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To cite this article: Weicheng Zhang, Libao Chen & Lunguang Yao (2018) Predicting skin sensitization potential of organic compounds based on toxicity enhancement to *Tetrahymena pyriformis*, fathead minnow, and *Daphnia magna*, Journal of Immunotoxicology, 15:1, 82-89, DOI: [10.1080/1547691X.2018.1465144](https://doi.org/10.1080/1547691X.2018.1465144)

To link to this article: <https://doi.org/10.1080/1547691X.2018.1465144>



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Published online: 26 Apr 2018.



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RESEARCH ARTICLE



Predicting skin sensitization potential of organic compounds based on toxicity enhancement to *Tetrahymena pyriformis*, fathead minnow, and *Daphnia magna*

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ABSTRACT

Skin sensitization is an important occupational health problem and immunotoxicity endpoint. Considering animal welfare and time and cost savings, many alternative approaches, such as those conducted *in vitro*, *in silico*, and *in chemo*, have been proposed and applied to predict skin sensitization of compounds. Toxicologically, sensitizers can elicit excess toxicity at greater levels than non-sensitizers due to their capacity to react with proteins/peptides. Based on this understanding, calculated toxicity enhancements (T_e) of 65 organic compounds from three *in vitro* bioassays, i.e. 48-hr ciliate (*Tetrahymena pyriformis*) growth inhibition, and both 96-hr fathead minnow and 48-hr *Daphnia magna* acute lethal toxicities, were employed to qualitatively and quantitatively predict skin sensitization potencies of the test agents. The sensitivity, specificity, and accuracy reaching 80% strongly suggested toxicity enhancement was an excellent parameter for predicting skin sensitization. Linear regressions of skin sensitization against toxicity enhancement were fitted for each bioassay, and they were improved after the sensitizers were categorized into different reaction mechanistic domains, which, in decreasing order of contribution from T_e to sensitization, were $S_{NAr} > S_{N1} > MA$. These results indicated that toxicity bioassays are useful tools and that T_e could be a useful parameter that might be applied to predict skin sensitization.

ARTICLE HISTORY

Received 31 January 2018
Revised 20 March 2018
Accepted 11 April 2018

KEYWORDS

Skin sensitization; toxicity enhancements; immunotoxicity; bioassays

Introduction

Skin sensitization (e.g. allergic contact dermatitis, ACD), is an important immunotoxicologic endpoint that results from the allergic reaction of sensitizers in the skin (Aeby et al. 2010). Including allergic and irritant contact dermatitis, ACD is the second most commonly reported occupational disease, accounting for 10–15% of all occupational diseases (Anderson et al. 2011). Currently, the only valid methods for identifying skin sensitization to compounds are *in vivo* assays, such as the guinea pig maximization test (GPMT) and local lymph node assay (LLNA) in mice (mOECD 1992, 2002). However, due to regulatory requirements to reduce animal testing, researchers have tried to develop alternative (*in vitro*, *in silico*, and *in chemo*) approaches to predict allergenic compound skin sensitization potency (Aptula et al. 2006; Arning et al. 2009; Roberts and Natsch 2009; Aeby et al. 2010; Jeong et al. 2013). Common to these additional approaches is a fact that a key step in skin sensitization is the formation of a covalent adduct between skin sensitizers and local proteins/peptides (Natsch and Gfeller 2008; Aleksic et al. 2009).

Although aquatic toxicity and skin sensitization are different endpoints, their chemical reaction mechanisms are partially the same (Aptula and Roberts 2006). From the skin sensitization perspective, the higher the sensitizer reactive capacity, the higher is its sensitization potency (Natsch and Gfeller 2008). Moreover, it is accepted that when a compound presents reactivity to a protein/peptide, it could be a potential sensitizer (Gerberick et al.

2007) regardless of bioavailability. From a toxicity perspective, the higher the reactive capacity of an organic agent, the greater the excess toxicity elicited by that agent (Böhme et al. 2009; Schramm et al. 2011). Chemically, the covalent binding of reactive compounds to DNA could lead to mutagenesis or genotoxicity (Thaens et al. 2012), binding to protein and/or peptides could lead to excess toxicity (Böhme et al. 2010), and binding to immuno-proteins in skin could lead to skin sensitization (Roberts et al. 2011). Based on this link between skin sensitization and excess toxicity, it has been hypothesized that a sensitizer can elicit excess toxicity because of its reactive capability but that a non-sensitizer will only show narcotic-level toxicity due to a lack of reactive capacity.

Toxicity enhancement (T_e), previously termed excess toxicity, is widely used to determine whether a compound can elicit reactive toxicity through a comparison with its baseline toxicity (von der Ohe et al. 2005; Schramm et al. 2011). $T_e = 10$ has been proposed as a threshold to discriminate reactive from non-reactive compounds. Similarly, one reasonably assumes that toxicity enhancement can be utilized to discriminate a sensitizer from a non-sensitizer. In the present study, data on the toxicity of 65 compounds to *Tetrahymena pyriformis*, fathead minnow, and *Daphnia magna* were compiled and collected, and toxicity enhancements of each in the three biosystems were calculated and applied to predict their sensitization potency. The three selected assays in the different species, toxic endpoints, and exposure durations demonstrated that toxicity enhancement was

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Table 1. Classification of skin sensitization potency through EC3 values.

EC3 value (%)	Potency
<0.1	Extreme sensitization
≥0.1–<1.0	Strong sensitization
≥1.0–<10	Moderate sensitization
≥10–<100	Weak sensitization
>100	Non-sensitization

Method from Gerberick et al. (2005).

a reliable parameter that could be used to predict skin sensitization potency and discriminate strong (and extreme) and moderate sensitizers from weak and non-sensitizers.

Materials and methods

Skin sensitization data

Skin sensitization estimates for the compounds of interest – based on local lymph node assay (LLNA) outcomes – were obtained from Gerberick et al. (2005) and Kern et al. (2010). Based on EC3 values, the compounds were categorized into five sub-categories (Table 1), i.e. extreme, strong, moderate, weak, and non-sensitizers. Log EC3 values of each were calculated (Equation (1); MW = molecular weight). Values were not calculated for non-sensitizers.

$$\log EC3 = \log(EC3/MW) \quad (1)$$

Aquatic toxicity data

Data on aquatic toxicity to three aquatic organisms by the test compounds were collected from the U.S. EPA ECOTOX database and QSAR Toolbox 2.2. Experimental toxicity data (48-hr EC₅₀, 50% growth inhibition concentration) were collected for the ciliate *T. pyriformis*, but data from minor 40-hr or 24-hr assays were also accepted. Additionally, data on 96-hr LC₅₀ (50% lethal concentration) experimental toxicities towards fathead minnows were used, as were experimental acute toxicity (48-hr LC₅₀, 50% lethal concentration) data with the cladoceran *Daphnia magna*. Only immobilization data (EC₅₀, 50% effective concentration) were obtained for the minority compounds in the present research. The data set of information on the 65 agents represents an overlap between the LLNA and ECOTOX databases. Some compounds were excluded from the set due to their very complex structures or they were not widely utilized in daily life. Still, although the set here did not comprise the full LLNA database, the number of selected compounds was in the range of that used in the Direct Peptide Reactivity Assay (DPRA), i.e., 38 compounds used in 2004 and 82 in 2007 (Gerberick et al. 2004, 2007). This limited pool of test agents would be in keeping with another OECD-accepted *in vitro* assay; assessments of the *Nrf2* pathway activation in keratinocytes also just examined 67 test agents in the model (Emter et al. 2010).

Reaction mechanistic domains

Previous studies suggested categorizing the sensitizers through their reaction mechanism domains (Aptula et al. 2005). Therefore, in the present research, these compounds had been categorized according to their reaction mechanisms, which were followed by QSAR Toolbox 2.2 suggestions. Accordingly, multiple reaction mechanisms had been proposed for some agents.

Table 2. QSAR equations used to describe basic toxicity of organic agents to each organism.

Test organisms	<i>n</i>	Baseline toxicity equations	<i>r</i> ²	<i>s</i>
<i>Tetrahymena pyriformis</i>	87	$\log EC_{50}[M] = -0.78 \log K_{ow} - 0.99$	0.96	0.20
Fathead minnow	68	$\log LC_{50}[M] = -0.85 \log K_{ow} - 1.41$	0.94	0.34
<i>Daphnia magna</i>	36	$\log LC_{50}[M] = -0.857 \log K_{ow} - 1.281$	0.90	0.44

Number of samples (*n*), correlation coefficients (*r*²), and standard errors (*s*).

For example, Compounds 8 and 10 act either as pro-Michael acceptors or via S_N1 reaction mechanisms, and compound 9 acts either Michael acceptor or S_N1 reaction mechanisms. Thus, the S_N1 reaction mechanisms were selected as the dominant type for these compounds based on QSAR Toolbox 2.2 suggestions. Considering the roles of bio- or chemical-transformations, pro-Michael acceptors were classified as using Michael addition (MA) reaction conditions. Chemically, the non-sensitizers had not been expected to bind with proteins/peptides; as such, the QSAR Toolbox 2.2 suggested these compounds had no reaction mechanisms.

Basic and excess toxicity

Basic toxicity (defined as minimal toxicity correlated with hydrophobicity) of the 65 compounds to the three aquatic organisms were calculated through log *K*_{ow} regression models (van Leeuwen et al. 1992; von der Ohe et al. 2005; Ellison et al. 2008). The baseline toxicity models for each organism are summarized in Table 2; hydrophobicity, log *K*_{ow}, was obtained using EPI Suite 4.1 software. Toxicity enhancements (*T*_e) of each compound were calculated using Equation (2) (comparing experimental toxicity with narcotic toxicity).

$$T_e = \frac{EC_{50}/LC_{50}(\text{narc})}{EC_{50}/LC_{50}(\text{exp})} \quad (2)$$

Results

Excess toxicity data for 65 compounds

Values for toxicity enhancements (log *T*_e) to the three organisms by the compounds of interest are shown in Table 3, which also summarizes the skin sensitization (log EC3), reaction mechanisms, as well as physicochemical properties of each agent. The toxicity enhancements of some compounds were unavailable because their toxicity data were missing. Figure 1 illustrates that the relationship between toxicity enhancement and sensitization potency presented a similar trend in all three organisms, i.e., extreme/strong sensitizers yielded the highest excess toxicities (average log *T*_e > 2.50), followed by the moderate sensitizers (average log *T*_e > 2.10). In comparison, weak and non-sensitizers showed only narcotic toxicity, with an average log *T*_e < 1.00 and < 0.50, respectively. Generally, a threshold of log *T*_e = 1 is applied to separate excess toxic compounds from narcotic-level compounds (Blaschke et al. 2010, 2011; Schramm et al. 2011). In the study here, log *T*_e = 1 was also used to discriminate strong, extreme, and moderate sensitizers from weak and non-sensitizers. All the bioassays yielded good sensitivity, specificity, and accuracy (of at least 80%) (Table 4). The fish model yielded the highest degree of accuracy (90.2%) and specificity (93.8%); the *D. magna* model presented the highest sensitivity (95.7%).

Table 3. The 65 test compounds evaluated in this study.

#	Name	CAS	log K_{ow}	EC3 (%)	Potency	log EC3	log T_e ciliate	log T_e fish	log T_e Daphnia	Reaction Mechanism
1	1,3-Benzenedicarbonitrile, 2,4,5,6-tetrachloro-	1897-45-6	3.05	0.004	Extreme	-4.82	2.64	3.25	2.42	S_NAr
2	5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	-0.34	0.009	Extreme	-4.22	N/A	4.50	4.93	S_N2
3	p-Benzoquinone	106-51-4	0.2	0.0099	Extreme	-4.04	4.18	N/A	3.98	MA
4	1-Chloro-2,4-dinitrobenzene	97-00-7	2.17	0.05	Extreme	-3.61	2.39	2.80	2.26	S_NAr
5	Benzene, 1-bromo-2,4-dinitro	584-48-5	2.52	0.085	Extreme	-3.46	2.35	1.14	2.01	S_NAr
6	1,4-Dihydroquinone	123-31-9	0.59	0.11	Extreme	-3.00	1.78	3.83	4.30	MA
7	Glutaraldehyde	111-30-8	-0.18	0.1	Strong	-3.00	2.15	2.85	4.00	SB
8	1,4-Phenylenediamine	106-50-3	-0.3	0.16	Strong	-2.83	2.40	N/A	4.49	S_N1
9	Iodopropenyl butylcarbamate	55406-53-6	2.45	0.9	Strong	-2.49	N/A	2.98	3.47	S_N1
10	2-Aminophenol	95-55-6	0.62	0.4	Strong	-2.44	2.47	N/A	3.18	S_N1
11	3-Phenylenediamine	108-45-2	-0.33	0.49	Strong	-2.34	1.69	N/A	3.73	S_N1
12	1-Naphthol	90-15-3	2.85	1.3	Moderate	-2.04	0.52	0.66	1.57	MA
13	2-Mercaptobenzothiazole	149-30-4	2.42	1.7	Moderate	-1.99	1.34	0.71	1.02	S_N2
14	2-Hydroxyethyl acrylate	818-61-1	-0.21	1.4	Moderate	-1.92	2.86	3.15	3.25	MA
15	2-Methyl-2H-isothiazol-3-one	2682-20-4	-0.83	1.9	Moderate	-1.78	4.30	5.08	5.24	S_N2
16	Formaldehyde	50-00-0	0.35	0.61	Strong	-1.69	2.68	1.35	1.18	SB
17	Tetramethylthiuram disulfide	137-26-8	1.73	5.2	Moderate	-1.66	3.94	3.90	4.62	S_N2
18	Cinnamic aldehyde	104-55-2	1.9	3	Moderate	-1.64	1.33	1.87	1.71	MA
19	3-Aminophenol	591-27-5	0.21	3.2	Moderate	-1.53	1.33	N/A	2.93	S_N1
20	Diethyl maleate	141-05-9	2.2	5.8	Moderate	-1.47	0.53	1.00	N/A	MA
21	Ethylenediamine (free base)	107-15-3	-2.04	2.2	Moderate	-1.44	5.63	2.76	3.82	SB
22	Resorcinol	108-46-3	0.8	5.5	Moderate	-1.30	0.74	1.16	3.17	MA
23	2-Ethylhexyl acrylate	103-11-7	4.09	10	Weak	-1.27	0.82	-0.99	-0.52	MA
24	Diethylenetriamine	111-40-0	-2.13	5.8	Moderate	-1.25	N/A	2.41	3.83	SB
25	1-Bromohexane	111-25-1	3.8	10	Weak	-1.22	-0.01	0.04	N/A	S_N2
26	α -Terpinene	99-86-5	4.25	8.9	Moderate	-1.18	N/A	-0.38	-0.05	NB
27	Pentachlorophenol	87-86-5	5.12	20	Weak	-1.12	0.22	0.27	0.71	S_NAr
28	Eugenol	97-53-0	2.27	13	Weak	-1.10	0.66	0.50	N/A	MA
29	Benzyl benzoate	120-51-4	3.97	17	Weak	-1.09	0.36	0.40	N/A	S_N2
30	Butyl acrylate	141-32-2	2.36	11	Weak	-1.07	0.69	N/A	1.09	MA
31	Benzene, 2,4-dichloro-1-nitro-	611-06-3	3.07	20	Weak	-0.98	0.61	N/A	0.75	S_NAr
32	Benzoic acid	65-85-0	1.87	20	Weak	-0.79	0.24	-0.58	-0.98	NB
33	Methyl acrylate	96-33-3	0.8	20	Weak	-0.63	1.94	1.97	2.55	MA
34	Ethyl acrylate	140-88-5	1.32	28	Weak	-0.55	1.50	0.07	1.95	MA
35	R(+)-limonene	5989-27-5	4.38	69	Weak	-0.30	N/A	0.15	0.13	NB
36	2-Propenoic acid, 2-methyl-, methyl ester	80-62-6	1.38	90	Weak	-0.05	-0.29	-0.03	-0.71	MA
37	Pyridine	110-86-1	0.65	72	Weak	-0.04	0.21	0.94	0.76	NB
38	Dimethyl sulfoxide	67-68-5	-1.35	72	Weak	-0.04	0.57	0.10	0.37	NB
39	Aniline	62-53-3	0.90	89	Weak	-0.02	1.35	0.77	1.56	S_N1
40	Benzaldehyde	100-52-7	1.48	NS	NS	NS	0.76	1.36	1.34	SB
41	Benzocaine	94-09-7	1.86	NS	NS	NS	1.23	0.67	N/A	NB
42	1-Butanol	71-36-3	0.88	NS	NS	NS	-0.11	-0.53	-0.44	NB
43	Chlorobenzene	108-90-7	2.84	NS	NS	NS	-0.33	-0.0005	0.14	NB
44	Diethylphthalate	84-66-2	2.42	NS	NS	NS	0.28	0.38	0.06	NB
45	Ethyl vanillin	121-32-4	1.58	NS	NS	NS	0.80	0.53	N/A	NB
46	Hexane	110-54-3	3.9	NS	NS	NS	N/A	-0.19	-1.39	NB
47	4-Hydrobenzoic acid	99-96-7	1.58	NS	NS	NS	-0.24	N/A	0.26	NB
48	Isopropanol	67-63-0	0.05	NS	NS	NS	0.09	-0.61	-0.54	NB
49	Methyl 4-hydroxybenzoate (methylparaben)	99-76-3	1.96	NS	NS	NS	0.56	N/A	0.67	NB
50	Propylene glycol	57-55-6	-0.92	NS	NS	NS	N/A	-0.44	1.39	NB
51	Salicylic acid	69-72-7	2.26	NS	NS	NS	-0.26	-0.47	0.04	NB
52	Vanillin	121-33-5	1.21	NS	NS	NS	1.04	0.82	N/A	NB
53	Vinylidene dichloride	75-35-4	2.13	NS	NS	NS	N/A	-0.36	0.67	NB
54	1,2-Benzenedicarboxylic acid, dibutyl ester	84-74-2	4.5	NS	NS	NS	0.1	0.21	-0.38	NB
55	Benzenemethanol	100-51-6	1.1	NS	NS	NS	0.28	0.03	0.45	NB

(continued)

Table 3. Continued

#	Name	CAS	log K_{ow}	EC3 (%)	Potency	log EC3	log T_e ciliate	log T_e fish	log T_e Daphnia	Reaction Mechanism
60	1,2-Benzenedicarboxylic acid, butyl phenylmethyl ester	85-68-7	4.73	NS	NS	NS	N/A	-0.26	-0.09	NB
61	Clofibrate	637-07-0	3.62	NS	NS	NS	N/A	-0.14	-0.44	NB
62	Benzamide, <i>N,N</i> -diethyl-3-methyl-	134-62-3	2.18	NS	NS	NS	N/A	-0.02	0.26	NB
63	2-Nitro-3-pyridinol	15128-82-2	0.72	NS	NS	NS	2.32	0.90	N/A	NB
64	1-Butanol, 3-methyl-	123-51-3	1.16	NS	NS	NS	0.07	N/A	-0.28	NB
65	Ethanol, 2,2-oxybis-	111-46-6	-1.47	NS	NS	NS	0.72	-0.01	0.50	NB

NS: non-sensitizer; N/A: not available; and NB: non-binding.

Data provided: CAS number; logarithmic octanol/water partition coefficient (log K_{ow}); skin sensitization potency and skin sensitization values; their logarithmic toxicity enhancements to *Tetrahymena pyriformis*, fathead minnow, and *Daphnia magna*; and, potential binding reaction mechanisms with proteins or peptides

Various prediction models for different reaction mechanism domains

Linear regressions of log T_e vs. log EC3 for the bioassays were calculated and examined (Figure 2, Equations (3)–(5) [Table 5]). Interestingly, the *T. pyriformis* bioassay model presented the steepest slope (−0.807) compared to the fish (−0.377) and *Daphnia* (−0.401) models. Previous research has strongly recommended that sensitizers be categorized according to their suspected reaction mechanisms but not their structure classes (Aptula et al. 2005). Due to limited toxicity and skin sensitization data, we only obtained three models of log EC3 plotted against log T_e for the S_N1 , S_NAr , and Michael addition (MA) reaction mechanisms. MA mechanism models showed negligible improvements; the slopes were not much steeper, and the r^2 values did not increase (compare Equation (3)–(5) with (11)–(13)). By comparison, each model of the S_N1 and S_NAr reaction mechanisms showed visible improvements, with much steeper slopes and increased r^2 values.

Contribution of bioavailability to skin sensitization

Regression equations including hydrophobicity as an additional descriptor to simulate bioavailability are summarized in Table 6. For all chemical compound groups, hydrophobicity clearly contributed to skin sensitization, though the r^2 decreased and s increased (see Equation (14)–(16)). The steeper slopes of log T_e and the negative slopes of log K_{ow} in the fish and *D. magna* models indicated that reactivity and bioavailability cooperatively contribute to skin sensitization. Contributions from bioavailability were also visible in the models for the different reaction mechanisms (S_N1 , S_NAr , MA). Based on the positive slopes associated with the *T. pyriformis* (S_N1 ; Equation (17) and the minnow (S_NAr ; Equation (20), hydrophobicity did not appear to contribute to skin sensitization potency.

Discussion

The key step in skin sensitization is formation of a covalent adduct between allergenic compounds and local proteins/peptides (Natsch and Gfeller 2008; Aleksic et al. 2009). Allergenic compounds could also elicit excess toxicities due to this kind of reactive capacity. In principle, skin sensitization and aquatic toxicity in part share chemical reaction mechanisms, but the endpoints differ (Aptula and Roberts 2006). A general understanding is that strong (and extreme) and moderate sensitizers can elicit excess toxicity due to high reactive capacity, but weak/non-sensitizers show only narcotic-level toxicity (due to lack of capacity). Therefore, it is possible toxicity enhancement (T_e) can be used to screen for skin sensitization potencies.

T_e has been successfully used for qualitative monitoring of skin sensitization (Aptula et al. 2006; Arning et al. 2009). The present research confirmed the suitability of T_e for this application. Here it seems the largest T_e values were observed with strong (and extreme) sensitizers (average log $T_e > 2.50$), followed by moderate sensitizers (average log $T_e > 2.10$); only narcotic toxicities were found with the weak (average log $T_e < 1.00$) or non- (average log $T_e < 0.50$) sensitizers. Compounds with a high reactive capacity could elicit excess toxicity and strong (or extreme) or moderate skin sensitization, while those with weak/no reactive capacity could only induce narcotic toxicity and weak or no skin sensitization. Thus, T_e could be an excellent parameter for use in predicting skin sensitization.

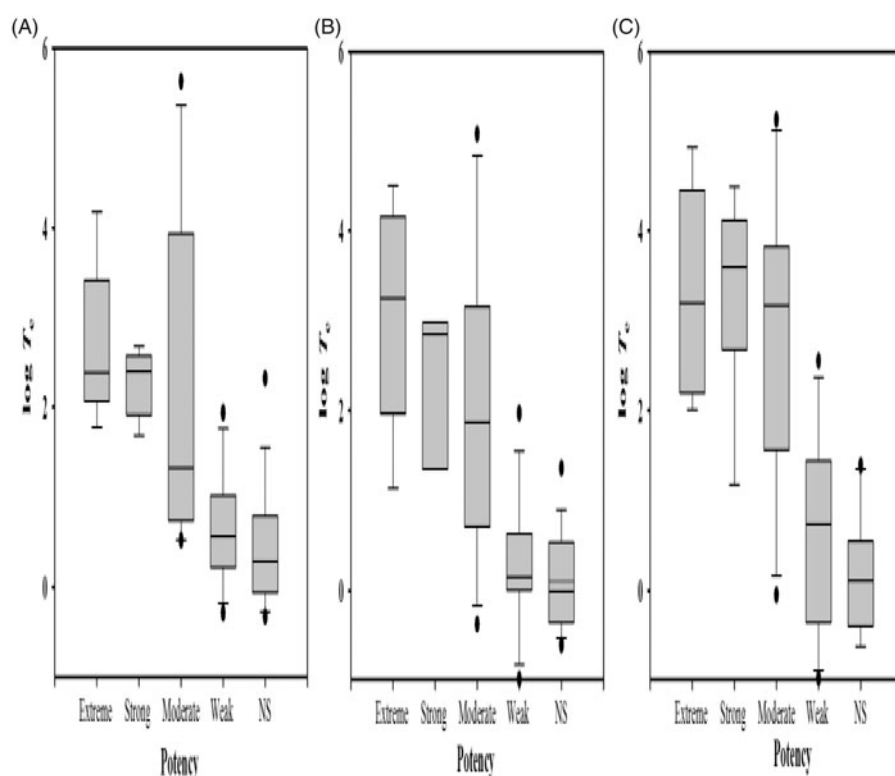


Figure 1. Box-plots of toxicity enhancement ($\log T_e$) against skin sensitization potency. Skin sensitization potency was identified through EC3 values: extreme <0.1%; strong 0.1–1.0%; moderate 1.0–10%; weak 10–100%; non-sensitizer >100%. (A) *Tetrahymena pyriformis*. (B) Fathead minnow. (C) *Daphnia magna*.

Table 4. Sensitivity, specificity and accuracy of *Tetrahymena pyriformis*, fathead minnow, and *Daphnia magna* bioassay models employing $T_e = 10$ as threshold.

Bioassay model	<i>n</i>	Sensitivity	Specificity	Accuracy
<i>Tetrahymena pyriformis</i>	50	85.0	80.0	82.0
Fathead minnow	51	84.2	93.8	90.2
<i>Daphnia magna</i>	53	95.7	80.0	86.8

A $\log T_e = 1$ as a threshold is widely accepted to discriminate excess toxic compounds from basic toxic compounds (von der Ohe et al. 2005; Schramm et al. 2011). In the current study, most of the weak sensitizers only showed narcotic toxicity (average $\log T_e < 1.0$). Aptula et al. (2006) proposed a “rule” that $\log T_e > 0.5$ be a threshold to distinguish sensitizers from non-sensitizers. The present study results were partially consistent with those in the literature indicating that some weak sensitizers yield only narcotic-level toxicities. Nevertheless, $\log T_e = 1$ was still used as a threshold here to separate strong (and extreme) and moderate sensitizers from weak/non-sensitizers.

Each bioassay had up to 80% sensitivity, specificity, and accuracy for use in predicting skin sensitization potency of the test compounds. The sensitivity, specificity, and accuracy levels in the Direct Peptide Reactivity Assay were up to 53% in the pioneer 2004 investigation and improved to 80% in the 2007 analyses. In comparison, another OECD-accepted *in vitro* assay applied in a test cell line also obtained a close test accuracy of 85.1% (Emter et al. 2010). The LuSens assay – based on 69 test compounds – also had up to 80% sensitivity, specificity, and accuracy (Ramirez et al. 2014). Among the three models evaluated here, the highest accuracy and specificity [at-least 90%] was with the fish system. Furthermore, each organism showed different sensitivity in predicting skin sensitization, with the pattern fish > *D. magna* > *T. pyriformis*. Notably, the assays utilized different toxicity endpoints (i.e., growth vs. survival) and

exposure durations (96 hr vs. 48 hr). Accordingly, such differences likely governed the sensitivity, specificity, and accuracy of each model to predicting sensitization potency. Nevertheless, the levels of consistency suggested that the bioassays evaluated here provided reliable outcomes, in many cases on par with the OECD-accepted reactivity assay (DPRA) and other established *in vitro* assays.

While 80% sensitivity, specificity, and accuracy of each bioassay is laudable, there were still some outliers in each case. For example, some moderate sensitizers showed only narcotic-level toxicities or were “non-sensitizers”. There are four possible explanations for such outliers. (A) Some sensitizers are too hydrophobic and fail to induce toxicity enhancement in bioassays; α -terpinene (26), a moderate sensitizer according to LLNA (EC3 = 8.9), is an excellent example. Here, it presented only narcotic-level toxicities ($\log T_e = -0.38$ and -0.05 to fish and *Daphnia*, respectively) because of its relatively high $\log K_{ow}$ of 4.25. Generally, the $\log T_e$ of a compound will decrease with an increase in $\log K_{ow}$ (Schramm et al. 2011), so the toxicity enhancement of α -terpinene is masked due to a high $\log K_{ow}$. (B) Some weak/non-sensitizers showed excess toxicities due to biotransformation in *in vitro* assays. Vanillin (Compound 55) fails to induce sensitization due to a high activation energy (carbonyl group has deactivation effect) (Patlewicz et al. 2001). However, if a demethylated methoxy group is present in the *meta* and *para* position, hydroxyl can then be oxidized to a carbon-oxygen double bond (Patlewicz et al. 2001; Fabjan and Hulzebos 2008) – as illustrated in Figure 3 – enabling a Michael addition. Similarly, 2-nitro-3-pyridinol (Compound 63) shows toxicity enhancement because of biotransformation (Roberts et al. 2007). Notably, biotransformation is important for some sensitizers (especially pro-haptens) to exert their effect (Aptula et al. 2006; Natsch and Haupt 2013), thus, biotransformation should be considered during sensitization risk assessment. (C)

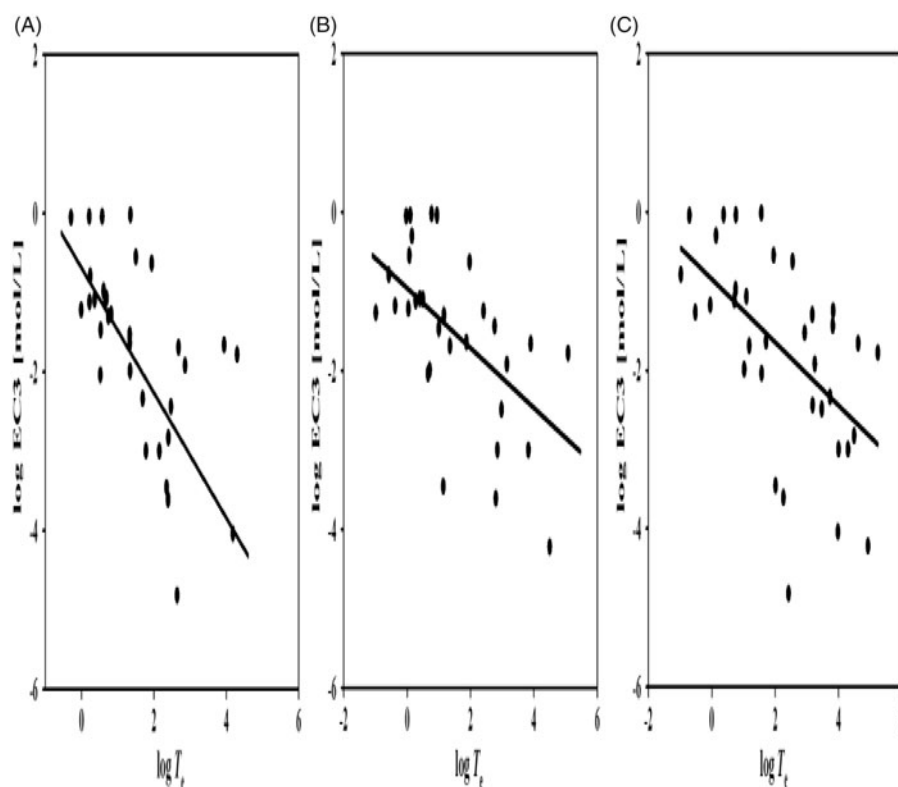


Figure 2. Linear regressions for toxicity enhancement ($\log T_e$) against skin sensitization ($\log EC_3$) for selected compounds (excluding non-sensitizers). Box-plots for (A) *T. pyriformis*. (B) Fathead minnow. (C) *D. magna*.

Table 5. Linear regressions of toxicity enhancements, $\log T_e$, against skin sensitization potency, $\log EC_3$, for different reaction mechanisms.

Mechanism domain	Test organism	n	$\log EC_3 = a \log T_e + b$		r^2	s	Equation
			a	b			
All compounds	<i>Tetrahymena pyriformis</i>	32	-0.789 (± 0.077)	-0.686 (± 0.127)	0.770	0.352	(3)
	Fathead minnow	29	-0.377 (± 0.071)	-0.959 (± 0.120)	0.511	0.367	(4)
	<i>Daphnia magna</i>	35	-0.401 (± 0.056)	-0.860 (± 0.150)	0.605	0.382	(5)
S_N1	<i>Tetrahymena pyriformis</i>	5	-1.024 (± 0.407)	-0.202 (± 0.581)	0.678	0.373	(6)
	<i>Daphnia magna</i>	6	-1.013 (± 0.147)	-1.390 (± 0.486)	0.922	0.293	(7)
S_NAr	<i>Tetrahymena pyriformis</i>	5	-1.317 (± 0.186)	-0.606 (± 0.268)	0.972	0.268	(8)
	Fathead minnow	4	-1.150 (± 0.202)	-0.527 (± 0.352)	0.944	0.346	(9)
	<i>Daphnia magna</i>	5	-1.928 (± 0.195)	-0.375 (± 0.324)	0.970	0.286	(10)
MA	<i>Tetrahymena pyriformis</i>	13	-0.792 (± 0.100)	-0.605 (± 0.168)	0.856	0.338	(11)
	Fathead minnow	10	-0.417 (± 0.109)	-0.856 (± 0.196)	0.618	0.365	(12)
	<i>Daphnia magna</i>	10	-0.492 (± 0.102)	-0.576 (± 0.250)	0.720	0.390	(13)

Slopes of $\log EC_3$ denoted by a ; b denotes intercepts of regression equations; n : number of compounds; r^2 : squared correlation coefficient; s : standard error of estimate.

Table 6. Linear regressions of toxicity enhancements ($\log T_e$) as well as hydrophobicity ($\log K_{ow}$) vs skin sensitization potency ($\log EC_3$) for different reaction mechanisms.

Chemical class	Test organism	n	$\log EC_3 = a \log T_e + b \log K_{ow} + c$			r^2	s	Equation
			a	b	c			
All compounds	<i>Tetrahymena pyriformis</i>	32	-0.761 (± 0.160)	-0.216 (± 0.120)	-0.201 (± 0.418)	0.440	0.914	(14)
	Fathead minnow	29	-0.631 (± 0.108)	-0.185 (± 0.085)	-0.307 (± 0.280)	0.576	0.711	(15)
	<i>Daphnia magna</i>	34	-0.614 (± 0.123)	-0.309 (± 0.119)	-0.013 (± 0.421)	0.444	0.960	(16)
S_N1	<i>Tetrahymena pyriformis</i>	5	-1.273 (± 0.439)	1.189 (± 0.446)	0.258 (± 0.865)	0.910	0.821	(17)
	<i>Daphnia magna</i>	6	-1.061 (± 0.181)	-0.201 (± 0.171)	1.600 (± 0.640)	0.921	0.868	(18)
S_NAr	<i>Tetrahymena pyriformis</i>	5	-1.845 (± 0.206)	-0.533 (± 0.204)	1.930 (± 0.943)	0.984	0.298	(19)
	Fathead minnow	4	-0.720 (± 0.573)	0.410 (± 0.606)	-3.227 (± 2.830)	0.854	1.022	(20)
	<i>Daphnia magna</i>	5	-2.232 (± 0.292)	-0.267 (± 0.213)	1.692 (± 1.079)	0.979	0.348	(21)
MA	<i>Tetrahymena pyriformis</i>	13	-0.770 (± 0.251)	-0.197 (± 0.248)	-0.210 (± 0.692)	0.538	0.786	(22)
	Fathead minnow	10	-0.750 (± 0.140)	-0.539 (± 0.158)	0.451 (± 0.420)	0.784	0.420	(23)
	<i>Daphnia magna</i>	10	-0.724 (± 0.255)	-0.302 (± 0.397)	0.395 (± 1.000)	0.588	0.875	(24)

Slopes of $\log EC_3$ and $\log K_{ow}$ denoted by a and b , respectively; c denotes intercepts of regression equations; n : number of compounds; r^2 : squared correlation coefficient; s : standard error of estimate.

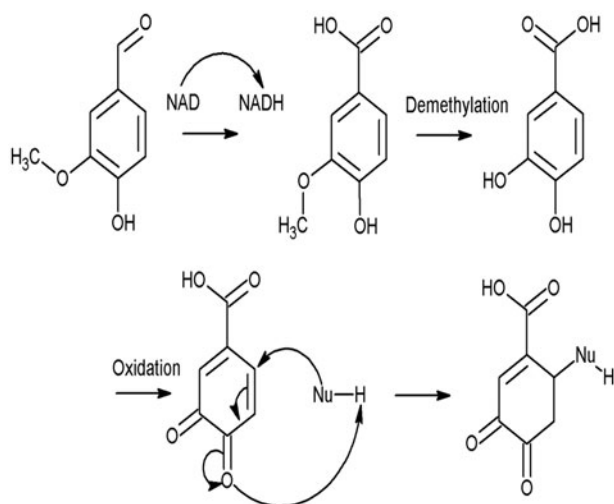


Figure 3. Metabolic transformation of vanillin and its possible reactions.

Some skin sensitization data were false-negatives. For example, benzaldehyde (Compound 41) is identified as a non-sensitizer in the LLNA, but human data (patch test) indicated it is a moderate sensitizer (EC3 value of 10%) (Natsch et al. 2012). Indeed, here, benzaldehyde elicited excess toxicity to the fish ($\log T_e = 1.36$) and *D. magna* ($\log T_e = 1.34$), and its toxicity to *T. pyriformis* was 6-times higher than its narcotic toxicity. Both explanations (B) and (C) suggest the importance of biotransformation in skin sensitization. In the three bioassays here, biotransformation of some sensitizing pro-haptens is possible. However, from past studies it is clear that in many cases, a presence of additional enzymes (such as various P_{450} s or those commonly found in S9 mixes prepared from rat liver) are needed to accurately measure reactive capabilities of test compounds and their skin sensitization potency (Natsch and Haupt 2013). (D) The potency of some sensitizers could be underestimated in LLNA due to compound loss via volatilization. By this, some reactive agents that should be strong or moderate sensitizers exhibit only weak sensitization potency. Roberts and Natsch (2009) found that loss by volatilization was an important factor that could result in low skin sensitization potency in an LLNA. Examples of this can be seen by comparing methyl (Compound 33) and ethyl (Compound 34) acrylates to 2-ethylhexyl acrylate (Compound 23). Sensitization potencies of each agent are comparably weak, but their excess toxicities differ; this could be explained by significant difference in vapor pressures, i.e., 1.59 vs. 1.94 vs. -0.75 (log values; EPI suite), respectively. Such volatilization loss was also noted in other bioassays and clearly impacted on accurate toxicity calculations (Schramm et al. 2011).

While T_e has been successfully applied in the qualitative monitoring of skin sensitization (Aptula et al. 2006; Arning et al. 2009), the current study established a quantitative relationship between $\log EC3$ and $\log T_e$ (excluding non-sensitizers because of their missing EC3 values) (Equations (3)–(5)). Furthermore, linear regressions were established for all compound classes as well as different reaction mechanisms. Interestingly, for all compound classes, the *T. pyriformis* model had the steepest slope (-0.807) compared with the fish (-0.377) and *Daphnia* (-0.401) models. This result suggested the *Tetrahymena pyriformis* model was the most sensitive to monitor skin sensitization potencies. Indeed, the $\log T_e$ of *T. pyriformis*, fathead minnow, and *D. magna* (5.96, 6.07, and 6.63-orders of magnitude against the same 4.80 log units of skin sensitization potency) might help

explain why the *T. pyriformis* model presented with the steepest slope.

The above conclusions were based on the degree of accuracy and specificity, which indicated the fish model was superior to the *D. magna* model. That the two model outcomes conflicted was possible because of discrepancies between qualitative and quantitative analyses and partly due to some false-negative $\log T_e$ values that could statistically affect their generated regressions. For example, hexane is structurally a narcotic-level compound and a non-sensitizer, but its false-negative $\log T_e$ value (-1.39) in *D. magna* suggested an experimental toxicity 10-times below baseline. Volatilization [and sorption] losses with different exposure durations could also be a reason for the false-negative toxicity data, as $>99\%$ of hexane can be lost in that manner (Schramm et al. 2011).

Based on the different slopes, the contribution of toxicity enhancement to skin sensitization potential followed a decreasing scale, i.e., $S_{NAr} > S_{N1} > MA$. This finding agreed with previous reports that the degree of increase in skin sensitization differed with each reaction mechanism. As Michael acceptors present the lowest slopes compared to other reaction mechanisms (Natsch et al. 2011), this suggests that great increases in toxicity enhancement of Michael acceptors only slightly increase sensitization potency (compared with sensitizers that act via S_{N1} and S_{NAr}). Thus, bioassays should be carefully used if monitoring Michael acceptor sensitization potencies.

Penetration of the stratum corneum is a first step in inducing skin sensitization (Aeby et al. 2010); this penetration process is usually modeled by hydrophobicity. Thus, a possible reason for failure of weak or non-sensitizers to induce skin sensitization could be a low bioavailability. Hydrophobicity showed a clear contribution to skin sensitization of the test compounds (though r^2 decreased while s increased). Slopes of bioavailability ($\log K_{ow}$) and reactivity ($\log T_e$) in the fish and *T. pyriformis* models strongly indicated bioavailability and reactivity were inter-dependent and cooperatively contributed to compound sensitization. However, $\log K_{ow}$ (which has a broad range) would be expected to less predictive of skin sensitization potency; this was confirmed by the steeper slopes of $\log T_e$ compared to those of $\log K_{ow}$ (Equations (14)–(16)).

Bioavailability also seemed to impact skin sensitization potency via each of the S_{N1} , S_{NAr} and MA reaction mechanisms. In the *T. pyriformis* model for S_{N1} (Equation (17) and the minnow model for S_{NAr} (Equation (20) mechanisms, potency likely only depended on chemical reactivity, not on hydrophobicity. *In silico* studies also suggested that skin sensitization via Schiff base (SB) and S_{NAr} mechanisms were independent of hydrophobicity (Roberts et al. 2006, 2011). For the Michael acceptors, a role for hydrophobicity in skin sensitization appeared in all three bioassays, as illustrated by negative $\log K_{ow}$ slopes and decreased r^2 and s values.

Conclusions

Toxicity enhancement (T_e) appears to be a useful parameter to rank skin sensitization potencies of test agents, and could be used to discriminate strong, extreme, and moderate sensitizers from weak or non-sensitizers. Still, such enhancements vary in their ability to be used to predict sensitization potency, due to their different reaction mechanisms. Nevertheless, these bioassays seem to be useful approaches to predict skin sensitization by a variety of compounds and could be applied to replace or reduce animal tests. However, these tools should be carefully applied

with non-sensitizers that could undergo biotransformation to agents that now impart excessive toxicity.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The authors acknowledge financial support from the Scientific Research and Service Platform Fund of Henan Province (2016151) and the funds of the scientific and technological innovation team of water ecological security (Water Source Region of Midline of South-to-North Diversion Project of Henan Province) (17454).

References

- Aeby P, Ashikaga T, Bessou-Touya S, Schepky A, Gerberick F, Kern P, Marrec-Fairley M, Maxwell G, Ovigne JM, Sakaguchi H, et al. 2010. Identifying and characterizing chemical skin sensitizers without animal testing: Colipa's research and method development program. *Toxicol In Vitro*. 24:1465–1473.
- Aleksic M, Thain E, Roger D, Saib O, Davies M, Li J, Aptula A, Zazzeroni R. 2009. Reactivity profiling: Covalent modification of single nucleophile peptides for skin sensitization risk assessment. *Toxicol Sci*. 108:401–411.
- Anderson S, Siegel P, Meade B. 2011. The LLNA: A brief review of recent advances and limitations. *J Allergy (Cairo)*. 2011:424203.
- Aptula A, Patlewicz G, Roberts D. 2005. Skin sensitization: Reaction mechanistic applicability domains for structure-activity relationships. *Chem Res Toxicol*. 18:1420–1426.
- Aptula A, Patlewicz G, Roberts D, Schultz T. 2006. Non-enzymatic glutathione reactivity and *in vitro* toxicity: A non-animal approach to skin sensitization. *Toxicol In Vitro*. 20:239–247.
- Aptula A, Roberts D. 2006. Mechanistic applicability domains for non-animal-based prediction of toxicological endpoints: General principles and application to reactive toxicity. *Chem Res Toxicol*. 19:1097–1105.
- Arning J, Matzke M, Stolte S, Nehen F, Bottin-Weber U, Bösch A, Abdulkarim S, Jastorff B, Ranke J. 2009. Analyzing cytotoxic effects of selected isothiazol-3-one biocides using the toxic ratio concept and structure-activity relationship considerations. *Chem Res Toxicol*. 22:1954–1961.
- Blaschke U, Eismann K, Böhme A, Paschke A, Schüürmann G. 2011. Structural alerts for the excess toxicity of acrylates, methacrylates, and propiolates derived from their short-term and long-term bacterial toxicity. *Chem Res Toxicol*. 25:170–180.
- Blaschke U, Paschke A, Rensch I, Schüürmann G. 2010. Acute and chronic toxicity toward the bacteria *Vibrio fischeri* of organic narcotics and epoxides: Structural alerts for epoxide excess toxicity. *Chem Res Toxicol*. 23:1936–1946.
- Böhme A, Thaens D, Paschke A, Schüürmann G. 2009. Kinetic Glutathione chemoassay to quantify thiol reactivity of organic electrophiles: Application to α,β -unsaturated ketones, acrylates, and propiolates. *Chem Res Toxicol*. 22:742–750.
- Böhme A, Thaens D, Schramm F, Paschke A, Schüürmann G. 2010. Thiol reactivity and its impact on the ciliate toxicity of α,β -unsaturated aldehydes, ketones, and esters. *Chem Res Toxicol*. 23:1905–1912.
- Ellison C, Cronin M, Madden J, Schultz T. 2008. Definition of the structural domain of the baseline non-polar narcosis model for *T. pyriformis*. *Environ Res*. 19:751–783.
- Emter R, Ellis G, Natsch A. 2010. Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers *in vitro*. *Toxicol Appl Pharm*. 245:281–290.
- Fabjan E, Hulzebos E. 2008. An evaluation of selected valid and mechanistically based SARs for skin sensitization. *Toxicol In Vitro*. 22:468–490.
- Gerberick F, Ryan C, Kern P, Schlatter H, Dearman R, Kimber I, Patlewicz G, Basketter D. 2005. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. *Dermatitis*. 16:157–202.
- Gerberick G, Vassallo J, Bailey R, Chaney J, Morrall S, Lepoittevin J. 2004. Development of a peptide reactivity assay for screening contact allergens. *Toxicol Sci*. 81:332–343.
- Gerberick G, Vassallo J, Foertsch L, Price B, Chaney J, Lepoittevin J. 2007. Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach. *Toxicol Sci*. 97:417–427.
- Jeong Y, An S, Shin K, Lee T. 2013. Peptide reactivity assay using spectrophotometric method for high-throughput screening of skin sensitization potential of chemical haptens. *Toxicol In Vitro*. 27:264–271.
- Kern P, Gerberick G, Ryan C, Kimber I, Aptula A, Basketter D. 2010. Local lymph node data for the evaluation of skin sensitization alternatives: A second compilation. *Dermatitis*. 21:8–32.
- mOECD. 1992. Guideline for Testing of Chemicals No. 406, Skin Sensitization. Adopted 17th July.
- mOECD. 2002. Guideline for Testing of Chemicals No. 429, Skin Sensitization: Local Lymph Node Assay. Adopted 24th April.
- Natsch A, Gfeller H. 2008. LC-MS-based characterization of the peptide reactivity of chemicals to improve the *in vitro* prediction of the skin sensitization potential. *Toxicol Sci*. 106:464–478.
- Natsch A, Gfeller H, Haupt T, Brunner G. 2012. Chemical reactivity and skin sensitization potential for benzaldehydes: Can Schiff base formation explain everything? *Chem Res Toxicol*. 25:2203–2215.
- Natsch A, Haupt T. 2013. Utility of rat liver S9 fractions to study skin-sensitizing pro-haptens in a modified KeratinoSens assay. *Toxicol Sci*. 135:356–368.
- Natsch A, Haupt T, Laue H. 2011. Relating skin sensitizing potency to chemical reactivity: Reactive Michael acceptors inhibit NF- κ B signaling and are less sensitizing than S_NAr - and S_N2 -reactive chemicals. *Chem Res Toxicol*. 24:2018–2027.
- Patlewicz G, Basketter D, Smith C, Hotchkiss S, Roberts D. 2001. Skin-sensitization structure-activity relationships for aldehydes. *Contact Dermatitis*. 44:331–336.
- Ramirez T, Mehling A, Kolle SN, Wruck CJ, Teubner W, Eltze T, Aumann A, Urbisch D, van Ravenzwaay B, Landsiedel R. 2014. LuSens: A keratinocyte based ARE reporter gene assay for use in integrated testing strategies for skin sensitization hazard identification. *Toxicol In Vitro*. 28:1482–1497.
- Roberts D, Aptula A, Patlewicz G. 2006. Mechanistic applicability domains for non-animal based prediction of toxicological endpoints. QSAR analysis of the Schiff base applicability domain for skin sensitization. *Chem Res Toxicol*. 19:1228–1233.
- Roberts D, Aptula A, Patlewicz G. 2011. Chemistry-based risk assessment for skin sensitization: Quantitative mechanistic modeling for the S_NAr domain. *Chem Res Toxicol*. 24:1003–1011.
- Roberts D, Natsch A. 2009. High throughput kinetic profiling approach for covalent binding to peptides: Application to skin sensitization potency of Michael acceptor electrophiles. *Chem Res Toxicol*. 22:592–603.
- Roberts D, Patlewicz G, Kern P, Gerberick F, Kimber I, Dearman R, Ryan CA, Basketter D, Aptula A. 2007. Mechanistic applicability domain classification of a local lymph node assay dataset for skin sensitization. *Chem Res Toxicol*. 20:1019–1030.
- Schramm F, Müller A, Hammer H, Paschke A, Schüürmann G. 2011. Epoxide and thiirane toxicity *in vitro* with the ciliates *Tetrahymena pyriformis*: Structural alerts indicating excess toxicity. *Environ Sci Technol*. 45:5812–5819.
- Thaens D, Heinzelmann D, Böhme A, Paschke A, Schüürmann G. 2012. Chemoassay screening of DNA-reactive mutagenicity with 4-(4-nitrobenzyl)pyridine - application to epoxides, oxetanes, and sulfur heterocycles. *Chem Res Toxicol*. 25:2092–2102.
- van Leeuwen C, van der Zandt P, Aldenberg T, Verhaar H, Hermens J. 1992. Application of QSARs, extrapolation and equilibrium partitioning in aquatic effects assessment. I. Narcotic industrial pollutants. *Environ Toxicol Chem*. 11:267–282.
- von der Ohe P, Kühne R, Ebert R-U, Altenburger R, Liess M, Schüürmann G. 2005. A new classification model to discriminate excess toxicity from narcotic effect levels of organic compounds in the acute daphnid assay. *Chem Res Toxicol*. 18:536–555.