

Read-across to rank skin sensitization potential: subcategories for the Michael acceptor domain

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Background: Eliminating animal testing for skin sensitization is a significant challenge in consumer safety risk assessment. To be able to perform resilient risk assessments in the future, one will need alternative approaches to fill the data gaps.

Objectives: To this end, we propose a subcategory-based read-across approach to estimate and rank skin sensitization potential of chemicals. The example described here is for the mechanism of Michael-type nucleophilic addition with subcategories being limited to carbonyl-containing compounds.

Patients/Methods: In this approach, *in silico* tools based on structural alerts were used to determine both the mechanism of protein binding and the relative subcategories within that mechanism.

Results: Fifty compounds previously evaluated in the *in vivo* mouse local lymph node assay (LLNA) were placed in 10 subcategories defined by their polarized α,β -unsaturated substructure. To offset the limitations and skewness of the published *in vivo* data, *in chemico* glutathione (GSH) depletion data also were included.

Conclusions: It was shown that the read-across approach can be successfully used to rank qualitatively skin sensitization potential of an untested carbonyl-containing Michael acceptor chemical by using subcategories. Moreover, the use of the more resilient *in chemico* GSH depletion data added further support to the read-across result.

Key words: categories; glutathione reactivity; LLNA; Michael acceptors; skin sensitization; subcategories. © Blackwell Munksgaard, 2008.

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There is a general agreement that any substance that binds irreversibly to proteins has the potential to be a sensitizer (1). Yet, in order to determine if a chemical will be an *in vivo* sensitizer, other issues such as bioavailability and other aspects of immunogenicity may have to be considered in a holistic approach (2). Currently, what these other issues are and how to measure or scale them is under debate (3). This debate reflects the fact that our current knowledge of skin sensitization is incomplete; however, it is clearly established that there are both qualitative and quantitative elements to sensitization.

For several reasons, including time, resources, animal welfare, and recent and impending legisla-

tion, there is an increasing importance on replacing *in vivo* testing for skin sensitization (4). A potential approach to limiting the need for animal testing, which has been proposed for skin sensitization, is mechanistic-based 'read-across' (5, 6). Read-across is a concept used to predict end-point information for one chemical using data from the same end-point from another chemical(s) that is considered to be 'similar' in some way (7, 8). Read-across methods work best for homologous series of chemicals where the metric for extrapolating from one chemical to another can be most easily linked to a particular property of the chemicals. This concept is explained further in the Organization for Economic Co-operation and

Development (OECD) guidance document on grouping chemicals (8).

In some cases, a toxic effect can be present for some, but not all, members of an analogue group. For example, covalent protein binding is considered as a common property for skin sensitizing agents, yet not all protein binders are skin sensitizing agents in the local lymph node assay (LLNA) [e.g. 2-hydroxypropyl methacrylate $RC_{50} = 28$]. We note that being a non-sensitizer in the LLNA does not necessarily mean that a compound is incapable of sensitizing. Thus, the specific compound mentioned above, although a non-sensitizer in the LLNA, is reported as a contact allergen in man* (9)]. In addition, those sensitizing agents, even ones with a common reactive mechanism (e.g. Michael addition), may not show a consistent trend when sensitizing potency is considered. In these cases, it is helpful to divide the chemical category into subcategories or more similar restrictive analogous sets. The term category is used here in the context described in the OECD guidance document (8). The concept of subcategories improves the practicality, flexibility, and predictivity of the analogue approach without altering the scientific basis, common mechanism of action, of the analogous series.

In the case of qualitative read-across, the presence or absence of an activity for the untested chemical of interest is inferred from the presence or absence of the same activity for the tested analogue(s). In the qualitative instance, the analogous category is often defined solely on the mechanism of action. However, in the case of quantitative read-across, a known parameter value for tested analogue(s) is used to estimate the value for the untested chemical with the assumption that the potency of the effect of interest is shared by both the tested and the untested analogue. We propose that meeting the assumption of shared potency in skin sensitization will require the use of subcategories within a given mechanistic category. This is consistent with the goal of moving assessment of skin sensitization from one of qualification to one of quantification (10).

A conceptual framework for predicting the toxicity of reactive chemicals, with an emphasis on modelling soft electrophilicity, was recently developed (11). Empirical measures of the chemical reactivity of xenobiotics with model nucleophiles

such as thiol can be used to simulate the relative rates at which a reactive chemical is likely to bind irreversibly to nucleophiles in cellular targets (12). Assuming the mechanism of action relating to the end point being predicted is governed by reactivity to proteins as part of the molecular initiating event, these data can be used to scale the trend across the members of the chemical mechanism of action domain and establish subcategories for the biological mechanism of action. Previous work has shown that empirical measures of intrinsic reactivity verify *in silico* rules of the chemical mechanism of action and establish the applicability domain (11, 13). In addition, reactivity measurements serve as quantitative descriptors of selected toxic effects for reactive mechanisms of toxic action (11). In an effort to exploit the link between chemicals that are covalent protein binders and their potential to sensitize, we propose using an analogue approach for read-across. To this end, we selected the Michael acceptor chemical mechanism of action as defined by Roberts et al. (7).

The hypothesis for this read-across investigation of skin sensitization is that chemical reactivity is the rate determining step. Therefore, we propose forming subcategories based on principles of reactivity. The specific aim of this study was to use structural fragments to form subcategories within the mechanistic category of Michael acceptor protein binders. LLNA data (14) were then used to rank skin sensitization potential within the subcategories. Using this hypothesis, *in chemico* reactivity data (15) were used in a supplementary fashion, especially where only limited *in vivo* data were available within a subcategory to support the ranking of skin sensitization potential. It is proposed that this combination of information (i.e. *in vivo* and *in chemico*) gives greater degree of confidence in read-across to predict the skin sensitization potential of untested compounds residing within each subcategory.

Materials and Methods

Subcategorizing Michael acceptors from an in vivo data set

Aptula et al. (16) described the reaction mechanistic applicability domains that relate to skin sensitization. Among these is the polarized, α,β -unsaturated structural domain of Michael type nucleophilic addition. Because the Michael acceptor domain is structurally diverse, it is difficult to model quantitatively; this suggests that there is a need for further subcategorization.

As described by Roberts et al. (7), 55 compounds of the 210 chemicals in the LLNA data set published in Gerberick et al. (14) were noted

*Although the LLNA has proved very useful since its introduction, it does not always agree with human and guinea-pig data. In fact, there are some indications that the threshold for a positive result in the LLNA may be somewhat higher. The threshold for the determination of a positive sensitizer in the LLNA was set to be equivalent to regulatory classification (R43), which means (as with the equivalent guinea-pig tests) that some substances shown to be sensitizers in man will be too weak in potency to be picked up in the LLNA.

as being in the Michael acceptor mechanistic domain. A number of subcategories of this mechanism each linked to a particular two-dimensional structural alert have been demonstrated (13). Applying these structural alerts to the LLNA *in vivo* skin sensitization data shows that the direct-acting Michael acceptors can be placed in one of a series of mutually exclusive subcategories. To account for metabolism and other conversions, this subcategorization scheme was expanded to include substances that can be transformed either biotically or abiotically to compounds possessing the polarized α,β -unsaturated substructure.

In chemico thiol reactivity

Expanding on the work of Schultz et al. (15) that examined *in chemico* reactivity data of α,β -unsaturated carbonyl compounds towards the model nucleophile glutathione (GSH), a further series of potential Michael acceptors were tested. Chemicals were selected to reflect each of the 10 subcategories noted in Table 1. In each test, the tripeptide GSH was used as a model nucleophile, and reactivity was quantified in a concentration–response scheme with free thiol being measured [following reaction with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB)] spectrophotometrically at 412 nm as described in (15). Abiotic thiol reactivity expressed as RC_{50} is the concentration of tested compound at which 50% of the thiol moiety of the tripeptide GSH is unbound after 120 min. Range finding experiments were conducted, followed by definitive experiments with the toxicant concentrations adjusted so that at least three partial effects and one partial effect on each side of the 50% effect concentration were observed. Definitive assays were repeated twice, each with fresh reduced GSH and toxicant stock solutions. No decrease of GSH was observed in controls who were spiked with dimethyl sulphoxide (DMSO) only. Initially, negative control experiments, ones without GSH, were conducted to make sure that the test substances did not interfere with the absorbance of DTNB-conjugates. In addition, two vials of buffer were included in each assay, one with thiol, the control, and the other without thiol, the blank.

Additional *in chemico* testing was performed on the compounds that were not reactive in the buffer-based method. In these cases, the reactivity assessment was performed in a medium of 50% methanol and 50% buffer with all other parameters being the same as described by Schultz et al. (15). This modification increased the solubility of test substances without altering inherent reactivity.

Test substances were purchased from commercial sources (e.g. Sigma-Aldrich, St. Louis, MO, USA) in the highest purity available (95% minimum) and were not further purified. Stock solutions of each were prepared by dissolving the agent in DMSO just prior to use. In each small glass vial, 1 ml GSH solution was added, followed by an aliquot of stock solution, and then the appropriate amount of phosphate buffer to bring the final volume to 10 ml. This resulted in a final thiol concentration of 0.1375 mM. Vials were capped, shaken gently, and left to stand for 120 min prior to the addition of 200 μ L of 50 mM DTNB. The 120-min time-point was selected to allow the greatest time for reacting while reducing the potential for oxidation of the GSH to GSSG, which would reduce the free thiol concentration by means other than the test chemical binding to the thiol. Absorbance was read at 412 nm and recorded with a Thermo electronics Genesys™ 6 spectrophotometer. The effect levels were determined from nominal toxicant concentrations, and the RC_{50} values were determined by Probit Analysis using SAS software (17) with absorbance normalized to control as the Y variable and toxicant concentration as the X variable.

Results

In vivo data

Historically, methods for identifying skin sensitizing chemicals utilized end points that show the onset of allergic contact dermatitis. Among these methods are the guinea-pig maximization test and the occluded patch test of Buehler (18). In recent decades, information has been acquired with the murine LLNA (19–21), which provides a substantial refinement over the guinea-pig assays. The LLNA is a well established and extensively evaluated approach that underwent significant validation before final acceptance as an OECD-approved method under test guideline 429 (22). This assay allows for a quantitative assessment of skin sensitization potency through the use of the EC3% value and broader grouping of sensitization as extreme, strong, moderate, weak, and non-sensitizers (23). Several other classification schemes are also available based on smaller number of groups (24). The decision to choose the one published in (23) was based on the assumption that if we could successfully demonstrate the applicability of the read-across concept using GSH reactivity data for the scheme with the most number of groups (extreme, strong, moderate, weak, and non-sensitizers), then it should readily be equally applicable also for the other classification schemes with smaller number of groups.

Table 1. Michael acceptor domain with LLNA data

No.	Chemical name	CAS no.	LLNA EC3 (%)	Potency group	Subcategory ^a
1	<i>p</i> -Benzoquinone	106-51-4	0.0099	Extreme	1
2	Hydroquinone	123-31-9	0.11	Strong	9
3	1,4-Phenylenediamine	106-50-3	0.16	Strong	9
4	2,5-Diamino toluene	95-70-5	0.2	Strong	9
5	Lauryl gallate (dodecyl gallate)	1166-52-5	0.3	Strong	9
6	2-Aminophenol	95-55-6	0.4	Strong	9
7	2-Nitro- <i>p</i> -phenylenediamine	5307-14-2	0.4	Strong	9
8	2-Methyl-5-hydroxyethylaminophenol	55302-96-0	0.4	Strong	10
9	3-Phenylenediamine	108-45-2	0.5	Strong	10
10	4-(<i>N</i> -Ethyl- <i>N</i> -2-methan-sulphonamido-ethyl)-2-methyl-1,4-phenylenediamine (CD3)	25646-71-3	0.6	Strong	9
11	Isopropyl isoeugenol		0.6	Strong	10
12	4-(Methylamino)phenol sulfate	55-55-0	0.8	Strong	9
13	2-Hydroxyethyl acrylate	818-61-1	1.4	Moderate	3
14	6-Methylisoeugenol	13041-12-8	1.6	Moderate	10
15	Vinyl pyridine	1337-81-1	1.6	Moderate	8
16	Isoeugenol	97-54-1	1.7	Moderate	10
17	2-Amino-6-chloro-4-nitrophenol	6358-09-4	2.2	Moderate	9
18	HC Red No. 3	2871-01-4	2.2	Moderate	9
19	<i>trans</i> -Anethol	104-46-1	2.3	Moderate	10
20	<i>trans</i> -2-Decenal	3913-71-1	2.5	Moderate	4
21	Methyl 2-nonynoate	111-80-8	2.5	Moderate	2
22	Cinnamic aldehyde	104-55-2	3.0	Moderate	4
23	3-Aminophenol	591-27-5	3.2	Moderate	10
24	3-Methylisoeugenol	186743-29-3	3.6	Moderate	10
25	Benzylidene acetone (4-phenyl-3-buten-2-one)	122-57-6	3.7	Moderate	5
26	2,4-Heptadienal	5910-85-0	4.0	Moderate	4
27	α -Methyl cinnamic aldehyde	101-39-3	4.5	Moderate	7
28	<i>trans</i> -2-Hexenal	6728-26-3	5.5	Moderate	4
29	2-Methoxy-4-methyl-phenol	93-51-6	5.8	Moderate	10
30	Diethyl maleate	141-05-9	5.8	Moderate	5
31	Dihydroeugenol	2785-87-7	6.8	Moderate	10
32	Safranal (1,1,3-trimethyl-2-formylcyclohexa-2,4-diene)	116-26-7	7.5	Moderate	7
33	Perillaldehyde	2111-75-3	8.1	Moderate	7
34	1-(<i>p</i> -Methoxyphenyl)-1-penten-3-one	104-27-8	9.3	Moderate	5
35	α -Hexyl cinnamic aldehyde	101-86-0	11	Weak	7
36	α -Amyl cinnamic aldehyde	122-40-7	11	Weak	7
37	α -Butyl cinnamic aldehyde	7492-44-6	11	Weak	7
38	Eugenol	97-53-0	13	Weak	10
39	5-Methyleugenol	186743-25-9	13	Weak	10
40	6-Methyleugenol	186743-24-8	17	Weak	10
41	4-Allylanisole	140-67-0	18	Weak	10
42	Cinnamic alcohol	104-54-1	21	Weak	10
43	Ethyl acrylate	140-88-5	28	Weak	3
44	Ethylene glycol dimethacrylate	97-90-5	28	Weak	6
45	3-Methyleugenol	186743-26-0	32	Weak	10
46	Isopropyl eugenol	51474-90-9	NC	Non-sensitizer	10
47	Coumarin	91-64-5	NC	Non-sensitizer	5
48	2-Hydroxypropyl methacrylate	923-26-2	NC	Non-sensitizer	6
49	6-Methylcoumarin	92-48-8	NC	Non-sensitizer	5
50	Resorcinol	108-46-3	NC	Non-sensitizer	10

NC, not calculable.

^aQuinones {1}, acetylenic esters and ketones {2}, vinyl esters and ketones {3}, vinylene aldehydes {4}, vinylene esters and ketones {5}, methacrylates {6}, methacrylate-like aldehydes {7}, vinyl pyridines {8}, pre-Michael acceptor aromatics with *o*-hydroxyl or *p*-hydroxyl- and/or amino groups {9}, and metabolically activated pro-Michael acceptors {10}.

A recently published data set (14) represents the depth and breadth of chemicals tested in the LLNA that are currently available in the public domain. For some chemicals, it is possible to find several different LLNA results (e.g. some hair dyes, 24). We should bear in mind that the error limits for the LLNA EC3 value are typically a factor of 2 (25).

More recent publications summarize the chemistry of the major chemical reaction domains (26). These reactions can be used to classify and rationalize skin sensitization data sets (13, 16, 27). Despite the significant number of chemicals tested in the LLNA data set (12), attempts to develop structure–activity relationships have had limited success (28). Previous efforts by our group and

others, even with local models restricted by mechanistic categories, result in models with unsatisfactory performance. These limitations reflect the fact that the data set was not designed for structure–activity modelling but rather to validate the assay with the intent of using it to evaluate substances of commercial interest. Realizing the limitations of the published data set (14), the current evaluation was undertaken.

A cursory examination of the 55 chemicals in the LLNA database identified as Michael acceptors (7) shows that while the majority are sensitizers, potency varies over several orders of magnitude. Structurally, the majority are olefinic carbonyl-containing chemicals or compounds that are likely to be metabolized into an olefinic carbonyl-containing chemical. Three compounds – 1,2-dibromo-2,4-dicyanobutane (methyldibromo-glutaronitrile), 3-bromomethyl-5,5-dimethyl-dihydro-2(3H)-furanone, and vinylidene dichloride – contain halogens and are thought to be classified better, as their structure suggests, in the S_N2 applicability domain (29). For 1,2-dibromo-2,4-dicyanobutane, Patlewicz et al. (30) also proposed an S_N2 mechanistic pathway. 5,5-Dimethyl-3-methylene-dihydro-2(3H)-furanone is an alpha substituted vinyl ester. As such, this compound is similar to a methacrylate; however, a key difference is that in a methacrylate, the α -substituent is an electron-donating (deactivating) methyl group, whereas in the lactone, the α -substituent is a β -acetoxyethyl group whose electronic effect is similar to that of a hydrogen atom (26).

The remaining 50 compounds formed the basis for our analyses and were placed in the subcategory defined by their polarized α,β -unsaturated substructure: quinones {1}, acetylenic esters and ketones {2}, vinyl esters and ketones {3}, vinylene aldehydes {4}, vinylene esters and ketones {5}, methacrylates {6}, and methacrylate-like aldehydes {7}. Additional subcategories included vinyl pyridines {8}, aromatics with *o*-hydroxyl or *p*-hydroxyl- and/or amino groups {9}, and metabolically activated pro-Michael acceptors {10}. These subcategories closely followed the categorization defined in (13) (see also Fig. 1). It is realized that subcategories {1} to {8} are readily identified from structure. Subcategory {9}, however, includes compounds that undergo transformations to quinones, quinoneimines, or quinone methides (26). Subcategory {10} is the most uncertain as our ability to predict enzymatic metabolism is currently incomplete. It is likely that the active metabolites would be placed in one of the other subcategories.

Table 1 shows the appropriate subcategory for each of the 50 Michael acceptors with LLNA

data. In addition, their LLNA EC3% value and potency group are listed. There is an uneven distribution of the *in vivo* data for LLNA-tested Michael acceptors between subcategories. Only one tested chemical belongs to subcategory {1}, namely *p*-benzoquinone. Subcategories {2} and {8} also have only one tested representative in the LLNA, methyl 2-nonynoate and vinyl pyridine, respectively. Two chemicals populate subcategory {3}, two chemicals represent subcategory {6}, four chemicals represent subcategory {4}, five compounds are in subcategory {5}, and six are in subcategory {7}. The majority of the LLNA-tested Michael acceptor compounds are classified in subcategory {9} or subcategory {10}, 10 and 18 chemicals, respectively.

The comparison of LLNA potency group and subcategory shows that each subcategory is populated with different potency groups. *p*-Benzoquinone in subcategory {1} is an extreme sensitizer. Methyl-2-nonynoate and vinyl pyridine, the lone representatives of subcategories {2} and {8}, respectively, both are moderate sensitizers in the LLNA. The four representatives of subcategory {4}, vinylene aldehydes, are classified as moderate sensitizers. Both moderate and weak sensitizers are found in subcategories {3} and {7}. Subcategory {3}, the vinyl esters and ketones, is represented in the LLNA data set by two structurally similar acrylates, ethyl and 2-hydroxyethyl. Yet, the EC3% values of these two acrylates are 20-times different. Subcategory {7} is the methacrylate-like aldehydes that are represented in the LLNA data set by six compounds, three of which are moderate sensitizers and three of which are weak sensitizers. The three weak sensitizers (α -butyl-, α -amyl-, and α -hexyl-cinnamic aldehyde) have EC3% values of 11, which is very close to the cut-off value of 10 that separates the moderate and weak potency categories in the LLNA (11). Three of the five compounds tested in the LLNA belonging to subcategory {5} are moderate sensitizers. The remaining two, coumarin and 6-methyl coumarin, are non-sensitizers. Subcategory {6}, methacrylates, is represented in the LLNA data set by ethylene glycol dimethacrylate and 2-hydroxypropyl methacrylate, which are reported as weak and non-sensitizer, respectively. This discrepancy in potency categorization is considered to be minor, as EC3% values are in the range of 10–100.

The majority of the compounds (8 of 10) in subcategory {9} are strong sensitizers. The other two compounds, 2-amino-6-chloro-4-nitrophenol and HC Red No. 3 [4-(2-hydroxyethyl)amino-3-nitroaniline] are moderate sensitizers. Sensitization potency subcategory {10} is the most diverse,

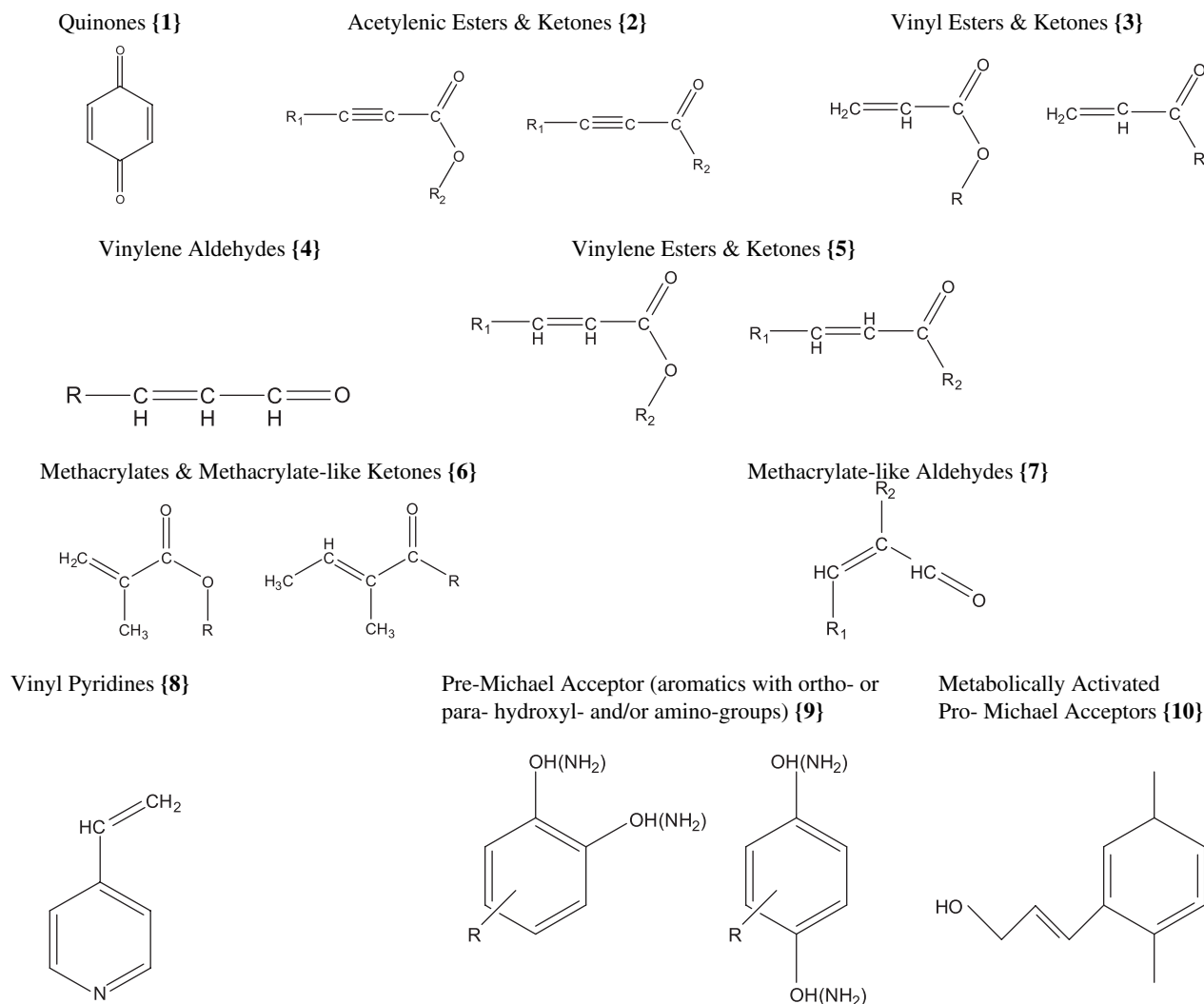


Fig. 1. Representative structure(s) for each subcategory.

being populated with three strong, seven moderate, six weak, and two non-sensitizers (Table 1).

In chemico data

The *in chemico* GSH reactivity RC_{50} values for all of LLNA-tested compounds in subcategories {1} to {8} along with some additional representative compounds are reported in Table 2. For the two larger subcategories, {9} and {10}, representative compounds were tested. In addition, for the subgroups with less than five LLNA-tested compounds, additional substances were evaluated so that each subcategory, with the exception of vinyl pyridines {8}, had five members tested for GSH reactivity. Most of the direct-acting electrophiles, subcategories {1} to {8}, are found to be reactive in the GSH assay. The exceptions – 2-ethylhexyl acrylate, ethyl-trans-2-octanoate, coumarin, 6-methyl coumarin, ethylene glycol dimethacrylate, ethyl methacrylate, L(-)-perillaldehyde, and

α -methyl, α -butyl, α -amyl, and α -hexyl cinnamic aldehydes – are the result of the aquatic solubility limitation of the method.

Subcategories {6} and {7} are the two subcategories where the slow rate of the reaction combined with low aquatic solubility affect results. In an effort to verify that the lack of GSH reactivity was a result of solubility limitations rather than an inherent lack of reactivity, selected compounds were evaluated in a methanol/buffer system that increased the amount of electrophile in solution over the standard buffer-based system (Table 2). Results show that in the cases of 2-ethylhexyl acrylate, ethyl-trans-2-octanoate, ethylene glycol dimethacrylate, ethyl methacrylate, L(-)-perillaldehyde, and α -methyl cinnamic aldehyde, reactivity could be quantified in the methanol/buffer system. However, coumarin as well as α -amyl cinnamic aldehyde were still not reactive.

Because vinyl pyridine is a sensitizer, it is likely that ethynyl pyridine is also a sensitizer; yet, to our

knowledge, it has not been tested *in vivo*. 4-Ethynyl pyridine, like the 2-vinyl and 4-vinyl derivatives, is reactive with GSH (Table 2). Compounds in subcategory {9}, aromatics with *o*-hydroxyl or *p*-hydroxyl- and/or amino groups, are abiotically reactive with GSH (Table 2).

A comparison of *in chemico* reactivity potency between esters and ketones within subcategories {2}, {3}, and {5} shows that the ketones are consistently more reactive than the esters (Table 2). These results lead to predicting that methacrylates would be less reactive than methacrylate-like ketones. An examination of the GSH reactivity data for subcategory {6} shows this to be the case (Table 2). If vinyl-substituted esters and ketones are sensitizers, it is likely that ethynylene esters and ketones are also sensitizers; yet, to our knowledge, none has been tested in the LLNA.

Discussion

Skin sensitization is an end point that is difficult to model (28). There is a growing agreement that modelling the structure-sensitizing space is easiest and most accurately done by treating each molecular mechanism as a separate category or domain (7). Within each domain, identifying analogues will aid in forming subcategories that may be used to fill data gaps by analogue analyses (6). However, *in vivo* data, such as those in the LLNA data set, often reflect an incomplete and skewed representation of the domain of a particular chemical mechanism (e.g. Michael acceptors). The same is valid for the other *in vivo* data sets (e.g. guinea-pig). Subcategories {1} to {7} recognize only the carbonyl moiety as the polarizing group in Michael addition reactions; yet, organic chemistry suggests that other substituents (e.g. nitro, cyano, or sulfone) will also serve as polarizing unsaturates in Michael addition reactions.

One way of getting around the lack of representivity in the *in vivo* data is to base the risk assessment on all the available relevant and reliable information. Specific to this exercise, we used estimated data in the form of mechanistically based, *in silico* qualitative predictions of protein binding (13, 16, 27), along with experimental *in chemico* GSH reactivity (13, 15) and *in vivo* skin sensitization data (14) to rank the sensitization potential of subcategories of Michael acceptors.

The structural alerts for Michael acceptors have been verified (13) and the rules encoded in software that is available for general dissemination in the Open Source project chemical evaluation framework accessible at the Source-Forge.net Web site (<http://sourceforge.net/projects/chemeval>).

Only one chemical in subcategory {1} quinone, *p*-benzoquinone, has been tested in the LLNA. The *in chemico* GSH reactivity measured for other quinones (Table 2) supports a ranking that quinones having at least one hydrogen-substituted C-atom will have the same sensitization potential (i.e. extreme sensitizers). Based on the proposed mechanism of action, we do not predict 9,10-anthraquinone or 2,3,5,6-tetramethyl-1,4-benzoquinone to be sensitizers because neither of the latter two compounds is reactive with GSH (data not shown in Table 2).

Following the same evidence used for subcategory {1}, we predict with a similar degree of confidence that untested acetylenic esters and ketones (subcategory {2}) and vinylene aldehydes (subcategory {4}) will be moderate sensitizers.

Subcategory {3}, vinyl esters and ketones, are represented in the LLNA data set by two structurally similar acrylates, ethyl and 2-hydroxyethyl. However, their markedly different EC3% values present us with a dilemma. An examination of the GSH reactivity data for this subcategory shows uniformly strong reactivity. These latter data support our contention that the 2-hydroxyethyl acrylate is the better candidate (i.e. gives the more conservative outcome) to use for analogue read-across predictions. Based on 2-hydroxyethyl acrylate, we would rank untested vinyl esters and ketones as moderate sensitizers.

Subgroup {5}, vinylene esters and ketones, has five compounds tested in the LLNA. Three of these compounds are moderate sensitizers. The remaining two, coumarin and 6-methyl coumarin, are non-sensitizers in the LLNA, although coumarin is a human contact allergen (31). While the latter two substances possess a substructure of a vinylene ketone, the aromatic and cyclic nature of the substructure may reduce their chemical reactivity to the extent that they are non-sensitizers at least in the LLNA (27). Supporting these *in vivo* findings is the lack of GSH reactivity for both coumarins. With the exception of when there is an aromatic and cyclic nature to the vinylene ketone structure, one can read-across from the available information that untested vinylene esters and ketones will be ranked as moderate sensitizers.

The methacrylate-like compounds in subcategories {6} and {7} all have an alkyl substitution on the alpha-carbon of the olefinic moiety. This alkyl substitution diminishes the electron-withdrawing ability of the polar unsaturated group and reduces the chemical reactivity (26). The net result is that the skin sensitization potential of seven of the eight compounds in these two subcategories is either at the lower end of the moderate sensitizers or the upper end of the weak sensitizers.

Table 2. Measured RC₅₀ values for Michael acceptor domain

	CAS no.	RC ₅₀ (mM) (buffer)	RC ₅₀ (mM) (MeOH/buffer)
Quinones {1}			
<i>p</i> -Benzoquinone (EC3 = 0.0099%)	106-51-4	0.047	NT
Methyl-1,4-benzoquinone	553-97-9	0.046	NT
Chloro-1,4-benzoquinone	695-99-8	0.025	NT
2,6-Dimethyl-1,4-benzoquinone	527-61-7	0.085	NT
1,2-Naphthoquinone	524-42-5	0.023	NT
Acetylenic esters and ketones {2}			
Ethyl-2-butyrate	4341-76-8	1.30	NT
Ethyl-2-hexynoate	16205-90-6	0.96	NT
Methyl 2-nonynoate (EC3 = 2.5%)	111-80-8	0.27	NT
3-Hexyn-2-one	1679-36-3	0.12	NT
4-Phenyl-3-butyne-2-one	1817-57-8	0.05	NT
Vinyl esters and ketones {3}			
Methyl acrylate	96-33-3	0.50	0.41
Ethyl acrylate (EC3 = 28%)	140-88-5	0.52	0.37
2-Ethylhexyl acrylate	103-11-7	NRAS	0.44
2-Hydroxyethyl acrylate (EC3 = 1.4%)	818-61-1	0.27	NT
1-Hexen-3-one	1629-60-3	0.052	NT
1-Penten-3-one	1629-58-9	0.051	NT
Vinylene aldehydes {4}			
<i>trans</i> -2-Pentenal	1576-87-0	0.33	NT
<i>trans</i> -2-Hexenal (EC3 = 5.5%)	6728-26-3	0.40	NT
<i>trans</i> -2-Decenal (EC3 = 2.5%)	3913-81-3	0.17	NT
<i>trans</i> -Cinnamic aldehyde	14371-10-9	1.00	NT
<i>trans,trans</i> -2,4-Heptadienal	4313-03-5	1.10	NT
Vinylene esters and ketones {5}			
Diethyl maleate (EC3 = 5.8%)	141-05-9	2.1	NT
4-Phenyl-3-buten-2-one (EC3 = 3.7%)	122-57-6	3.7	NT
Methyl-2-hexenoate	2396-77-2	1.2	NT
Ethyl- <i>trans</i> -2-octanoate	7367-82-0	NRAS	3.2
4-Hexen-3-one	2497-21-4	0.34	NT
Coumarin (NC)	91-64-5	NRAS	NRAS
6-Methyl coumarin (NC)	92-48-8	NRAS	NT
Methacrylates and methacrylate-like ketones {6}			
Ethylene glycol dimethacrylate (EC3 = 28%)	97-90-5	NRAS	5.6
2-Hydroxypropyl methacrylate (NC)	923-26-2	28	NT
Methyl methacrylate	80-62-6	70	43
Ethyl methacrylate	97-63-2	NRAS	42
3-Methyl-3-penten-2-one	565-62-8	10	NT
Methacrylate-like aldehydes {7}			
α -Methyl cinnamic aldehyde (EC3 = 4.5%)	101-39-3	PRAS	25
L(-)-Perillaldehyde	18031-40-8	NRAS	5.5
α -Butyl cinnamic aldehyde (EC3 = 11%)	7492-44-6	NRAS	NT
α -Amyl cinnamic aldehyde (EC3 = 11%)	122-40-7	NRAS	NRAS
α -Hexyl cinnamic aldehyde (EC3 = 11%)	101-86-0	NRAS	NT
Vinyl pyridines {8}			
4-Vinyl pyridine	100-43-6	0.31	NT
2-Vinyl pyridine	100-69-6	2.8	NT
4-Ethynyl pyridine	352530-29-1	4.5	NT
Pre-Michael acceptor (aromatics with <i>o</i> -hydroxyl or <i>p</i> -hydroxyl- and/or amino groups) {9}			
1,4-Dihydroxybenzene (EC3 = 0.11%)	123-31-9	4.4	NT
1,2-Dihydroxybenzene	120-80-9	12	10
1,4-Phenylenediamine (EC3 = 0.16%)	106-50-3	21	NT
2-Aminophenol (EC3 = 0.4%)	95-55-6	23	NT
3-Methyl-1,2-dihydrobenzene	488-17-5	1.04	NT
Metabolically activated pro-Michael acceptors {10}			
2-Methoxy-4-methylphenol (EC3 = 5.8%)	93-51-6	NRAS	NRAS
Cinnamic alcohol (EC3 = 21%)	104-54-1	NRAS	NRAS
Isoeugenol (EC3 = 1.7%)	97-54-1	NRAS	NT
3-Aminophenol (EC3 = 3.2%)	591-27-5	NRAS	NT
4-Allylanisole (EC3 = 18%)	140-67-0	NRAS	NT
Resorcinol ^a	108-46-3	NRAS	NT

NRAS, not reactive at saturation; NT, not tested; PRAS, partial response at saturation.

^aBasketter et al. (35).

At first, GSH reactivity data were limited as in the buffer-only system, 6 of the 10 members of subcategories {6} and {7}, specifically ethylene glycol dimethacrylate, ethyl methacrylate, L(-)-perillaldehyde, α -butyl, α -amyl, and α -hexyl cinnamic aldehydes, were not reactive with GSH (Table 2). As demonstrated by the methanol/buffer testing, which allows for increased solubility of the electrophile, the lack of GSH reactivity in the standard buffer GSH method reflects the solubility limitation of these compounds where the slow rate of reactivity due to the deactivating alkyl group requires a concentration of electrophile in excess of aquatic solubility in order to reach the RC_{50} values (Table 2).

One non-sensitizer in the LLNA of subcategory {6}, which is active in the GSH method, is 2-hydroxypropyl methacrylate. While the available information for subcategories {6} and {7} are less consistent than that for other subcategories, based on the available data, we predict a ranking for untested methacrylate-like compounds as moderate at worst and more likely weak sensitizers.

Subcategory {8}, the vinyl pyridines, is a structurally limited group. Only vinyl pyridine, CAS number 1337-81-1, was tested in the LLNA with a sensitization ranking of moderate. While 2-vinyl and 4-vinyl pyridines were tested in GSH and found to be reactive, conventional organic chemistry suggests that 3-vinyl pyridine would not be reactive. Unfortunately, the latter chemical is commercially unavailable and has not been tested. Structurally, this subclass is very limited; we predict a ranking for untested *o*-unsaturated and *p*-unsaturated pyridines as moderate sensitizers.

Subcategory {9} has 10 LLNA-tested chemicals and 8 of them are strong sensitizers. This subcategory includes Michael acceptor compounds that undergo transformations to more reactive quinones, quinoneimines, or quinone methides (32, 33). For example, urushiol, the allergenic component of poison ivy and poison oak sap, is a mixture of long chain 3-alkenyl catechols, which are thought to sensitize by oxidation (which may well be abiotic) to Michael electrophilic 3-alkenyl *o*-quinones (34).

While this subcategory could be divided into three, we have elected to keep it as one. This reflects the facts that sensitization is consistently rated as strong and few *o*-hydroxyl or *p*-hydroxyl- and/or amino-substituted aromatics have been tested with the GSH method with all being reactive. The GSH data show that the dihydroxyl derivatives are more reactive than those containing an amino group (Table 2). The overall reduction in GSH potency of compounds in this subcategory may reflect the rate of the oxidative conversion necessary for reactivity.

An explanation for the potency difference between 2-amino-6-chloro-4-nitrophenol and HC Red No. 3 [4-(2-hydroxyethyl)-2-nitroaniline] and the other compounds in subgroup {9} is currently lacking. However, it can be argued that 4-(2-hydroxyethyl)-2-nitroaniline is similar to 2-nitro-1,4-phenylenediamine, and to a lesser extent, 2-amino-6-chloro-4-nitrophenol is similar to 2-aminophenol. All other available information suggests that the untested aromatics with *o*-hydroxyl or *p*-hydroxyl- and/or amino groups should be ranked as strong sensitizers.

The approach used here is of no value in predicting the sensitization group of metabolically activated pro-Michael acceptors, subcategory {10}. Again, it should be emphasized that this subcategory is the most uncertain one. The variation in LLNA potency may reflect our inability to fully understand skin metabolism and identify what chemical structures are candidates for skin metabolism. For example, 2-methoxy-4-methyl-phenol is thought to be metabolized to a reactive electrophilic quinone methide, and subsequently, the quinone methide is the sensitizer (26).

The lack of GSH reactivity reflects the selective nature of the reaction. In the case of subcategory {10}, the parent substances, those tested in GSH, were not reactive. Because there is no enzymatic capability with the *in chemico* GSH method, it would not be expected that this method would provide potency data for subcategory {10} compounds. The net result is that one should be extremely careful in using analogue read-across in predicting the sensitizing potential of pro-Michael acceptors.

Due to a variety of factors, including metabolism, abiotic transformation, lengths of the assays, and skewness of the data, we made no effort directly to compare LLNA potency with GSH reactivity. We feel that this is fraught with problems. For some subcategories, the GSH data are very successful in modelling sensitization potency, while for others, it is less successful and in the case of subcategory {10} unsuccessful. So an overall statistical fit between LLNA and GSH would depend on how many chemicals from each subcategory were in the data set evaluated. Nevertheless, the GSH data are very useful, provided one knows which subcategories of chemicals are well and which are poorly predicted.

One can assign new or untested chemicals within the Michael acceptor applicability domain to a subcategory. For subcategories {1} to {9}, this should be possible by an inspection of molecular structure. For other subcategories, this should be possible by GSH evaluation and molecular structure-based subcategorization (13).

In most cases, one can use this information with a high degree of confidence, and, as importantly, in other cases, one knows not to use the information.

A crucial element of the conceptual framework for predicting the toxicity of reactive chemicals (11) is that what happens downstream from the molecular initiating event is dependent on the extent to which the event has occurred. So in the Jowsey et al. approach (3), if one measures the ability of a chemical to produce, for example, a dendritic cell response, then one is indirectly measuring that chemical's ability to bind covalently to proteins or peptides.

As things presently stand, chemistry either *in silico* or *in chemico* can sometimes predict sensitization very well, and often, we can be highly confident in a chemistry-based prediction (6). However, compounds exist where we have a gap in our chemical knowledge, so the possibility of false predictions is a reality. At the same time, without *in vivo* testing, it would be prudent to assume all substances that are GSH reactive have the potential to be skin sensitizers in man.

When the LLNA was developed, it provided an objective numerical index of potency, the EC3 value (22). This stood in contrast to the guinea-pig results that were achieved by subjective scoring. So to replace the LLNA EC3 value with a subjective ranking (extreme, strong, moderate, and weak) appears to be a step backwards in this respect. While our knowledge of sensitization potential in some subcategories may warrant objective numerical indexing, that knowledge in other subcategories is still too limited and/or inconsistent.

In summary, in this article, we used estimated data in the form of mechanistically based, *in silico* qualitative predictions of protein binding (13, 16, 27) along with rapid and inexpensive experimental data, measuring *in chemico* GSH reactivity (13, 15), and *in vivo* skin sensitization data (14) to rank the sensitization potential of subcategories of compounds that are classified as Michael acceptors. To offset the limitations and skewness of the LLNA *in vivo* data, we used *in chemico* GSH reactivity data in an analogue approach based on subcategories. We have shown that the read-across approach can be used to qualitatively rank skin sensitization potential of a new untested carbonyl-containing Michael acceptor chemical using subcategories.

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References

- Landsteiner K, Jacobs J L. Studies on the sensitisation of animals with simple chemicals III. *J Exp Med* 1936; 64: 625–639.
- Maxwell G, Aleksic M, Aptula A et al. Assuring consumer safety without animal testing: a feasibility case study for skin sensitisation. *Altern Lab Anim* 2008 (in press).
- Jowsey I R, Basketter D A, Westmoreland C, Kimber I. A future approach to measuring relative skin sensitising potency: a proposal. *J Appl Toxicol* 2006; 26: 341–350.
- Basketter D A, Maxwell G. Identification and characterization of allergens: in vitro alternatives. *Expert Rev Dermatol* 2007; 2: 471–480.
- Enoch S J, Cronin M T D, Schultz T W, Madden J C. Quantitative and mechanistic read across for predicting the skin sensitization potential of alkenes acting via Michael addition. *Chem Res Toxicol* 2008; 21: 513–520.
- Roberts D W, Aptula A O, Patlewicz G, Pease C. Chemical reactivity indices and mechanism-based read-across for non-animal based assessment of skin sensitisation potential. *J Appl Toxicol* 2008; 28: 443–454.
- Roberts D W, Patlewicz G, Kern P S et al. Mechanistic applicability domain classification of a local lymph node assay dataset for skin sensitization. *Chem Res Toxicol* 2007; 20: 1019–1030.
- Organization for Economic Co-operation and Development (OECD). *OECD Environment Health and Safety Publications Series on Testing and Assessment No. 80: Guidance Document on Grouping of Chemicals ENV/JM/MONO (2007)28*. Paris, France, 2007.
- Kanerva L, Jolanki R, Estlander T. 10 years of patch testing with the (meth)acrylate series. *Contact Dermatitis* 1997; 37: 255–258.
- Organization for Economic Co-operation and Development (OECD). *OECD Environment Health and Safety Publications Series on Testing and Assessment No. 88: Workshop on Integrated Approaches to Testing and Assessment ENV/JM/MONO (2008) 10*. Paris, France, 2008.
- Schultz T W, Carlson R E, Cronin M T D et al. A conceptual framework for predicting toxicity of reactive chemicals: models for soft electrophilicity. *SAR QSAR Environ Res* 2006; 17: 413–428.
- Gerberick F, Aleksic M, Basketter D et al. Chemical reactivity measurement and the predictive identification of skin sensitizers. *Altern Lab Anim* 2008; 36: 215–242.
- Schultz T W, Yarbrough J W, Hunter R S, Aptula A O. Verification of the structural alerts for Michael acceptors. *Chem Res Toxicol* 2007; 20: 1359–1363.
- Gerberick G F, Ryan C A, Kern P S, Schlatter H, Dearman R J, Kimber I, Patlewicz G Y, Basketter D A. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. *Dermatitis* 2005; 16: 157–202.
- Schultz T W, Yarbrough J W, Johnson E L. Structure-activity relationships for reactivity of carbonyl-containing compounds with glutathione. *SAR QSAR Environ Res* 2005; 16: 313–322.
- Aptula A O, Patlewicz G, Roberts D W. Skin sensitization: reaction mechanistic applicability domains for structure-activity relationships. *Chem Res Toxicol* 2005; 18: 1420–1426.
- SAS Institute Inc. *SAS/STAT User's Guide, Version 6*, Vol. 2, 4th edition. STATS Publishing Inc., Cary, NC, 1989: pp. 846.
- Maurer T, Arthur A, Bentley P. Guinea-pig contact sensitization assays. *Toxicology* 1994; 93: 47–54.
- Basketter D A, Gerberick G F, Kimber I, Loveless S E. The local lymph node assay: a viable alternative to currently accepted skin sensitization tests. *Food Chem Toxicol* 1996; 34: 985–997.
- Basketter D A, Evans P, Fielder R J, Gerberick G F, Dearman R J, Kimber I. Local lymph node assay – validation, conduct and use in practice. *Food Chem Toxicol* 2002; 40: 593–598.

21. Kimber I, Dearman R J, Basketter D A, Ryan C A, Gerberick G F. The local lymph node assay: past, present and future. *Contact Dermatitis* 2002; 47: 315–328.
22. Organisation for Economic Co-operation and Development (OECD). *Guidelines for Testing of Chemicals. No. 429: Skin Sensitisation: The Local Lymph Node Assay*. Paris, France, 2002.
23. Kimber I, Basketter D A, Butler M et al. Classification of contact allergens according to potency: proposals. *Food Chem Toxicol* 2003; 41: 1799–1809.
24. SCCP (Scientific Committee on Consumer Products) *Memorandum on Hair Dye Substances and Their Skin Sensitising Properties*, 2006, (http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_s_05.pdf) accessed 06 December 2008.
25. Basketter D A, Blaikie L, Dearman R J et al. Use of the local lymph node assay for the estimation of relative contact allergenic potency. *Contact Dermatitis* 2000; 4: 344–348.
26. Aptula A O, Roberts D W. Mechanistic applicability domains for non-animal based toxicological endpoints. General principles and application to reactive toxicity. *Chem Res Toxicol* 2006; 19: 1097–1105.
27. Roberts D W, Aptula A O, Patlewicz G. Electrophilic chemistry related to skin sensitization. Reaction mechanistic applicability domain classification for a published dataset of 106 chemicals tested in the mouse local lymph node assay. *Chem Res Toxicol* 2007; 20: 44–60.
28. Roberts D W, Aptula A O, Cronin M T D, Hulzebos E, Patlewicz G. Global (Q)SARs for skin sensitization – assessment against OECD principles. *SAR QSAR Environ Res* 2007; 18: 343–365.
29. Schultz T W, Ralston E, Roberts D W, Veith G D, Aptula A O. Structure–activity relationships for abiotic thiol reactivity and aquatic toxicity of halo-substituted carbonyl compounds. *SAR QSAR Environ Res* 2007; 18: 21–29.
30. Patlewicz G, Roberts D W, Uriarte E. A comparison of reactivity schemes for the prediction skin sensitization potential. *Chem Res Toxicol* 2008; 21: 521–541.
31. Frosch P J, Johansen J D, Menné T et al. Further important sensitizers in patients sensitive to fragrances. *Contact Dermatitis* 2002; 47: 78–85.
32. Franot C, Roberts D W, Smith R G, Basketter D A, Benezra C, Lepoittevin J -P. Structure-activity relationships for contact allergenic potential of γ,γ -dimethyl- γ -butyrolactone derivatives. 1. Synthesis and electrophilic reactivity studies of α -(ω -substituted-alkyl)- γ,γ -dimethyl- γ -butyrolactones and correlation of skin sensitization potential and cross-sensitization patterns with structure. *Chem Res Toxicol* 1994; 7: 297–306.
33. Franot C, Roberts D W, Basketter D A, Benezra C, Lepoittevin J-P. Structure, activity relationships for contact allergenic potential of γ,γ -dimethyl- γ -butyrolactone derivatives. 2. Quantitative structure-skin sensitization relationships for α -substituted- α -methyl- γ,γ -dimethyl- γ -butyrolactones. *Chem Res Toxicol* 1994; 7: 307–312.
34. Roberts D W, Lepoittevin J-P. Hapten-protein interactions. In: *Allergic Contact Dermatitis. The Molecular Basis*, Lepoittevin J-P, Basketter D A, Goossens A, Karlberg A-T (eds): Heidelberg, Springer-Verlag, 1998: pp 81–111.
35. Basketter D, Sanders D, Jowsey I R. The skin sensitization potential of resorcinol: experience with the local lymph node assay. *Contact Dermatitis* 2007; 56: 196–200.

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