Skin sensitization non-animal risk assessment

Determination of a NESIL for use in risk assessment

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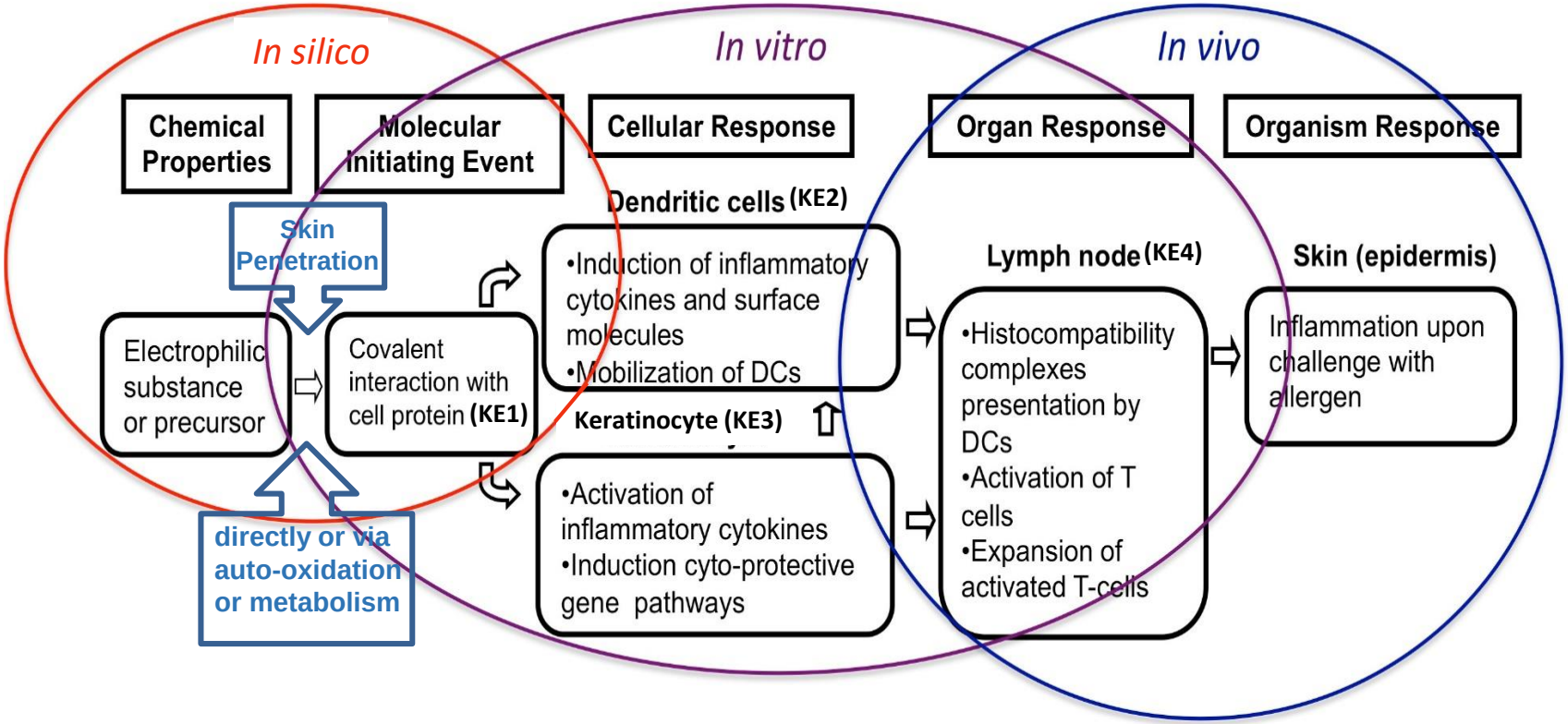
Ingredient specific data from literature

Chemical	**LLNA EC3**	**HRIPT** [µg/cm²] (RIFM)	**Metabolism**	**Skin penetration** (Shen et al 2014; RIFM DB)	**Human elicitation** *Prevalence rates of contact allergy in general population, EU* (Diepgen et al., 2015)
Coumarin	19.7 % *(commercial grade?)* 4925 µg/cm² (Vocanson et al ., 2006)	NOEL: 3543 LOEL: 8858	remains metabolically unchanged during absorption (Beckley-Kartey SA et al., 1997)	57.9% but no context	0.1% patch test with 5%
Eugenol	13 % 3250 µg/cm² (Loveless et al., 2010)	NOEL: 5906	bio-activation, pro-hapten in PPRA (Gerberick et al 2009); possibly via a dimethylation pathway followed by oxidation to the o-quinone (Bertrand et al., 1997)	22.6 % but no context	0.2% patch test with 2%
Isoeugenol	1.3 % 325 µg/cm² (Loveless et al., 2010)	NOEL: 250 LOEL: 775	radical oxidation (enzymatic or non-enzymatic) may lead to reactive species e.g. direct oxidation to the p-quinone methide (Bertrand et al., 1997)	38.4% but no context	0.7% patch test with 2%

Approaches used



Available data/in silico predictions	Availability in epidermis/ activation	DC-ITS
		COCAT *(dose response)*

HaCaT/THP-1 coculture (**COCAT**) model to estimate potency

- Keratinocytes may crucially modulate the strength of chemical-induced DC activation by providing xenobiotic metabolism and releasing DAMPs as well as (pro-inflammatory and anti-inflammatory) cytokines.
- increased dynamic range (dose response) after exposure to sensitizers compared to THP-1 alone.
- Adaptation of protocol to reconstructed human epidermis (RHE) model

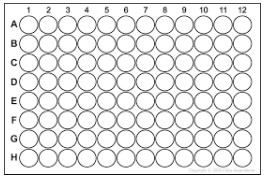
COCAT and RHE/THP-1 results are kindly provided by Brunhilde Blömeke, Trier University, Germany

Toxikologie
Mensch
Gesundheit
Umwelt

DAMP: damage-associated molecular patterns

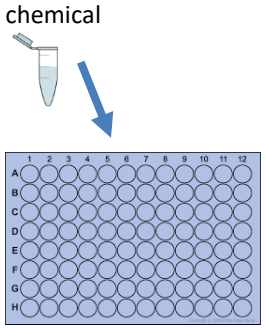
COCAT protocol

Day 1



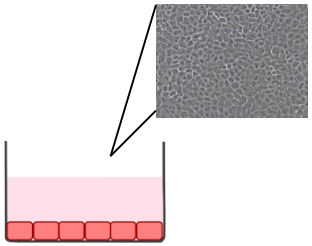
Seed HaCaT keratinocytes in 96 well plates

Day 2

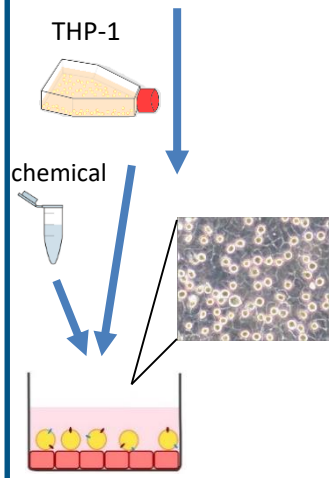


Testing of solubility

Day 3

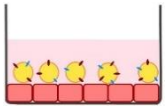


Confluent HaCaT keratinocytes



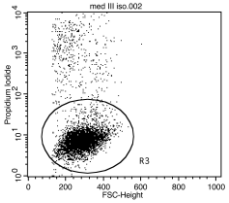
Change to exposure medium, addition of THP-1 cells and test chemicals

Day 4



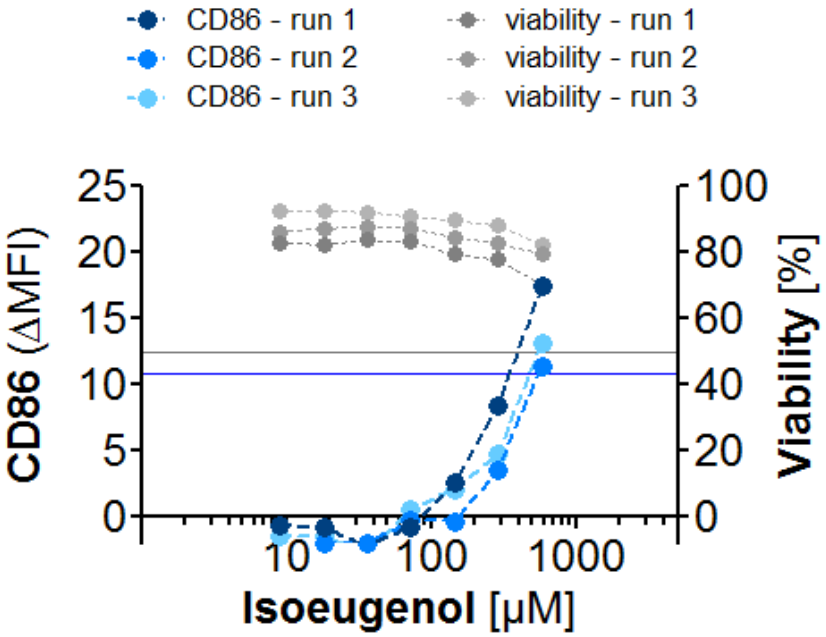
Harvest THP-1, analyse CD86, CD54 and cell viability by flow cytometry

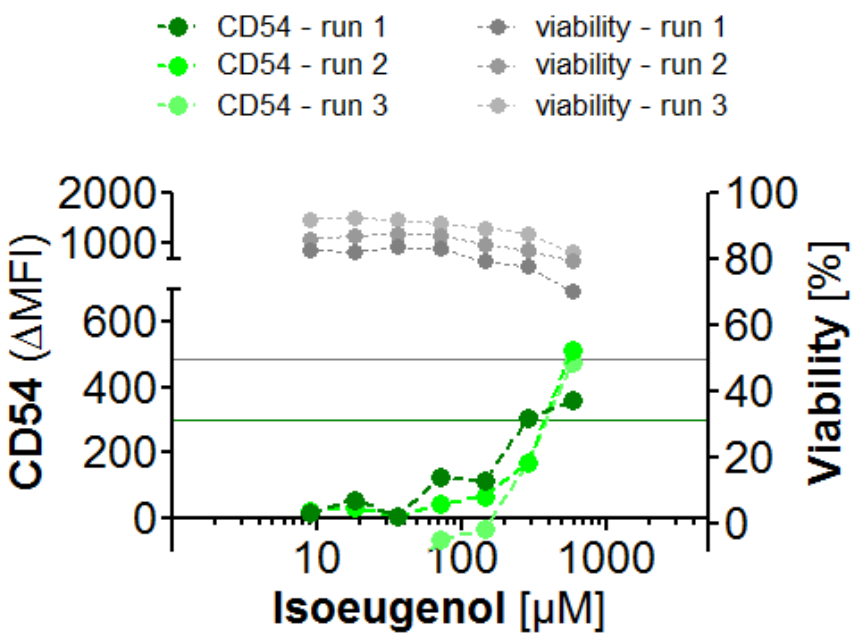
Day 5



Data analysis

Results: Example for concentration dependent responses in COCAT: Isoeugenol

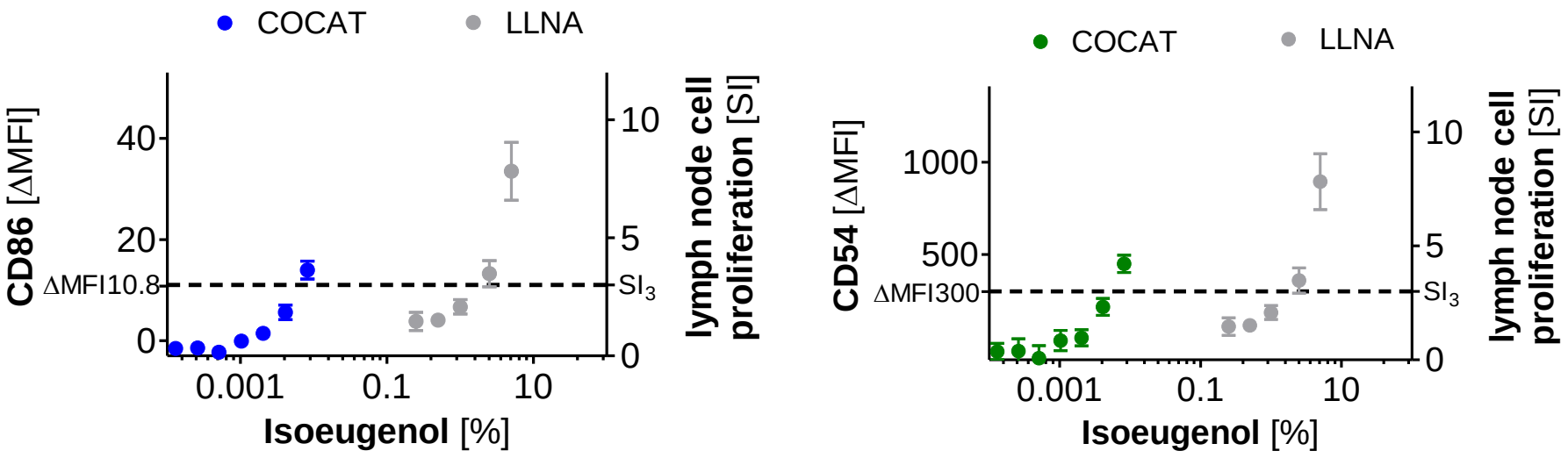




- Concentration-dependent increase of CD86 and CD54 in 3/3 runs
- Reaches thresholds for positivity for CD86 and CD54 at >50% cell viability
- Considered as sensitizer in COCAT

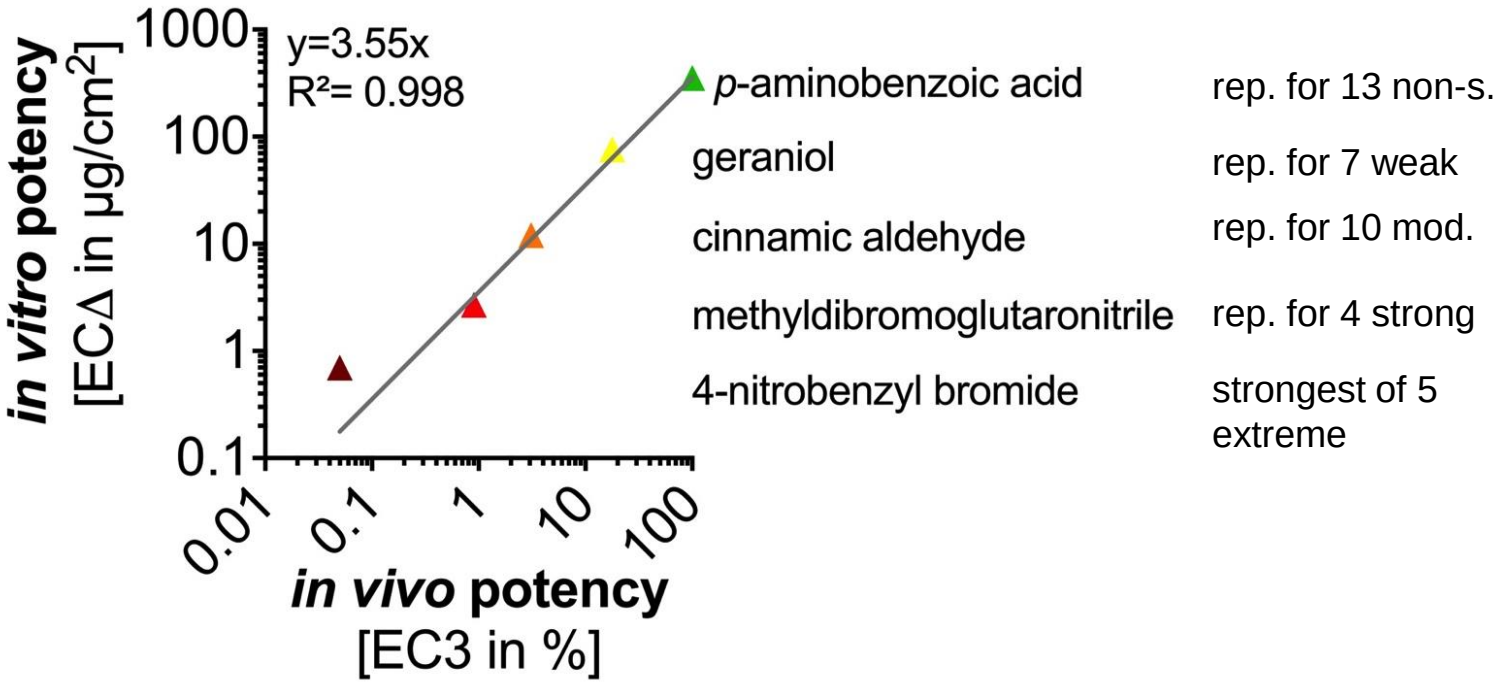
t=24h, 3 individual runs, each shown as mean of triplicates

Comparison of the estmated skin senisitizing potency in COCAT (ECΔ values) with skin sensitizing potency in LLNA



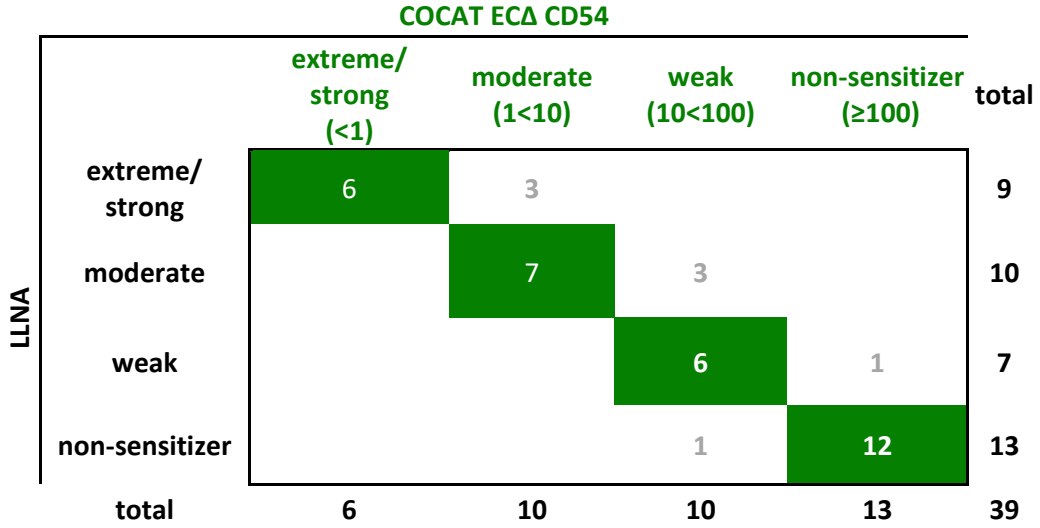
Isoeugenol dose response to estimate skin sensitizing potency in COCAT (expressed as **Δ**MFI (mean ± SEM), n=3) and LLNA (n=5, expressed as SI). COCAT concentration range is 0.00015-0.00945% (9 µM-576 µM), LLNA concentration range is 0.25; 0.5; 1.0; 2.5 and 5.0 % in AOO.
The horizontal dashed line represents the threshold for COCAT at **Δ**MFI=10.8 for CD86 and **Δ**MFI=300 for CD54 as well as the stimulation index of 3 (SI_3) for the LLNA (Loveless et al., 1996). MFI, mean fluorescence intensity.

Comparison of the potency in COCAT (ECΔ values) with skin sensitizing potency *in vivo* (LLNA)

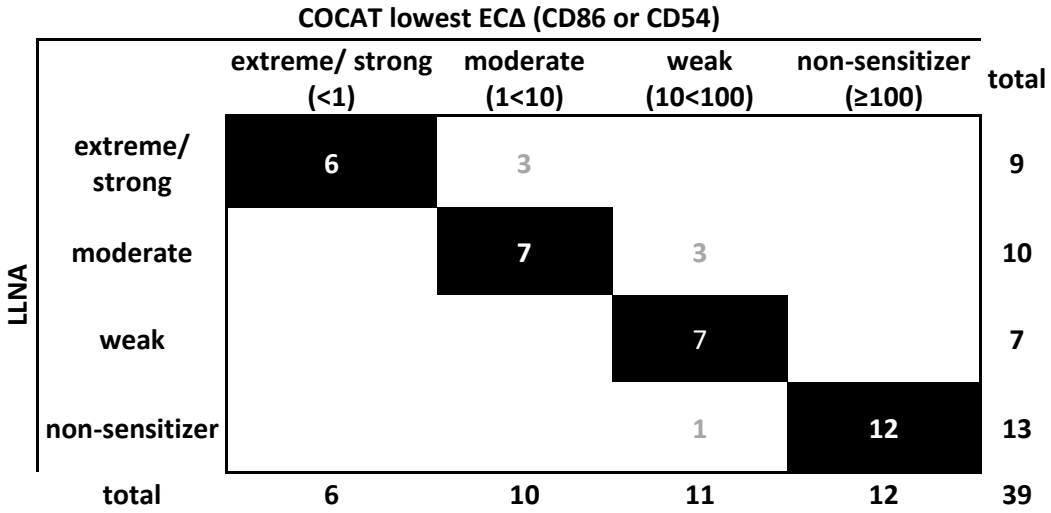


Association between *in vitro* COCAT ECΔ of CD54 and *in vivo* LLNA
(n=5 representative for 26 sensitizers and 13 non-sensitizers).

Classification of sensitizers based on predicted EC3 using ECΔ of CD54 or lowest ECΔ in COCAT

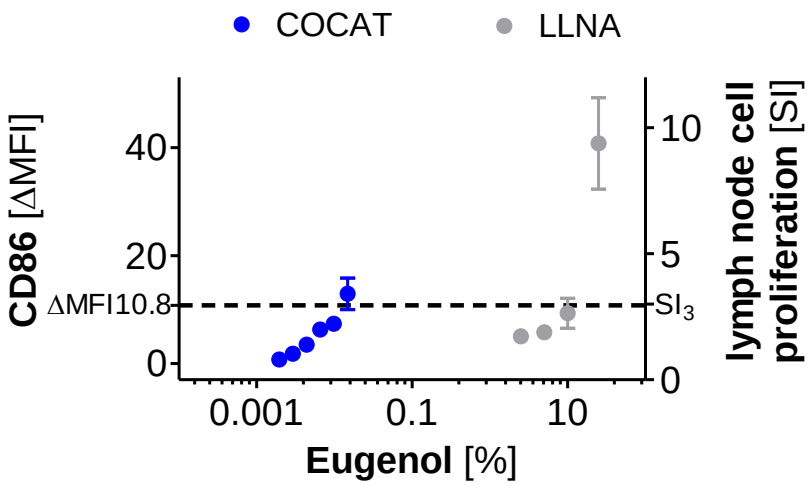


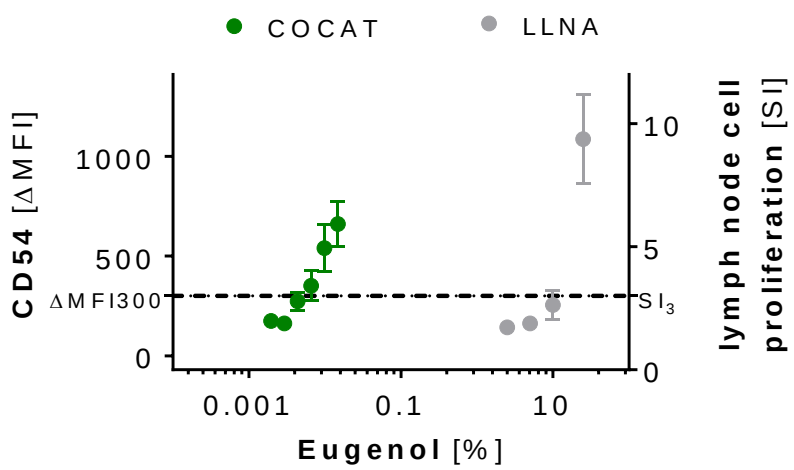
Concordance:
correctly predicted/total = 31/39 = 0.79



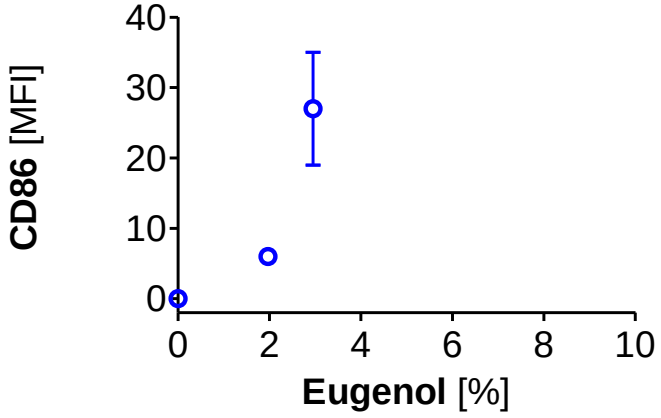
DRAFT caculation
Concordance:
correctly predicted/total = 32/39 = 0.82

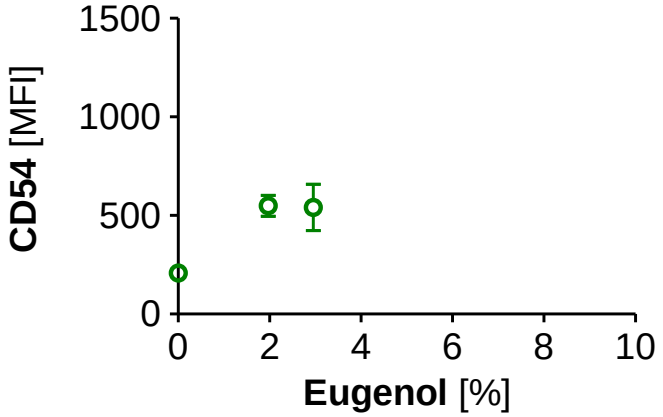
Eugenol comparison of the estimated skin senisitizing potency in COCAT, LLNA and RHE/THP-1 coculture model





Reconstructed human epidermis (RHE)





Dose-dependent upregulation of CD86 and CD54 on THP-1 cocultured with RHE after topical exposure of eugenol as mean fluorescent intensity (MFI), values for 2 % (120 mM) and 3 % (180 mM) eugenol (n=2, t=24h, mean ± SEM, RHE: SkinEthic, 0.5 cm² provided by Episkin). Chemicals were dissolved in 4:1 acetone:olive oil (AOO).

Calculation of surrogate EC3 values from COCAT

Compound information			COCAT results							
Compound Name	CAS no.	MW [g/mol]	ECΔ [µM]	SEM	**Marker for ECΔ**	Positive runs	ECΔ [µg/cm²]	ECΔ [µg/ml]	**Predicted EC3 [%]**	**Predicted category**
Eugenol (optimisation phase)	97-53-0	164.2	362	107	**CD54**	3 out of 3	36.9	59.5	**10.4**	**weak**
Isoeugenol (blind study)	97-54-1	*175**	315	35	**CD54**	3 out of 3	34.2	59.6	**9.6**	**moderate**
Coumarin (blind study)	91-64-5	*150**	938	370	**CD86**	3 out of 3	87.4	140.8	**24.6**	**weak**

Calculation ECΔ300 of CD54 in µM for isoeugenol

ECΔ is calculated from the ΔMFI of the highest concentration below the threshold (a ΔMFI of 300) and the lowest concentration above the threshold by linear interpolation. The mean of three valid runs is calculated

Run1: Highest tested conc. with ΔMFI < 300 at 125 µM, ΔMFI: 114.0

Lowest tested conc. with ΔMFI > 300 at 250 µM , ΔMFI: 307.3

ECΔ300 = 125 µM + [(300-114.0) : (307.3-114.0)] x (250 µM-125 µM) = 245.3 µM

Run2 ECΔ300 = 343.7 µM, Run3 ECΔ300 = 356.4 µM

mean: (254.3+343.7+356.4)/3 = 315 µM

**molecular weight adjustment as provided with coded chemicals for blind study (MW are 164.2 and 146.15 for Isoeugenol and Coumarin repectively)*

COCAT References

- Eskes C, Hennen J, Hoffmann S, Frey S, Goldinger-Oggier D, Schellenberger MT, Peter N, van Vliet E, Blömeke B. The HaCaT/THP-1 Cocultured Activation Test (COCAT) for skin sensitization: intra-laboratory predictive capacity and blind reproducibility study (manuscript expected to be submitted in May-June).
- Hennen J, Blömeke B. 2017. Keratinocytes improve prediction of sensitization potential and potency of chemicals with THP-1 cells. ALTEX **34**(2):279-288.
- Hennen J, Blömeke B. 2017. Assessment of skin sensitization potency of hair dye molecules *in vitro*. Contact Dermatitis **77**(3):179-180.
- Hennen J, Aeby P, Goebel C, Schettgen T, Oberli A, Kalmes M, Blömeke B. 2011. Cross Talk between Keratinocytes and Dendritic Cells: Impact on the Prediction of Sensitization. Toxicol Sci **123**(2):501-10.

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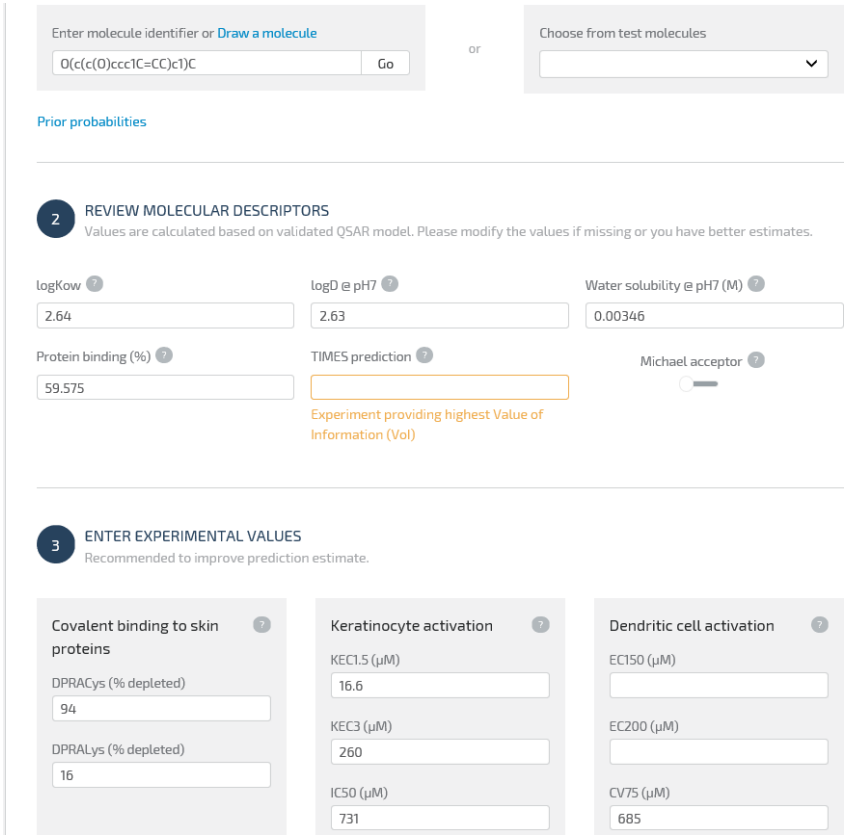
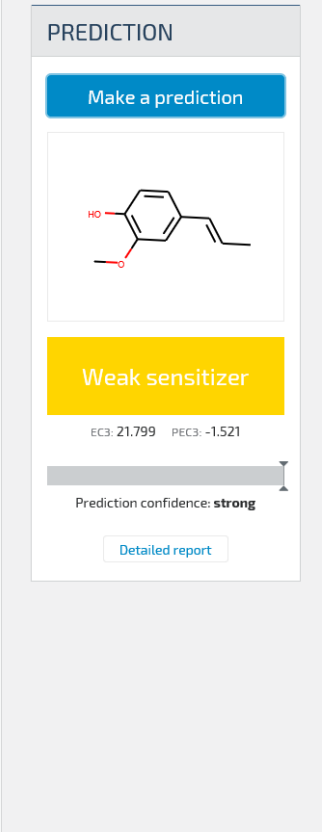
DC-ITS to estimate potency

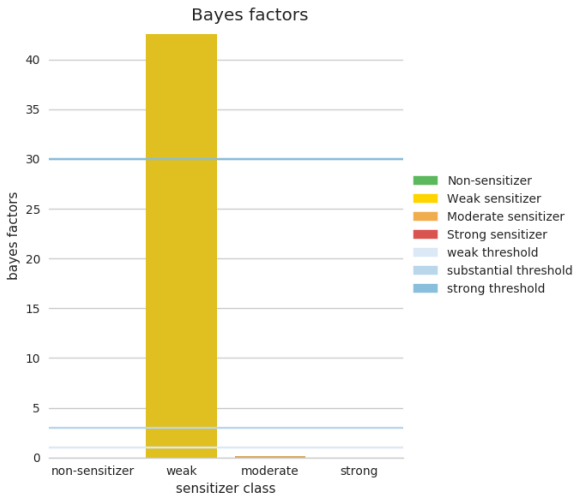
- DC ITS SkinSens online tool is publicly available at http://its.douglasconnect.com and refers to Jaworska et al., 2015
- Bayesian integrated testing strategy using
- physical-chemical properties
- *in silico* predictions for bioavailability
- *in vitro* data from DPRA, KeratinoSens and/or h-CLAT.
- TIMES predicted sensitization potential of 3 classes (non-sensitizer, weak, or moderate/strong) based on the most potent among parent compound and metabolites including consideration of activation (pro-haptens) as well as auto-oxidation (pre-haptens) and protein binding alerts
- builds a hypothesis also with partial data only
- provides prediction confidence ranges including an assessment of the evidence for acceptance by Bayes factors (Bayes factor of 3 indicates that the empirical data is 3 times more probable to fall in one sensitizer potency class compared to the others)

- Application examples in Goebel et al., 2017 in COTOX https://www.sciencedirect.com/science/article/pii/S2468202017300360?via%3Dihub

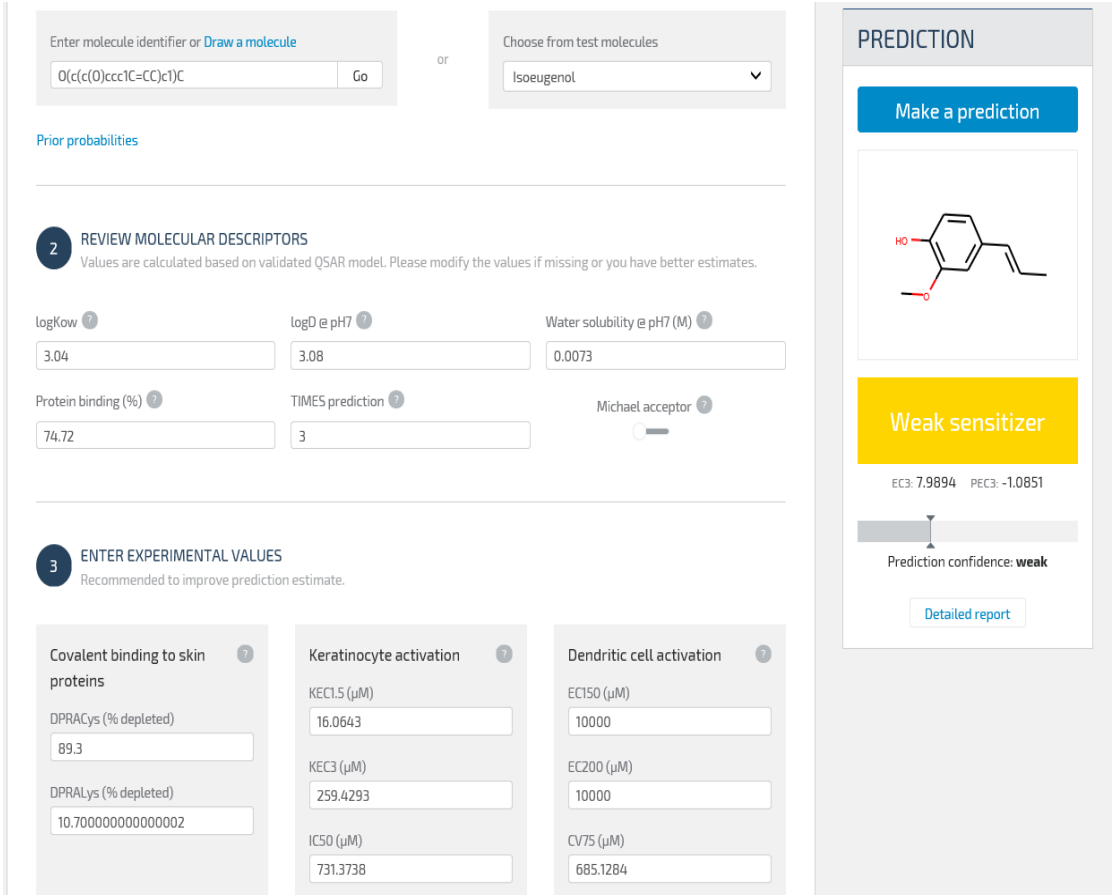
DC-ITS prediction for Isoeugenol based on *calculated molecular descriptors*

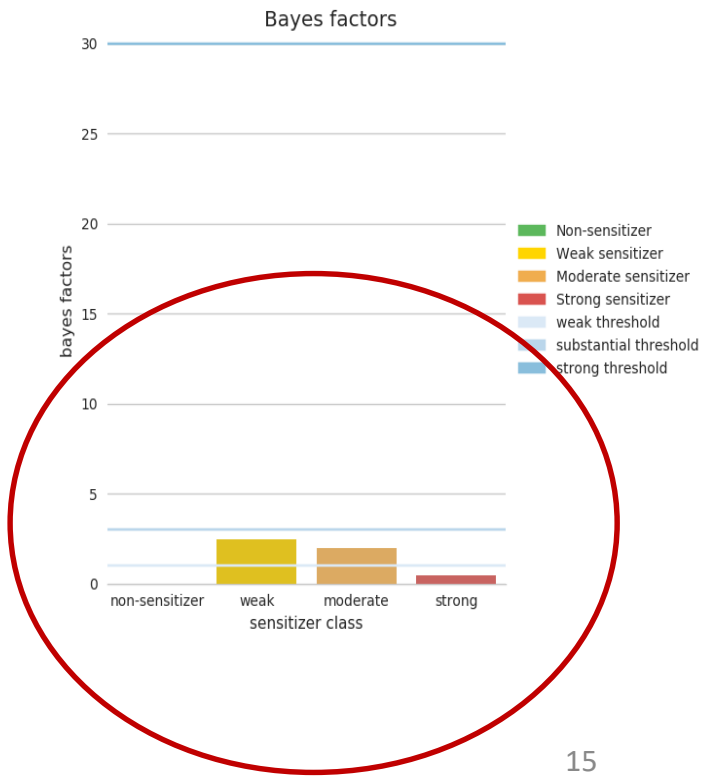




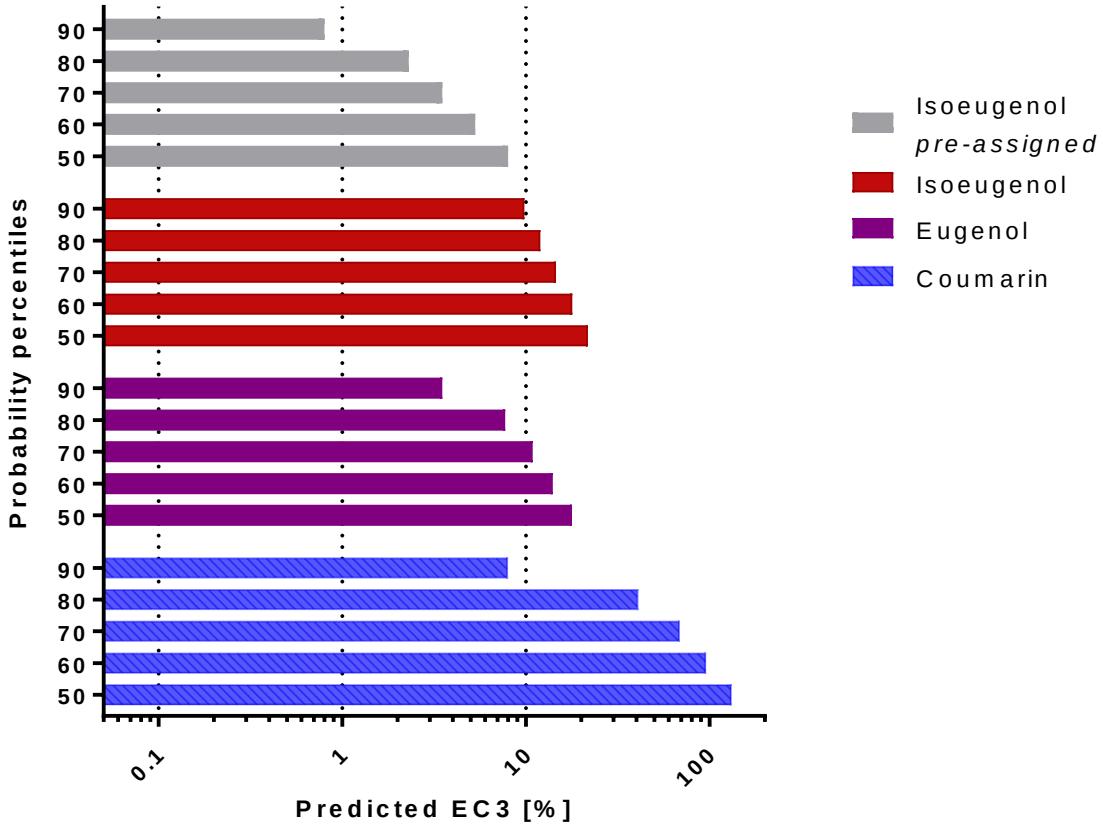


DC-ITS prediction for Isoeugenol based on *pre-assigned* test-set data

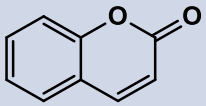
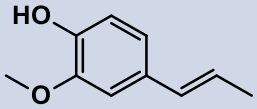
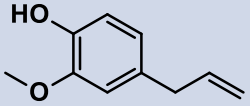




DC-ITS prediction probability



Calculation of surrogate EC3 values from DC-ITS

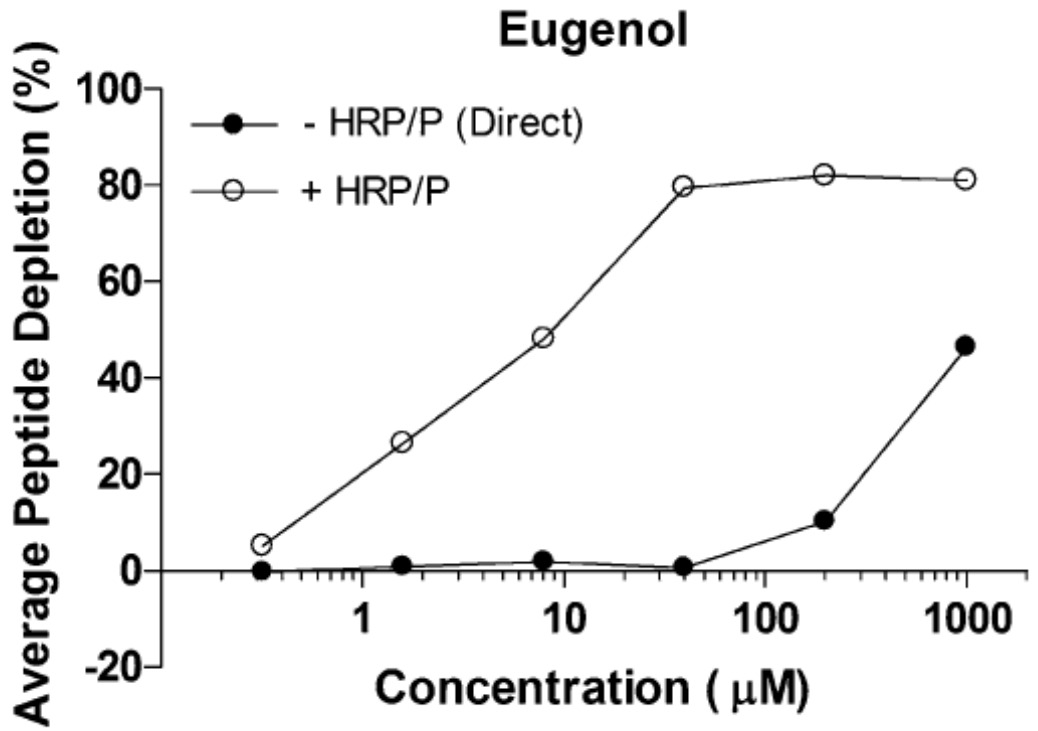
Chemical	Coumarin	Isoeugenol		Eugenol
Structure				
Molecular descriptor input	calculated	calculated	pre-assigned	calculated
TIMES class	not considered	not considered	3 preassigned (test set chemical)	not considered
In vitro input data	**DPRA, h-CLAT, Keratinosens** (Urbisch et al., 2015)		preassigned	**DPRA, h-CLAT, Keratinosens** (Urbisch et al., 2015)
EC3 value [%] percentile 50^{th} 90^{th}	 **131.9** 8.9	 **21.8** 9.8	 **8.0** 0.8	 **17.8** 3.5
Predicted potency class	**Non-sensitizer**	**weak**	**weak**	**weak**
Prediction confidence / Bayes factor	strong 10.0	strong 42.6	weak 2.4	substantial 9.8

Ingredient specific potency estimate

Chemical	COCAT (Trier University) Induction threshold		BN ITS-3 (Douglas Connect/P&G)		Alert OECD*	Activation*	Mechanistic uncertainties	Uncertainty factor
	Category default µg/cm² ECETOC 2008	Estimated EC3 [%; µg/cm²]	Category default µg/cm² ECETOC 2008	Estimated EC3 [%; µg/cm²]				
Coumarin	2500 (weak)	**23.7** 5925	Non-sensitizer	132%	Micheal acceptor/ acetylating	Unlikely, no activation observed in human skin	Not indicated, in line with read-across from 6-Methylcoumarin (EC3>25, Ashby et al., 1995)	-
Eugenol	2500 (weak)	**10.3** 2575	2500 (weak)	17.8 4450	No	Pro-hapten, PPRA activation, pro-Michael acceptor, limited activation in DPRA	Does bio-activation occur in human skin at max use concentration?	If substantiated consider as LOEL
Isoeugenol	250 (moderate	**8.9** 2225	2500 (weak)	21.8 5450 or	No	Pre-hapten, high depletion in DPRA/PPRA; pre/pro-Michael acceptor	Do human exposure conditions promote oxidation/bio-activation?	If substantiated consider as LOEL

* Urbisch et al., 2015; Beckley-Kartey SA et al., 1997

Activation of Eugenol in PPRA



DPRA: 24% Cys; 12.5 Lys depleted,
5 or 25 mM of the test chemical, 24 h

References: Gerberick et al., 2009, Urbisch et al., 2016

Summary

- Overall substantial prediction if mechanistical uncertainties are considered
- Dose response consideration in COCAT, PPRA considered relevant for interpretation
- Under-prediction for isoeugenol to be adjusted by
  - consideration of (bio)-activation
  -read across from data rich analogs (human data)
  - human patch test information
- Uncertainty consideration to be further explored

Questions?

???

Back up slides

Calculation of surrogate EC3 values from COCAT (please see notes)

Compound information			COCAT results							
Compound Name	CAS no.	MW [g/mol]	ECΔ [µM]	SEM	**Marker for ECΔ**	Positive runs	ECΔ [µg/cm²]	ECΔ [µg/ml]	**Predicted EC3 [%]**	**Predicted category**
Eugenol (optimisation phase)	97-53-0	164.2	362	107	**CD54**	3 out of 3	36.9	59.5	**10.4**	**weak**
Isoeugenol (blind study, exact MW)	97-54-1	164.2	363	40	**CD54**	3 out of 3	37.0	59.6	**10.4**	**weak**
Coumarin (blind study exact MW)	91-64-5	146.15	963	378	**CD86**	3 out of 3	87.4	140.7	**24.6**	**weak**

Calculation ECΔ300 of CD54 in µM for isoeugenol

ECΔ is calculated from the ΔMFI of the highest concentration below the threshold (a ΔMFI of 300) and the lowest concentration above the threshold by linear interpolation. The mean of three valid runs is calculated

Run1: Highest tested conc. with ΔMFI < 300 at 143.9 µM, ΔMFI: 114.0

Lowest tested conc. with ΔMFI > 300 at 287.8 µM , ΔMFI: 307.3

ECΔ300 = 143.9 µM + [(300-114.0) : (307.3-114.0)] x (287.8 µM-143.9 µM) = 282.4 µM

Run2 ECΔ300 = 395.6 µM, Run3 ECΔ300 = 410.3 µM

mean: (282.4+395.6+410.3)/3 = 362,7 µM

Conversion µM to % for eugenol (M=164.2 g/mol)

100 µM Eugenol (M=164.2 g/mol)

100 µM x 164.2 g/mol = 16420 µg/L

16420 µg/L : 10^6 = 0.01642 g/L

0.01642 g/L /1000 x 100 = 1.6 x 10^{-3} %

Conversion µM to µg/cm² explained by the example of eugenol (M= 164.2 g/mol)

The assay volume in the 96 well is 0.18 ml and the growth area is 0.29 cm²

100 µM Eugenol (M=164.2/mol)

100 µM x 164.2 g/mol = 16420 µg/L

16420 µg/L : 1000 = 16.42 µg/ml

16.42 µg/ml x 0.18 ml : 0.29 cm² = 10 µg/cm²