Deriving a no expected sensitization induction level for fragrance ingredients without animal testing: An integrated approach applied to specific case studies

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engage your senses

Agenda

1. **Defined approach (DA) + Data Interpretation procedure (DIP)**
   1. Potency based on kinetic peptide reactivity and quantitative KeratinoSens data and Regression models
2. **Domain and global assessments**
3. **IATA: Targeted additional testing**
4. **Uncertainty assessment**
5. **Adjustment of NESIL based on uncertainty assessment**
6. **Types of case studies**
7. **Case study Citral**
8. **Case studies: Molecules with high quality LLNA and human data**
9. **Case studies new molecules**

Overall approach

- Determine «**most likely LLNA EC3 value**» as **Point of departure** (PoD) with a defined approach (DA) using a data integration procedure (DIP)
  - Global model for all chemicals
  - Use a domain-model for prediction if available

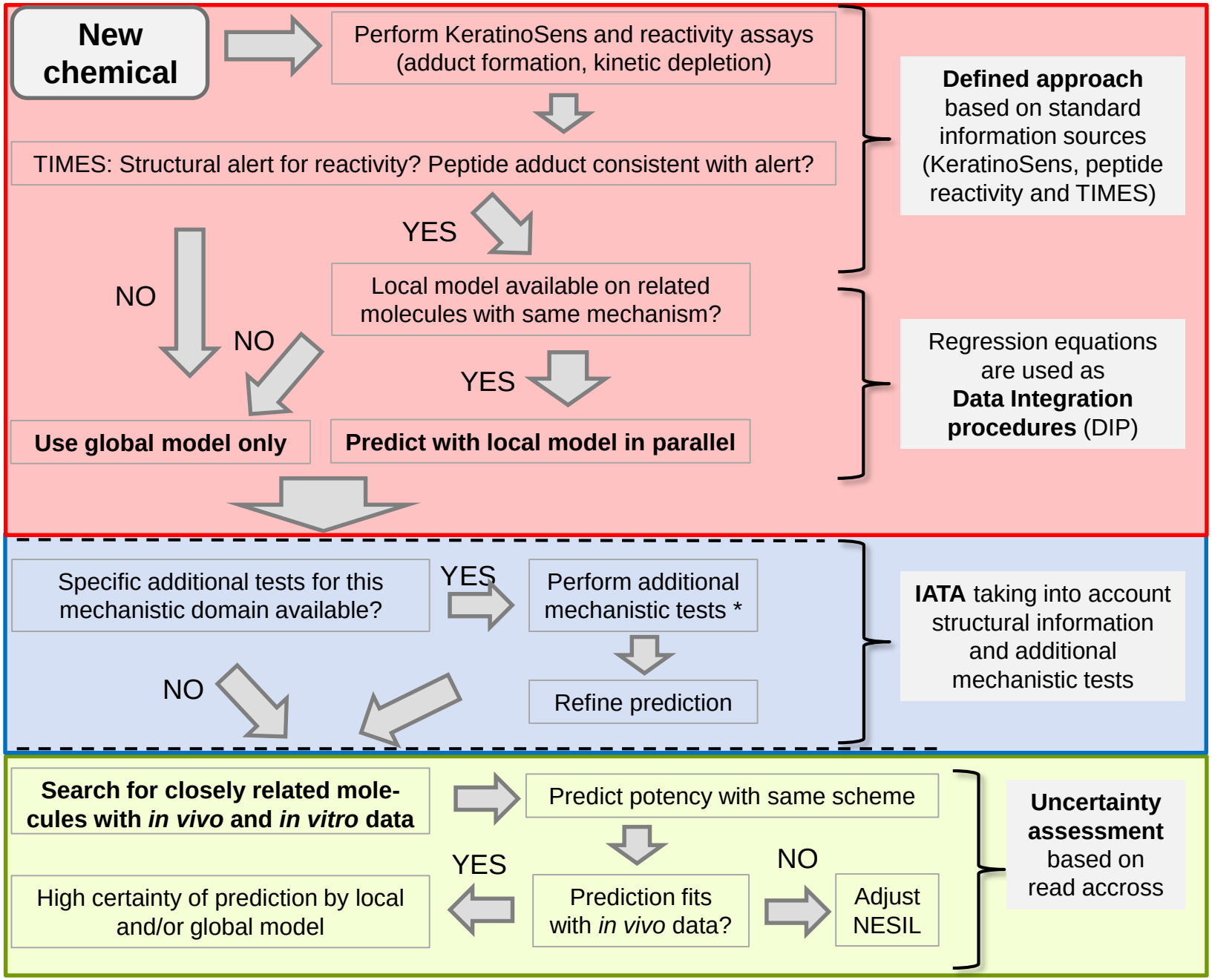
- (Opt:) Refine prediction with targeted **additional testing** based on domain of molecule : Integrated approach for testing and assessment (IATA), requires some expert input

- Search for analogues in database with *in vitro* and *in vivo* data: Predict with same approach
  - Determine **uncertainty** based on prediction accuracy

- Determine an **adjustment factor** based on uncertainty analysis

- **Divide PoD by adjustment factor to arrive at a final NESIL**

Overall approach: Schematic – details to follow…..



Defined approach (DA) : Potency based on kinetic peptide reactivity and quantitative KeratinoSens data

- **Standard input data for all molecules in DA:**
  - Dose response from KeratinoSens: EC1.5, EC3, IC50
  - Kinetic peptide reactivity (Rate constant for depletion)
  - Peptide adduct formation for reaction mechanism
  - TIMES for attribution to structural domains

} Continous variables

- Data interpretation procedure (DIP): Regression equations to predict **Likely LLNA EC3** as point of departure (PoD)

Global model:

$$pEC3 = 0.04 + 0.38 \times \text{Log } K_{norm} + 0.25 \times \text{Log } EC1.5_{norm} + 0.25 \times \text{Log } IC50_{norm} - 0.19 \times \text{Log } VP_{norm}$$

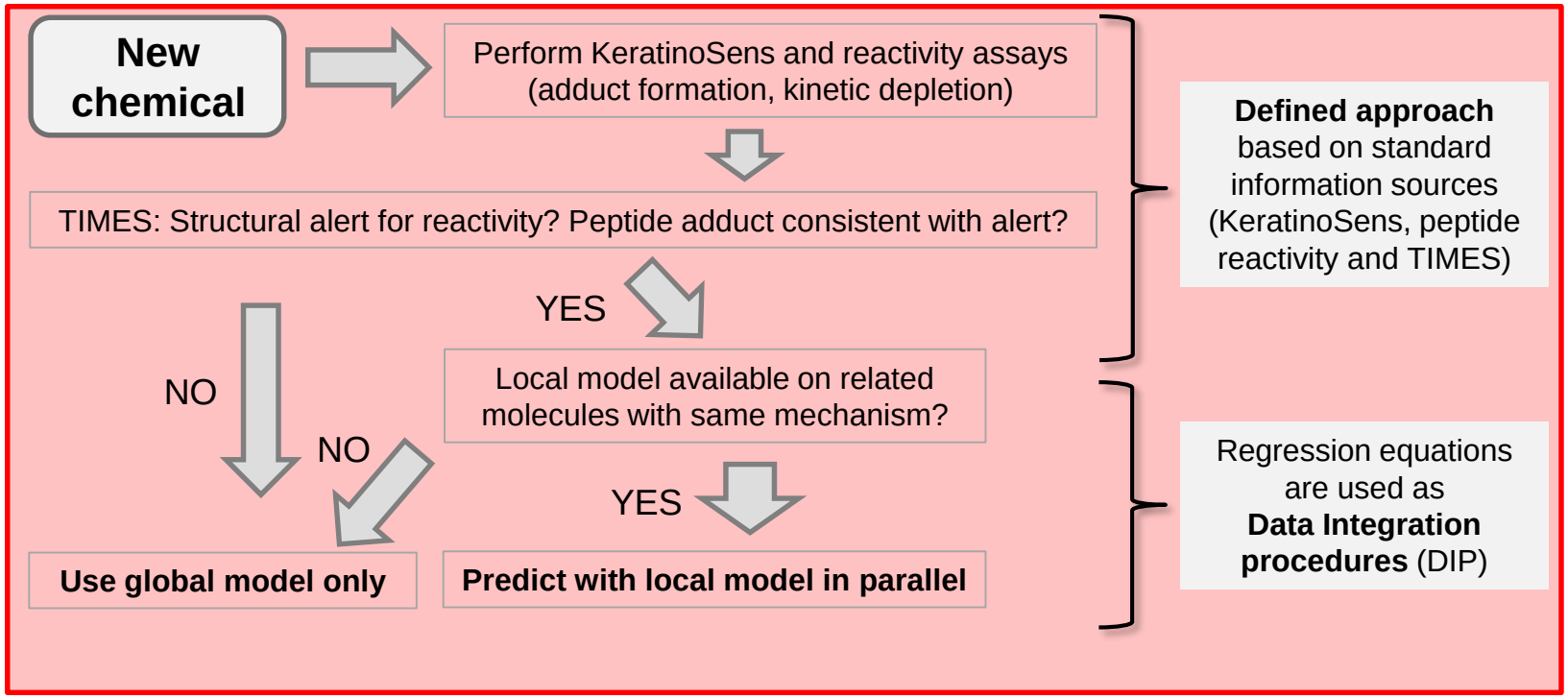
Peptide reactivity **KeratinoSens** **Volatility**

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

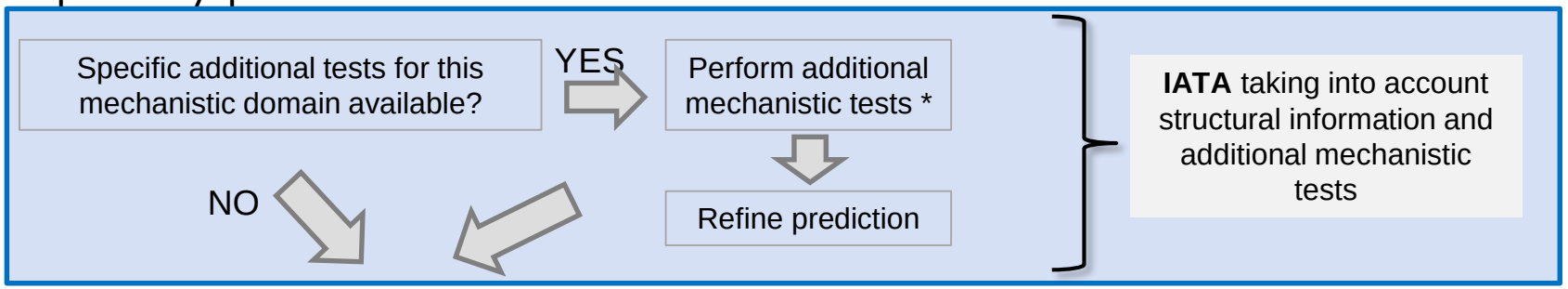
Domain and global assessments

- Based on TIMES SS and experimental peptide adduct data: Attribute chemicals to a domain (if applicable)
  - Global model for all chemicals
  - Use a domain-model for prediction if available

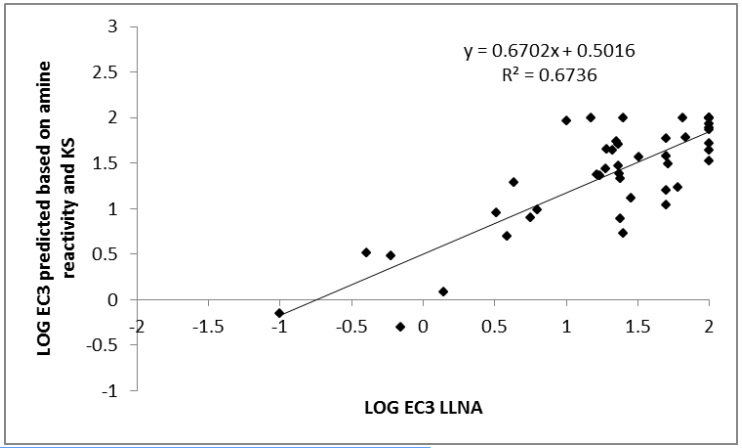


IATA: Targeted additional testing

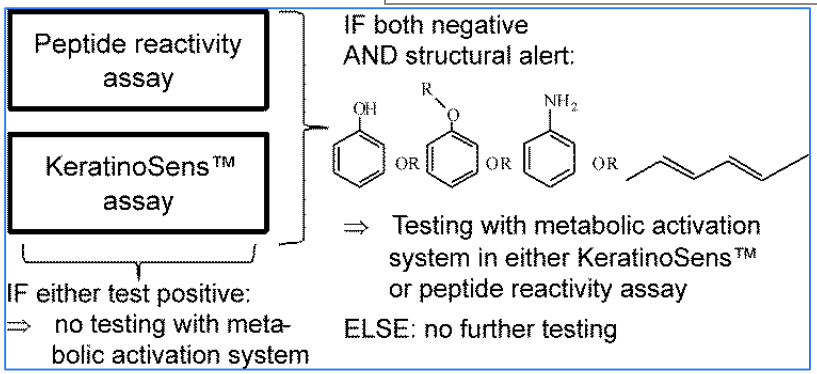
- Depending on the structure / domain, specific tests may help to refine the potency prediction.



- Examples:
- A) **Aldehydes:** Reactivity test using butylamine to measure rate of SchiffBase formation
  ⇒ Local model combined with KS data

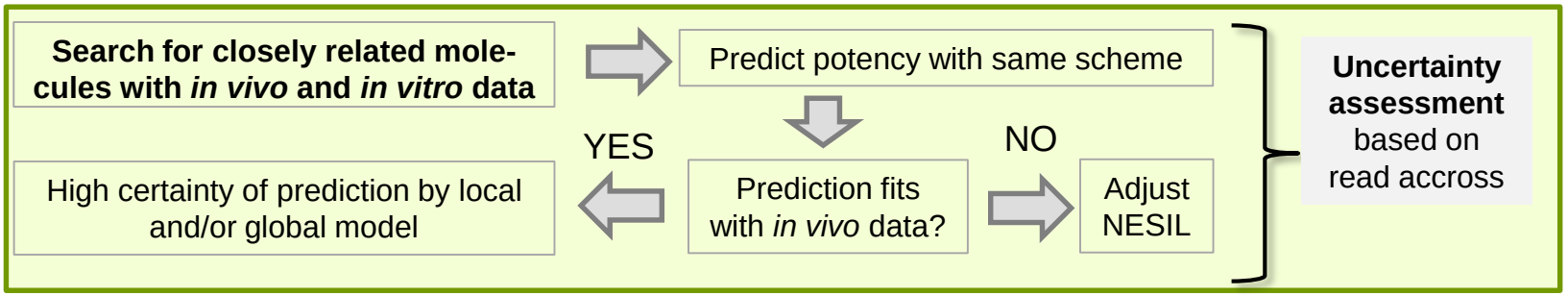


- B) **Phenolic prohaptens**: KS or peptide reactivity with activation system



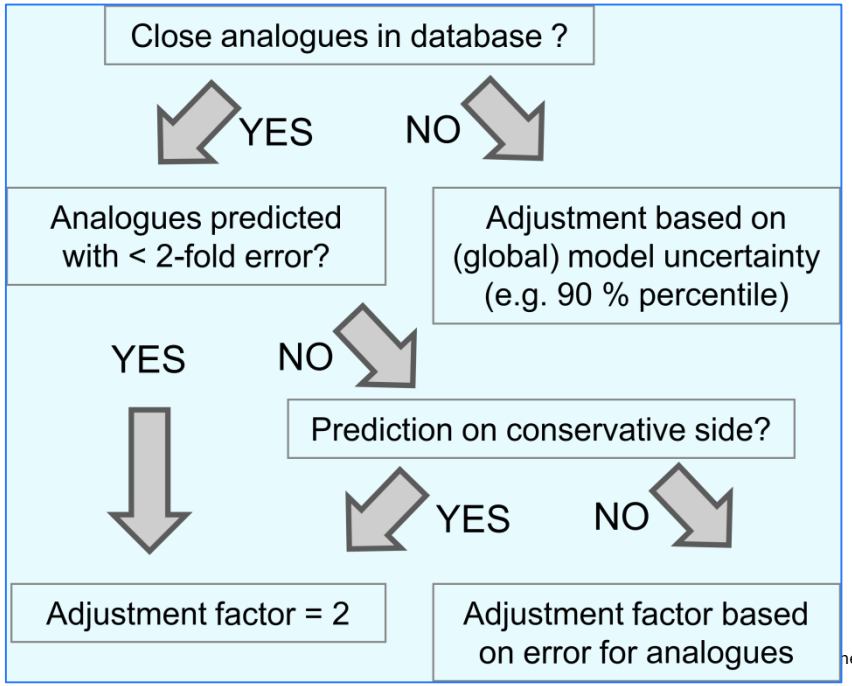
Uncertainty assessment

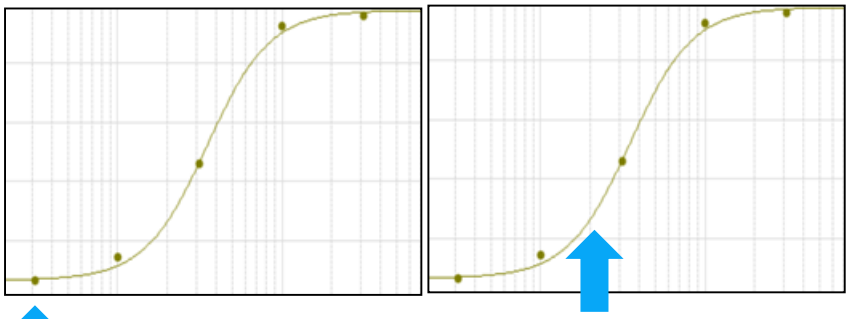
- Search for closely related molecules **with existing *in* vivo data** in database with **similar substructure** for the putative reactive part of the molecule
- Perform same assessment (DA / DIP /IATA)
- **Compare outcome to *in vivo* situation**
  - This helps to assess uncertainty for the very specific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



Adjustment of NESIL based on uncertainty assessment

- The predicted PoD (likely EC3 value) is transformed into a NESIL
- If uncertainty is low ⇒ Proposed 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
- If no uncertainty assessment possible – adjust based on precision of global model





Four types of case studies done:

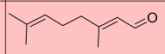
- 15 molecules with mainly congruent LLNA and human data, with human NOEL and LOEL (No /Lowest observed effect dose) data
  - Allows direct comparison of derived NESIL with human and animal derived NESIL
- 7 molecules with partly discordant human and LLNA data / missing human LOEL values
  - Indicates how DA /IATA compares against LLNA or human data for difficult cases
- 3 new molecules – tested as case studies and later challenged by LLNA
  - Molecules tested when REACH still considered LLNA as mandatory, unique opportunity to challenge predictions by *in vivo* data
- 4 new molecules, no LLNA data available nor currently planned
  - Demonstrates approach to risk assessment in absence of animal data

Case study Citral

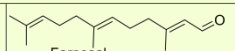
- One infocard covers all steps for each molecule; same info card generated for each molecule to be assessed

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:	 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	

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.

DA and DIP results

IATA: additional tests and results

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%)
- IATA: SchiffBase formation alternative MoA
  - Amine reactivity would indicate weaker activity – Michael acceptor MoA confers stronger reactivity and sensitization: **Use local MA model**

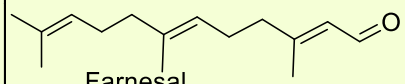
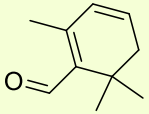
TIMES indicates MA acceptor, which is verified by LC-MS based protein binding test

Name:			Cys-depletion: 85.7 % Lys-depletion : 16.9 % Positive in high category
Structure:		**s:**	EC 1.5: 2 IC 50: 183 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 amine groups to for Schiff Base M
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.

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.

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
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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

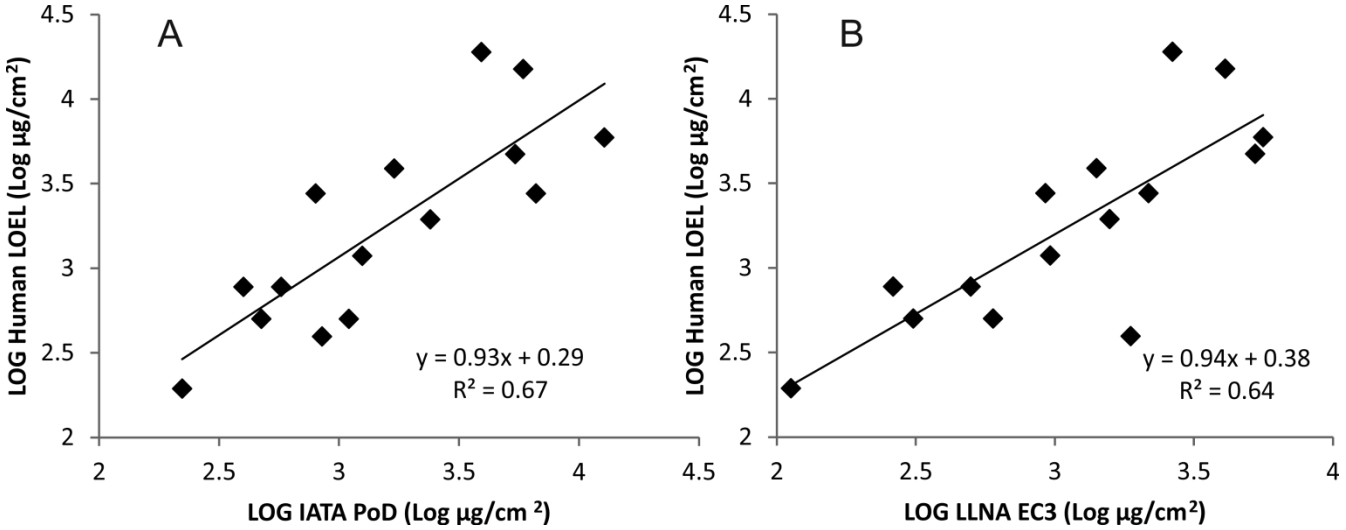
- **IATA assessment and discussion**

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/cm²**
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/cm², weighted average 11 studies[16]), **9.3%** (Median 6 studies[31]), **PoD LLNA and human: 1400 µg/cm²**, LOEL human 3870 µg/cm²
Discussion: *In vitro* prediction vs. *in vivo* data: **PoD derived from *in vitro* tests close to LLNA and human PoD, below human LOEL.**

- **Final NESIL: PoD / adjustment factor of 2: 850 µg/cm²**
- **NESIL human data: 1400 µg/cm²**
- **NESIL LLNA data: 1400 µg/cm²**

Case studies: Molecules with high quality LLNA and human data

- 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 IATA PoD with Human LOEL, PoD 0.29 Log units (=2-fold) below LOEL
  - Similar correlation between LLNA EC 3 and human LOEL



Case studies: Molecules with high quality LLNA and human data

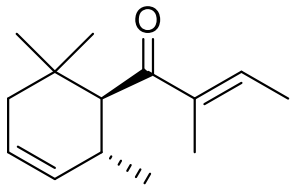
- For illustration: Summary of seven case studies

Table 1. Case studies 1- 7 on sensitizers with congruent human and LLNA data leading to similar NESIL [1) 2)]

Chemical	NESIL human (human NOEL) (µg/cm²)	Human LOEL (µg/cm²)	NESIL/ EC3 LLNA (µg/cm²)	PoD IATA (µg/cm²)	Uncertainty assessment IATA PoD	Adjustement factor to derive NESIL	IATA derived NESIL (µg/cm²)
Citral	**1400**	3876	1414	1700	high certainty	2	**850**
Phenylacetaldehyde	**590**	1180	962	1250	high certainty	2	**625**
Cinnamic aldehyde	**591**	775	262	575	high certainty	2	**288**
Cinnamic alcohol	**3000**	4724	5250	5425	high certainty, predictions of analogues on conservative side	2	**2712**
Isoeugenol	**250**	775	498	400	limited; analogues well predicted	2 if taking conservative model	**200**
2-phenyl-propionaldehyde	**388**	1938	1575	2400	high certainty	2	**1200**
2-hexyliden cyclopentanone	**300**	500	600	1100	high certainty	2	**550**

Case studies on new molecules: α-methyldamascone

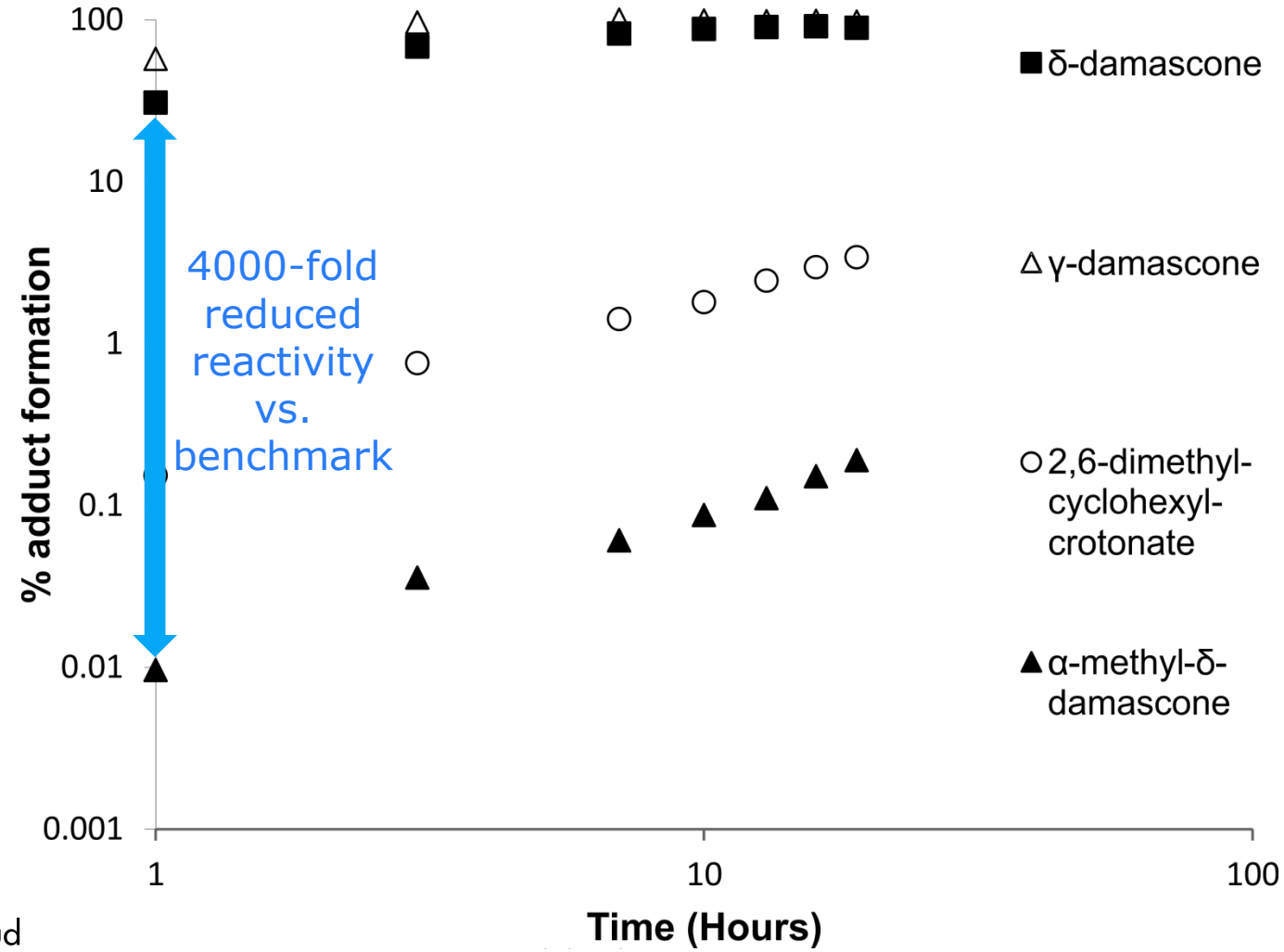
a) Data, assessment with DIP and additional mechanistic tests

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.

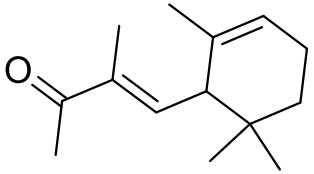
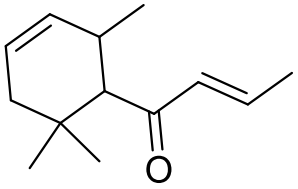
α-methyldamascone: Kinetic adduct formation

- 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**	

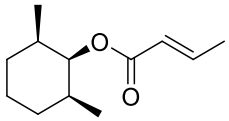
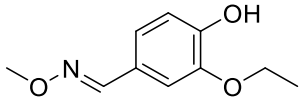
α-methyldamascone: IATA assessment and discussion

- 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

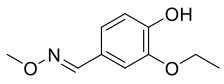
Table 3. Risk assessment for three new molecules without animal data – later challenged by LLNA [1)]

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

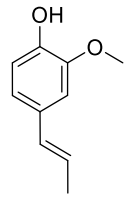
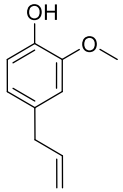
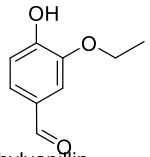
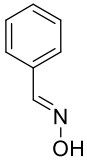
Case study: Oxime ether, potential prohapten

•**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

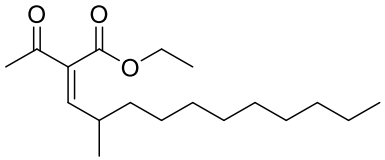
- **Analysis of close analogues for uncertainty assessment**

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

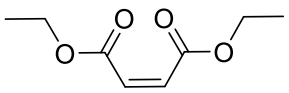
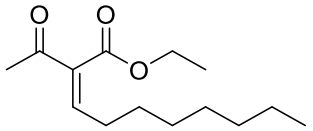
a) Data, assessment with DIP and additional mechanistic tests

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

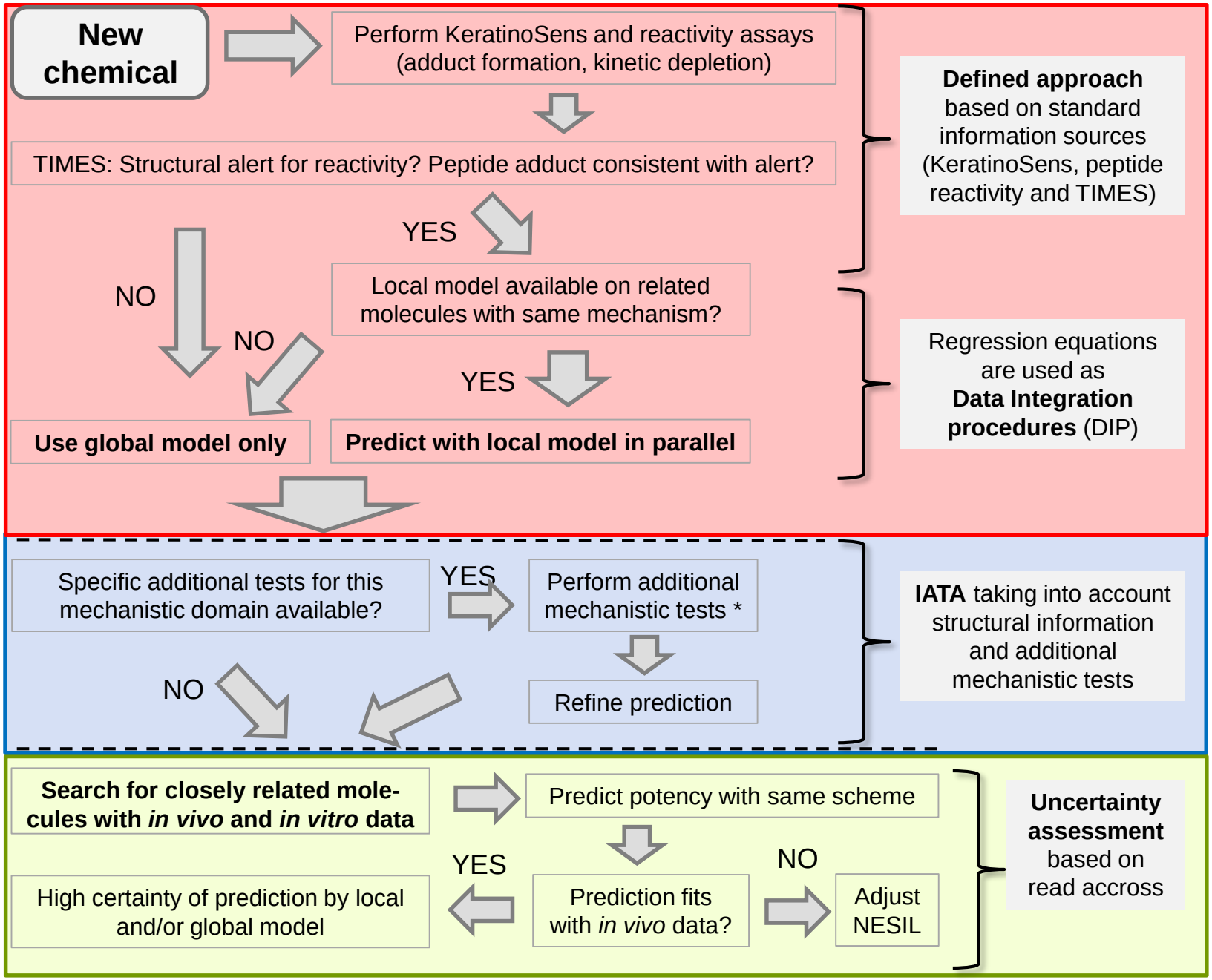
Close analogue:	 Diethylmaleate	 ethyl (Z)-2-acetyldec-2-enoate
Rationale for selecting close analogue:	Double activated MA-ester	Double activated MA-ester, substructure of target
Prediction close analogue global model:	**EC3 1.4%**	**EC3 3%**
Prediction close analogue local model (MA):	**EC3 3.8 %**	**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**

Overall approach: Hopefully clear by now …..



Discussion and Conclusion

- Structured approach with clearly defined data sources
- Takes chemical information into account
- Uses continous variables from *in vitro* tests
- Read accross to chemicals with known *in vivo* and *in vitro* data helps to assess uncertainty
  - Clearly possible in the data-rich domain of fragrance molecules – may be more difficult in other use sectors!
- Adjustment based on uncertainty assessment to transform PoD into NESIL for risk assessment
- Good prediction for fragrance molecules with high quality animal and human *in vivo* data
- Good prediction for three new molecules which were only later tested in LLNA
- **Approach deemed fit-for-purpose and now used on our latest four market candidates with no animal data**

Thank you

Contact