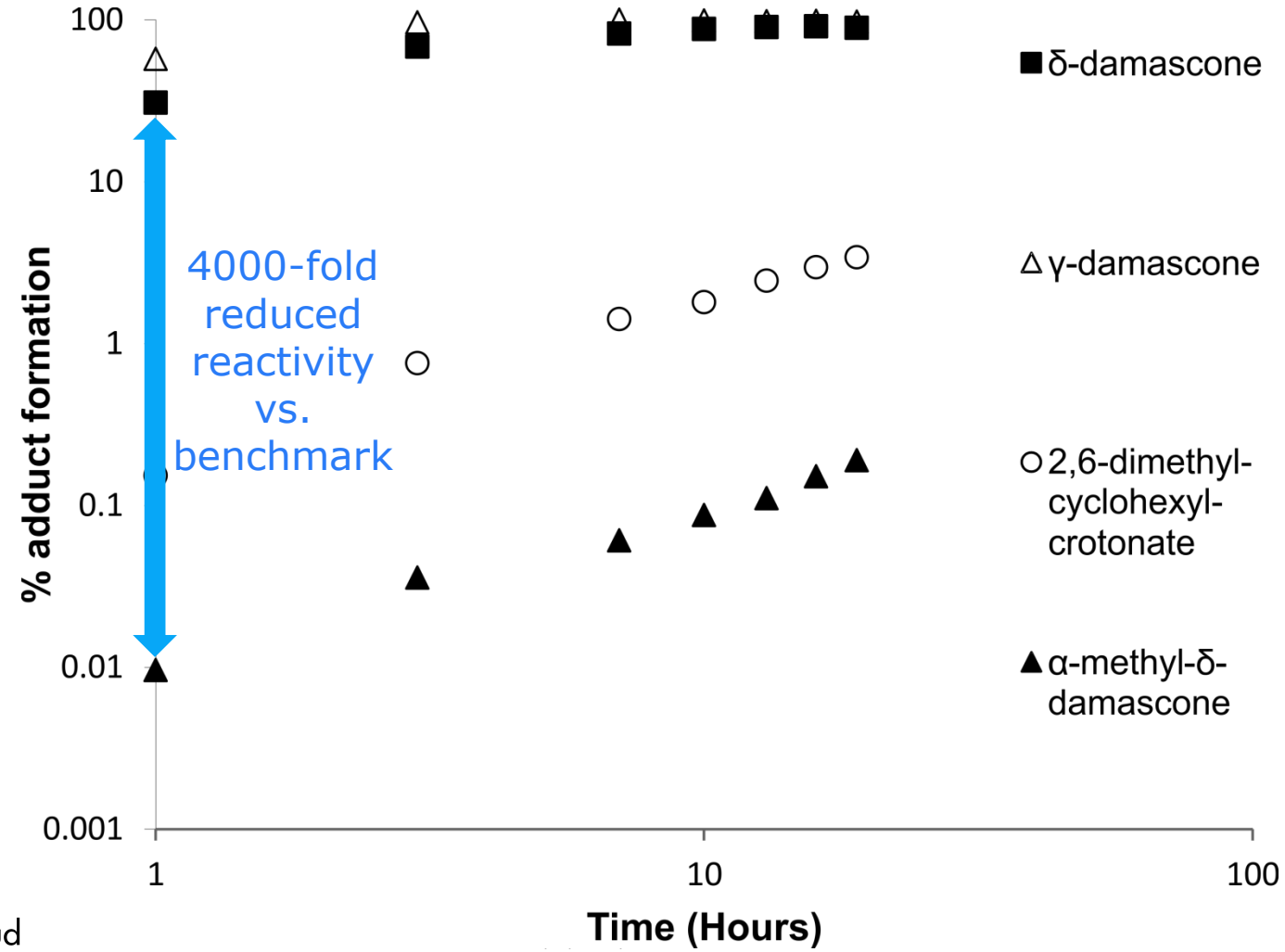
a) Data, assessment with DIP and additional mechanistic tests

Name:	**α-methyl-δ-damascone [(E)-2-methyl-1-((1S,2R)-2,6,6-trimethylcyclohex-3-en-1-yl)but-2-en-1-one]**	**DPRA:**	Cys-depletion: 4.4 % Lys-depletion : 0.2 % **Negative in minimal category, <0.1%** peptide adduct
Structure:		**KeratinoSens:**	EC 1.5: >1000 µM IC50: 69.6 µM **Negative**
TIMES parent:	**strong sensitizer, α,β-Carbonyl compounds** with polarized double bonds	**Prediction global model:**	EC
TIMES metabolite:	strong sensitizer, αβ-Carbonyl compounds with polarized double bonds	**Prediction Local model:**	EC3 58
LC-MS:	Cor1C420 depletion: 6.8 %; Adduct: **trace (< 0.5%) direct MA adduct**	**Additional mechanistic tests:**	**Kinetic profiling of adduct formation** vs. benchmarks, see Figure 4 main document
Domain attribution:	Michael acceptor	**Results mechanistic tests:**	4000-fold reduction in kinetic reaction rate vs. damascones

Better characterize reactivity of close damascone analogue.

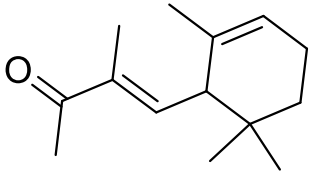
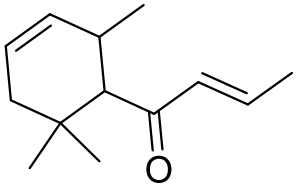
α-methyldamascone: Kinetic adduct formation

- Low reactivity cannot be accurately quantified based on depletion
- Additional test to quantify and verify low reactivity: Kinetic adduct formation



Case studies on new molecules: α-methyldamascone

a) **Analysis of close analogues for uncertainty assessment**

Close analogue:	 Methylionone	 Delta-damascone
Rationale for selecting close analogue:	α,β-Carbonyl compounds with polarized double bonds	α,β-Carbonyl compounds with polarized double bonds
Prediction close analogue global model:	***Negative, EC3 34.6% by cytotoxicity***	EC3 1%
Prediction close analogue local model (MA):	***Negative, EC3 63.3 % by cytotoxicity***	**EC3 2.7 %**
In vivo results close analogue:	EC3 21.8 % **HRIPT > 70'866 µg/cm^2**	**EC3:** 9.6/0.9/5.2; **Median 5.2%** HRIPT **LOEL 500 µg/cm^2**
Prediction accuracy analogues:	**Good prediction with local model, esp. for human data**	

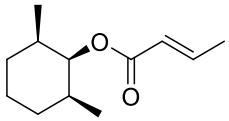
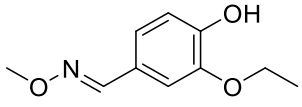
α-methyldamascone: IATA assessment and discussion

- Weight of evidence assessment:
  - Hazard assessment 2 out of 3: Negative (Negative KS and negative DPRA)
  - Very low residual reactivity observed by adduct formation
  - predicted very weak sensitizer, EC3 60%; PoD 15'000 µg/cm^2
- Uncertainty assessment based on close analogues: Prediction with local model for close analogues indicate high certainty, esp. for human data
  - Note: Methylionone has equal cytotoxicity (IC50 = 58 µM), highly similar structure
  - Methylionone is non-reactive and negative in human tests at high conc.; positive LLNA at EC3 21% could be due to irritation.
- *In vivo* results: **Negative, EC3 >25%**
  - LLNA performed after this prediction was made
- Discussion
  - *In vivo* data congruent with prediction and observation of very low reactivity
  - *In vitro* and *in vivo* data overrule the TIMES alert: TIMES sees 2D alerts, steric effects not taken into account!

Case studies: Two other new molecules, later challenged by LLNA

- Two molecules:
  - A) Crotonate: Predicted weak sensitizer, low direct reactivity observed
  - B) Oxime ether: Parent non sensitizer, weak sensitizer predicted due to metabolic activity

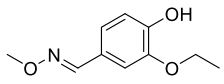
Table 3. Risk assessment for three new molecules without animal data – later challenged by LLNA [1)]

Chemical structure	**TIMES prediction**	**KS result**	**Peptide reactivity**	**PoD IATA (µg/cm²)**	**Uncertainty assessment IATA PoD**	**Adjustement factor to derive NESIL**	**IATA derived NESIL (µg/cm²)**	**LLNA result [1)]**
 2,6-dimethylcyclohexyl-crotonate	weak sensitizer, α,β-Carbonyl / polarized double bonds	**negative**	**Cor1C420: 5% direct MA adduct; DPRA low category**	**EC3 30 – 40%**; 11'000 µg/cm²	low uncertainty	2	5500	**Positive,** EC3 21%; 5450 µg/cm²
 (E)-3-ethoxy-4-hydroxybenzaldehyde O-methyl oxime	Parent: Non-sensitizer **Metabolite :** Strong sensitizer, **Quinoide oxime structure**	**negative**	Cor1C420: 5.7 % depletion; no adduct; **DPRA negative**	**EC3 30 – 50** %, 7500 µg/cm².	High certainty for four tested analogues; Remaining uncertainty due to metabolic activation	2	3750	**Negative,** EC3 >25%; >6250 µg/cm²

[1)] Determined after IATA assessment was made

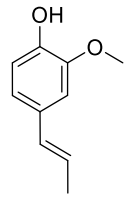
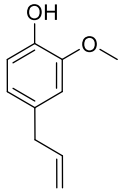
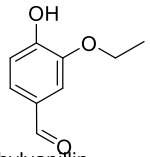
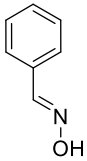
Case study: Oxime ether, potential prohapten

•**Data, assessment with DIP and additional mechanistic tests**

Name:	**(E)-3-ethoxy-4-hydroxybenzaldehyde O-methyl oxime**	**DPRA:**	Cys-depletion: 7.3 % Lys-depletion : 2.9 % **Negative** in minimal category, no adduct
Structure:		**KeratinoSens:**	EC 1.5: >1000 µM IC50: >1000 µM **Negative**
TIMES parent:	Non-sensitizer	**Prediction global model:**	Non-sensitizer; EC3 >100 %
TIMES metabolite:	**Strong sensitizer ; Quinone methide(s)/imines, Quinoide oxime structure, Nitroquinone**	**Prediction Local model:**	
LC-MS:	Cor1C420 depletion: 5.7 % Adduct: no adduct	**Additional mechanistic tests:**	**Test in presence of metabolic system (LC-MS and KS)**
Domain attribution:	Quinone methide precursor	**Results mechanistic tests:**	**Small trace of peptide adduct in presence of microsomes, positive in KeratinoSens with S9**

Case study: Oxime ether, potential prohapten

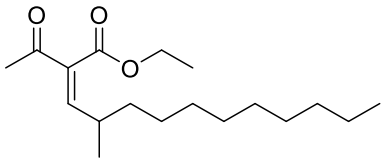
- **Analysis of close analogues for uncertainty assessment**

Close analogue:	 Isoeugenol	 Eugenol	 Ethylvanillin	 Benzaldoxime
Rationale for selecting close analogue:	Quinone methide precursor	Quinone methide precursor	Substructure of target	Aromatic oxime; Substructure of target
Prediction close analogue global model:	**EC3 1.6 %**	**EC3 14.1 %**	**EC3 41 %**	**EC3 29.8%**
Prediction close analogue local model:	EC3 7.9 %	EC3 16.2 %	EC3 49 %; >100% model with BA-test	No model
In vivo results close analogue:	**EC3 1.8 %**	**EC3 12.9 %**	**> 50%**	**> 20%**
Prediction accuracy analogues:	Good prediction with local and global model, better accuracy for global model in case of isoeugenol			

Case study on new material: Risk assessment without LLNA

- New molecule predicted as sensitizer by TIMES, KeratinoSens, DPRA and LC-MS assay

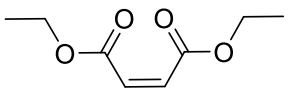
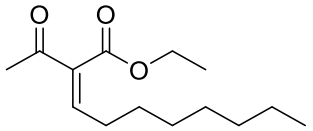
a) Data, assessment with DIP and additional mechanistic tests

Name:	**ethyl (Z)-2-acetyl-4-methyltridec-2-enoate**	**DPRA:**	Cys-depletion: 27.8 % Lys-depletion : 1.3 % **Positive in low category**, ca. **6.6% direct adduct** with Cys-peptide
Structure:		**KeratinoSens:**	EC 1.5: 7.95 µM EC3 not reached due to cytotoxicity IC50: 13.2 µM **Positive**
TIMES parent:	**strong sensitizer, αβ-Carbonyl compounds with polarized double bonds**	**Prediction global model:**	**EC3: 5.1 %**
TIMES metabolite:	strong sensitizer, αβ-Carbonyl compounds with polarized double bonds	**Prediction Local model:**	**EC3: 14 %**
LC-MS:	Cor1C420 depletion: 14 % Adduct: **direct MA adduct** Peptide oxidation predominant	**Additional mechanistic tests:**	Not needed
Domain attribution:	Michael acceptor	**Results mechanistic tests:**	n/a

Case study on new material: Risk assessment without LLNA

- Uncertainty assessment:
  - Related analogues: Michael acceptors with the double bond activated by two carbonyl groups
  - Well predicted by global and local model, here global model more accurate and on conservative side
  - Use global model for conservative assessment

a) Analysis of close analogues for uncertainty assessment

Close analogue:	 Diethylmaleate	 ethyl (Z)-2-acetyldec-2-enoate
Rationale for selecting close analogue:	Double activated MA-ester	Double activated MA-ester, substructure of target
Prediction close analogue global model:	**EC3 1.4%**	**EC3 3%**
Prediction close analogue local model (MA):	**EC3 3.8 %**	**EC3 5.6 %**
In vivo results close analogue:	**EC3 2.1 %**	**EC3 2.6 %**
Prediction accuracy analogues:	**Good prediction with local and global model, better accuracy for global model for these double activated MA-esters**	

ethyl (Z)-2-acetyl-4-methyltridec-2-enoate: IATA assessment and discussion

- Weight of evidence assessment:
  - Hazard assessment 2 out of 3: Positive (Positive KS and positive DPRA)
  - Directly reactive Michael acceptor
  - Conservative assessment takes EC3 from global model
  - EC3 = 5.1%; PoD 1250 µg/cm^2
- Uncertainty assessment based on close analogues:
  - Prediction with global model for close analogues indicates high certainty
  - adjustment factor to derive NESIL = 2, since conservative assessment from global model taken

  In vivo results:
  - No LLNA planned, use NESIL from this assessment
  - **NESIL = 625 µg/cm^2**

kDPRA pending publication – Case studies and approach published in detail with lots of supporting information

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Predicting Skin Sensitizer Potency Based on *In Vitro* Data from KeratinoSens and Kinetic Peptide Binding: Global Versus Domain-Based Assessment

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Research Article

Deriving a No Expected Sensitization Induction Level for Fragrance Ingredients Without Animal Testing: An Integrated Approach Applied to Specific Case Studies

Andreas Natsch,*,[1] Roger Emter,* Tina Haupt,* and Graham Ellis†

Conclusions

- Seven tests covering three key events in skin sensitization AOP are in OECD guidelines
- Defined approaches allow hazard ID
- Individual tests parameters correlated to LLNA potency
- Potency assessment possible based on integration of data
- Taking chemical domain into account improves predictivity
- Read-across anchored by *in vitro* and *in vivo* data helps for uncertainty assessment

- Deriving a NESIL for risk assessment without animal testing has become possible

Thank you

Contact