

Chemical reactivity indices and mechanism-based read-across for non-animal based assessment of skin sensitisation potential

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ABSTRACT: The skin sensitisation potential of chemicals is currently assessed using *in vivo* methods where the murine local lymph node assay (LLNA) is typically the method of first choice. Current regulatory initiatives are driving the impetus for the use of *in vitro/in silico* alternative approaches to provide the relevant information needed for the effective assessment of skin sensitisation, for both hazard characterisation and risk assessment purposes. A chemical must undergo a number of steps for it to induce skin sensitisation but the main determining step is formation of a stable covalent association with carrier protein. The ability of a chemical to react covalently with carrier protein nucleophiles relates to both its electrophilic reactivity and its hydrophobicity.

This paper focuses on quantitative indices of electrophilic reactivity with nucleophiles, in a chemical mechanism-of-action context, and compares and contrasts the experimental approaches available to generate reactivity data that are suitable for mathematical modelling and making predictions of skin sensitisation potential, using new chemistry data correlated against existing *in vivo* bioassay data. As such, the paper goes on to describe an illustrative example of how quantitative kinetic measures of reactivity can be usefully and simply applied to perform mechanism-based read-across that enables hazard characterisation of skin sensitisation potential. An illustration of the types of quantitative mechanistic models that could be built using databases of kinetic measures of reactivity, hydrophobicity and existing *in vivo* bioassay data is also given. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS: kinetics; electrophiles; nucleophiles; protein binding; contact allergy

Introduction

Under current regulatory frameworks, the identification of skin sensitisation potential of new substances is assessed through *in vivo* testing (e.g. the murine local lymph node assay (LLNA) or guinea-pig maximisation test (GPMT)) (Gerberick *et al.*, 2000; Magnusson and Kligman, 1969). However, with the advent of two new separate pieces of European legislation, namely the 7th Amendment to the EU Cosmetics Directive (EU, 2003) and the recently adopted Registration Evaluation, Authorisation of Chemicals (REACH) (Commission of the European Communities, 2001, 2003, 2006) there is a drive within industry to develop and apply alternative non-animal based approaches wherever possible. New methods should be aimed at generating data for the purposes of (a) chemical ‘screening’ for potential toxicity, (b) classification and labelling for regulatory purposes or (c) performing human health risk assessments; all of which

enable supported decision-making about a chemical’s prospective ‘safe’ use upon human exposure. In this paper, we aim to exemplify how chemistry information can be used to best effect in the future to make predictions on skin sensitisation without the generation of new animal data, primarily in the context of (a) and (b) above.

In Vivo Testing: The Mouse Local Lymph Node Assay

In the past, there have been several means of generating information required for the evaluation and assessment of skin sensitisation potential. In recent decades information has been acquired from *in vivo* testing, for example using the murine LLNA (Basketter *et al.*, 1996, 2000, 2002; Kimber *et al.*, 2002), which has provided a substantial refinement of methods over previous guinea-pig assays. It is important here, when using this quantitative bioassay data in predictive models, to describe what the measurements represent in biological terms. In the LLNA mice are treated with the test compound applied topically, and the stimulation of proliferative responses by draining

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lymph node cells is measured and compared with the response in control animals. A test/control ratio (referred to as the stimulation index, SI) of 3 or more is taken as an indication of sensitisation, and potency is quantified as EC₃, (EC for Effect Concentration), the test concentration which gives a stimulation index of 3 (Kimber *et al.*, 2002). Thus, for a given test chemical, the measurement of lymph node cellular proliferation is considered a dose-dependent phenomenon, and within the context of the assay, a threshold can be drawn above which the chemical is classed as a sensitizer. Comparisons between chemicals, of this rate of cellular proliferation measured over a snapshot in time of the biological response, are relative and need to be carefully drawn. The LLNA is now a well established and extensively evaluated approach that has undergone significant validation before its final acceptance as an OECD method under Test guideline 429 (OECD, 2002). In addition, this method allows for a quantitative assessment of relative skin sensitisation 'potency' through the use of the EC₃ value and broad classification of sensitizers as non, weak, moderate or strong. Whilst it is now becoming the first choice test for regulatory programmes, notably REACH, there are pressures to reduce and ultimately eliminate dependence on it as an animal assay.

Avenues of research have been pursued now for many years to try and develop non-animal alternative predictive methods of skin sensitisation potential (Basketter *et al.*, 2005). It can be envisaged that if successful these methods could lead to a prediction of whether a chemical is or is not a sensitizer, i.e. can provide a yes/no call. It is harder to see how some of these alternative methods could in isolation yield a measure of how 'potent' a sensitizer is likely to be in man, given that this is characterised more by a holistic biological response (e.g. as is provided in the context of the LLNA) and in current *in vivo* assays is a 'relative' rather than 'absolute' phenomenon.

***In Vitro* Assays**

In vitro cell-based assays (typically using dendritic-like cells or T cells) are being researched with a view to predicting sensitisation potential. This has been an active area of research for many years (Python *et al.*, 2007), but no robust methods have yet been developed, and it is proving to be a significant technical challenge. In particular, extensive work is now on-going under the EU Framework VI Sens-it-iv project (see website <http://www.sens-it-iv.eu> for more details).

Statistically Based QSARs

Another active area of research is the prediction of sensitisation potential from chemical structure, using sta-

tistically based QSARs (Quantitative Structure-Activity Relationships) as derived from empirical correlations, using *in vivo* test datasets, aiming to relate potency measures (e.g. EC₃ from LLNAs) and combinations of parameters calculated from structure (Miller *et al.*, 2005; Fedorowicz *et al.*, 2005). The currently available models have recently been re-evaluated with respect to the OECD principles for (Q)SAR validation. These are the principles envisaged to be used in the evaluation and assessment of a (Q)SAR model's validity and its applicability of its use under REACH. More details can be found in Roberts *et al.* (2007a) and Patlewicz *et al.* (2007). The evaluations conducted found that the models, whilst demonstrating high sensitivities, were poor at predicting non-sensitizers, lacked mechanistic insights and were typically only able to model a secondary endpoint of skin sensitisation presence or absence or else a crude potency classification.

The Mechanistic Chemistry Approach

Prediction of skin sensitisation potential using chemical properties is, however, a valuable area of research when performed within the domain of a chemical mechanism-of-action based understanding of the biological effect. The fundamental chemical basis of skin sensitisation is quite well understood (Landsteiner and Jacobs, 1936) although some gaps remain, in particular with respect to the nature and location of carrier proteins (Roberts *et al.*, 2007c). In brief, the ability of a chemical (either as such or after *in cutaneo* activation) to react covalently with 'carrier protein' is a major determinant factor in its ability to act as a skin sensitizer (Aptula and Roberts, 2006; Roberts and Aptula, 2007; Roberts *et al.*, 2007b; Roberts and Lepoittevin, 1998). The process has been successfully mathematically modelled (Roberts and Williams, 1982) and the model has since been verified against many sets of experimental data (Aptula *et al.*, 2006b; Basketter and Roberts, 1990; Roberts and Basketter, 1997, 2000; Patlewicz *et al.*, 2003, 2004). The ability of the causative chemical species to react covalently with carrier protein is related both to electrophilic reactivity and hydrophobicity.

Thus a skin sensitising chemical is either directly reactive (electrophilic) with protein nucleophiles or is a pro-electrophile (Aptula *et al.*, 2007) requiring activation (usually either metabolic or autoxidative) to make it into a reactive electrophile.

To predict and characterise the sensitisation potential of a chemical (i.e. make a yes/no prediction and provide some quantitative measure of relative potency), the following 'chemistry-based' information is required.

Mechanism of Action

The chemical nature of its electrophilic reactions, i.e. assignment to its reaction mechanistic domain. In many

cases, for direct acting electrophiles, this is immediately obvious to an organic chemist from inspection of the chemical structure; in other cases *in chemico* experimental studies (treating the chemical with model nucleophiles) may be necessary to determine the reaction chemistry. Recent publications summarised the chemistry of the major reaction mechanistic domains (Aptula and Roberts, 2006) and showed how this concept can be successfully applied to classify and chemically rationalise published skin sensitisation datasets (Roberts *et al.*, 2007b, 2007c; Aptula *et al.*, 2005).

Hydrophobicity

The hydrophobicity of a chemical governs the extent to which it partitions between lipid and aqueous parts of the tissue/cell. In terms of epidermal disposition of a chemical, the hydrophobicity will influence whether the chemical prefers to reside in layers of the lipid rich stratum corneum, aqueous epidermis, hydrophobic dermis, or fat layer beneath the dermis and whether it partitions into the blood in the microvasculature beneath the epidermis. Thus it models bioavailability at the location where the protein-binding reaction leading to sensitisation occurs. Hydrophobicity is usually expressed as the partition coefficient between octanol and water or more usually its logarithm, log P. However, more sophisticated estimates of epidermal disposition can also be achieved empirically and via modelling (Basketter *et al.*, 2007).

Reactivity

Electrophilic reactivity (ideally kinetic) models the protein binding of the bioavailable sensitizer. This, together with mechanism of action, is the subject of the present paper i.e. how can we best take learnings from *in chemico* kinetics experiments with skin sensitizers and interpret the data in the context of the biological effect.

Reactivity to What?

Skin sensitisation involves reaction of the sensitizing electrophile with nucleophilic groups on the carrier protein. The nature of the carrier protein, and its location in the skin, are not known (Smith Pease, 2003; Aleksic *et al.*, 2007a, 2007b; Divkovic *et al.*, 2005). Technological advances in analytical mass spectrometry and imaging

methods could allow this to be investigated in theory, but practically the area provides significant challenges and there appear to be no immediate prospects for realising this information in the near future. At one extreme, one could envisage a protein which has been 'designed' by natural selection to be especially nucleophilic, and hence be a protective entity against invading reactive electrophiles, perhaps in a specific location such as the membranes of cells. At the other extreme one could envisage that any protein which the sensitizer happens to encounter in the epidermis could potentially serve as a carrier protein. This lack of knowledge has implications in determining the approach for measuring electrophilic reactivity for the purposes of predicting skin sensitisation potential.

Firstly, since the nature of the carrier protein nucleophile cannot be specified, no assumption can be made about whether one model nucleophile (e.g. representing a cysteine or a lysine amino acid unit) is a more realistic representation of the *in cutaneo* effects than another. Secondly, since the location of the carrier protein cannot be specified, it cannot be assumed that any one model solvent system is more representative of the in tissue environment, in which the chemical reacts, than any other. Fortunately, neither of these two knowledge gaps presents major difficulties in using and interpreting *in chemico* data.

Different nucleophiles can differ very substantially in their absolute reactivity towards a given electrophile. However, their relative reactivities are usually well correlated over a range of electrophiles within the same mechanistic domain. As an illustration, Table 1 shows reactivity data for thiol and amino nucleophiles with the Michael acceptor electrophiles (Aptula and Roberts, 2006) methyl acrylate, acrylonitrile and acrylamide (Friedman *et al.*, 1965). Such correlation of relative reactivities is well recognised in organic chemistry, and is the basis of the long established utility of LFER (Linear Free Energy Relationships), e.g. the use of Hammett and Taft constants, in physical organic chemistry (Isaacs, 1995).

Deviations from this principle can occur, but these are usually the result of a change in mechanism or due to steric effects. For example, with the sensitizing 2,4-dinitro-halobenzenes, DNXB, reactivities towards methoxide ion, piperidine and thiophenoxide ion are all well correlated with Taft σ^* values (Bartoli and Todesco, 1977; Bunnett *et al.*, 1957) of the X substituents, except

Table 1. Rate constants for nucleophilic addition of thiolate and amine nucleophiles to acrylic acid derivatives

CH ₂ =CH-	k(thiolate)	k(amine)	k(thio)/k(am)	log k(thio)/k(am)
CO ₂ Me	11.0	76.0	145	2.16
CN	2.70	20.4	132	2.12
CONH ₂	0.46	2.60	178	2.25

k(thiolate) values shown are $10^3 \times$ true values; k(amine) values shown are $10^5 \times$ true values; units are [l-mol⁻¹.s⁻¹].

that the fluoro-compound shows an anomalously high reactivity with methoxide and piperidine. This is attributed to the small size of the fluoro-substituent, which gives a steric benefit, compared with the larger halogen substituents, in the case of methoxide and piperidine as nucleophiles. With the softer electrophile thiophenoxide the transition state is formed with the two reactants at a greater distance, so that there is little steric hindrance even with the larger halogen substituents. Consequently the steric benefit of the fluoro-substituent is minimal. Interestingly, the relative potencies of the DNXB compounds, including DNXB, as measured in the LLNA, are well correlated with the relative reactivities towards thiophenoxide in particular, suggesting that, at least for the S_NAr electrophiles, carrier protein reacts via a soft electrophilic group (Roberts and Aptula, unpublished).

Despite such exceptions, the general principle of comparing *in chemico* chemical reactivities illustrated in Table 1 is widely applicable, which means that relative reactivities determined with one nucleophile can be used to model the relative reactivities of electrophiles towards the unknown nucleophiles of carrier protein. In terms of predictive value, aniline, butylamine and propanethiolate are just as good as lysine, cysteine or peptides based on them, when comparing relative reactivities of direct acting electrophiles within a mechanistic domain.

Reaction rates *in chemico* can be strongly influenced by the nature of the reaction medium, particularly when the charge distribution changes between the reactants and the transition state — which is the case in most, if not all, electrophile–nucleophile reactions relevant to sensitisation. However, provided comparisons are restricted to reactions which undergo similar changes in charge distribution between reactants and transition state, relative reactivities of different electrophiles towards a given nucleophile are not significantly affected by the reaction medium.

The significance of the foregoing is that relative reactivity of an electrophile to the nucleophiles of a carrier protein can just as well be modelled by one nucleophile as by another. This is provided both are reactive and interpreted in the context of the mechanistic domain to which the electrophile belongs. Furthermore, the nature of the solvent is not critical.

Measurement and Quantification of Reactivity

In most cases, whether or not a ‘parent’ compound has any electrophilic reactivity is clear to an organic chemist from the chemical structure. If it is not obvious, then the simplest approach is to treat the compound with a nucleophile under forcing conditions and determine whether any reaction takes place. An early example is provided by the work of Landsteiner and Jacobs

published in 1936 (Landsteiner and Jacobs, 1936). They examined 20 benzene derivatives with halogen and nitro substituents. To determine which of these compounds were capable of reacting (acting as what are now referred to as S_NAr electrophiles — in accordance with the reaction domains defined by Aptula and Roberts (2006)), they heated each one with aniline in ethanol at ca. 100 °C for 2 h. Ten of the compounds reacted, ten did not. In guinea-pig tests, the ten reactive compounds were found to be sensitisers and the ten unreactive compounds were non-sensitisers. In retrospect we can see that to some extent Landsteiner and Jacobs were probably fortunate to obtain this complete correlation between reactivity and sensitisation. Their criteria for reaction or failure to react were, respectively, <5% of test compound remaining or >95% of test compound remaining. In no cases did they observe intermediate degrees of reaction. However, if they had chosen a lower reaction temperature some of the sensitisers would probably have failed to react, and if they had used a higher temperature some of the non-sensitisers would probably have reacted.

Such empirical reactions between chemicals of unknown properties and a range of representative nucleophiles under forcing conditions, may provide a good basis to determine a possible/unlikely call for sensitisation potential with a certain degree of confidence to enable predictions on sensitisation to be made for a new chemical. However, if we are to use chemistry to get information on the extent of reactivity and whether it can be related to the overall ‘potency’ properties of sensitisation in man, through integrating different types of chemical and biological information together, we will need more sophisticated information on chemical mechanism and kinetics.

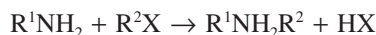
Reactivity of an electrophile can be quantified in terms of the rate constant k , or its logarithm $\log k$, for its reaction with a nucleophile. Sometimes it is convenient to use relative reactivity values, k_{rel} or $\log k_{rel}$, where the rate constant for the electrophile under consideration is divided by the rate constant for a standard compound. Rate constants (absolute or relative) or their logarithms are what physical organic chemists normally use to express reactivity quantitatively.

To be comparable, k or $\log k$ values for a range of electrophiles should be determined with the same nucleophile in the same solvent and at the same temperature. However, there is no requirement for the initial concentrations of the electrophile and the nucleophile to be constant. For situations where it is not possible to use the same temperature, the Arrhenius equation can be used to correct for temperature differences. Approximate methods are available for correcting for solvent differences (Hine, 1962), as we have illustrated recently in an analysis of sensitisation data for H-polar S_N2 electrophiles (Roberts *et al.*, 2007b) but require a high level of expertise and are best avoided where possible.

There are various approaches for determining rate constants. In order of decreasing accuracy they are.

Full Kinetics

In a typical kinetics experiment the extent of reaction, either in terms of depletion of one of the reactants or in terms of formation of a reaction product, is measured at several recorded time intervals, and from these the rate constant k is found. For example, in an experiment where the electrophile El is in excess over the nucleophile Nu , a plot of $\ln[Nu]$ against time* should give a straight line whose slope is $-k[El]$. Dividing by the initial concentration of El gives the rate constant k . A more rigorous method, which eliminates any error due to Nu depletion by a competing side reaction, is to carry out kinetic experiments at several initial concentrations of El , and to plot the slopes against $[El]$. The slope of this second plot is the rate constant k and the intercept is the (pseudo) first order rate constant for the side reaction. If formation of a reaction product is measured, this need not necessarily be the adduct formed between the nucleophile and the electrophile, but can be a co-product such as the acid generated when an amine nucleophile reacts with an S_N2 or S_NAr electrophile:



When the reaction is followed by measuring the formation of a reaction product P , the pseudo first order rate constant is obtained by plotting $\ln\{[P]_\infty - [P]_t\}$ against time t . The value of $[P]_\infty$ is determined by taking a measurement after the reaction has had time to go to completion (7 half-lives, corresponding to >99% reaction, is sufficient) or can be calculated from the reaction stoichiometry.

Concentration giving 50% Reaction in a Fixed Time (RC_{50})

Recently Schultz *et al.* (2005) have developed a method based on this approach, for measuring electrophilic reactivity to glutathione (GSH). The RC_{50} can be defined in kinetic terms as the concentration of electrophile which, if constant throughout the reaction, gives a defined half-life for the nucleophile.

Experimentally there are two ways of keeping the electrophile concentration constant throughout the reaction:

- Replenish the electrophile as it is consumed.
- Use an excess of electrophile so that the change in its concentration during the reaction is negligible.

Method (b) is what is used in the Schultz method. The defined half-life is 120 min and normally, $[GSH]_0$ is 0.1375 mM. This $[GSH]_0$ value is experimentally convenient from the viewpoint of the sensitivity of the analytical method, but there is no absolute requirement for it to be fixed. It is a mathematical consequence of the first order rate law that the RC_{50} is independent of the initial GSH concentration. An RC_{50} determination for 0.05 mM GSH going to 0.025 mM would give the same result as for 0.2 mM GSH going to 0.1 mM.

RC_{50} is inversely related to k :

$$k = [\ln 2]/t.RC_{50} \quad (1)$$

This relationship can also be expressed:

$$\log k = \log(1/RC_{50}) + \text{constant} \quad (2)$$

Thus $\log(1/RC_{50})$, conveniently abbreviated pRC_{50} (p for $-\log$, as in pH), can be used directly as a surrogate for $\log k$ in quantitative mechanistic modelling.

Extent of Reaction after a Fixed Time

Another method is to determine the extent of reaction (which can be expressed as % depletion, DP of one of the reactants) after a fixed time. Although not as accurate as a kinetics experiment, since only two data points are used, this method can be used to estimate rate constants, using the expression:

$$k = [\ln((100/(100 - \%DP)))/[El]_0]t \quad (3)$$

This expression applies when $[El]_0$ is greater than $[Nu]_0$ and depletion of Nu is measured. The method has been used by Freidig *et al.* (1999) to determine rate constants for reactions of Michael acceptors with GSH.

More recently Gerberick *et al.* (2004, 2007) have described a depletion assay approach based on this method, in which a broad range of chemicals have been investigated for their ability to deplete glutathione (or other nucleophilic peptides). In the peptide reactivity assay of Gerberick *et al.* (2004), the percent depletion ($\%DP$) of glutathione or a synthetic nucleophilic peptide when treated with an excess of the potentially electrophilic test compound, El , is measured. In the current version of the Gerberick peptide assay, the initial concentrations of peptide and test compound El are always 0.2 mM and 20 mM, and the reaction time t is always 15 min. The $\%DP$ observed under these conditions is used as the reactivity index. It is important to note two

* Strictly, $\ln([Nu] - [Nu]_\infty)$ should be plotted against time, $[Nu]_\infty$ being the remaining nucleophile concentration when the reaction reaches its maximum extent. It cannot necessarily be assumed that $[Nu]_\infty = 0$. One of the reasons why it is recognised good kinetics practice to follow the reaction only up to about 50% or less is that errors in assuming incorrectly that $[Nu]_\infty = 0$ have less significance. However, this is not always possible, for example in analysing data which have not been generated specifically for the purpose of determining rate constants.

things here: (a) that the %DP parameter cannot be used directly for modelling purposes, since it is not linearly related to k and (b) that the assay is performed generically for all chemicals and not within the context of pre-defined mechanistic domains. It can be seen from Equation (3) that when %DP is close to 0 or close to 100, small errors in %DP give rise to large errors in k . A way to overcome this deficiency in such a case would be to repeat with a different initial electrophile concentration and/or reaction time, so as to get a %DP nearer to the middle of the range, from which a more accurate estimate of k can be made. Natsch *et al.* (2007) have recently applied this principle in a modified version of the Gerberick peptide assay, by measuring %DP for a range of EI concentrations, so that from a dose-response plot of %DP against [EI] an RC_{50} value ([EI] which gives 50% DP) can be obtained. As we now show, such dose response data can be used to obtain rate constants directly.

The integrated pseudo first order rate equation (Frost and Pearson, 1961) for disappearance of the nucleophile Nu in the presence of excess electrophile EI, is:

$$\ln([Nu]_0 - [Nu]_{\infty}) - \ln([Nu] - [Nu]_{\infty}) = t(k[EI] + k_0) \quad (4)$$

The rate constant k_0 refers to depletion of Nu by side reactions not involving EI.

In 'conventional' kinetics, [EI] would be constant and [Nu] would be measured at various time intervals. In this case t is constant (24 h) and [Nu] is measured at various [EI] values. In this situation, it follows from Equation (4), that a plot of $\ln([Nu]_0 - [Nu]_{\infty}) - \ln([Nu] - [Nu]_{\infty})$ against [EI] should have a slope equal to tk and an intercept equal to tk_0 . On this basis, we can analyse the Natsch *et al.* (2007) dose-response data as follows. From the dose-response curves in Fig. 1 of Natsch *et al.* (2007) we read off the % DP (in the following paragraphs we will refer to % DP simply as DP) for each concentration of the electrophile. These figures are shown in Table 2.

Clearly the reaction of Acetyl-Cys is still far from completion after 24 h, even with the highest electrophile concentration. In this case it is therefore appropriate to plot $\ln 100 - \ln(100 - DP)$ against [EI] to obtain the rate constant. This plot is shown in Fig. 1a. For the other peptides it is clear from Fig. 1 of Natsch *et al.* (2007)

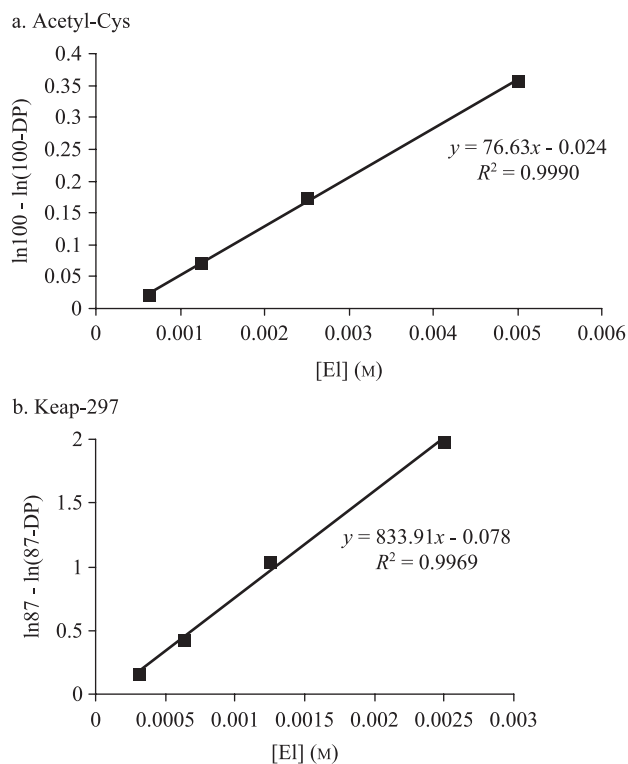


Figure 1. Kinetic plot for reaction of peptides with β -damascone

that after 24 h with the highest electrophile concentration the reactions are close to completion and the curves will level out below DP = 100. For these peptides therefore, we proceed as follows.

Taking the [EI] = 5 mM %DP values, which we abbreviate as DP_5 , as the basis for infinity values ($DP_5 = [Nu]_0 - [Nu]_{\infty}$), we plot $\ln DP_5 - \ln(DP_5 - DP)$ against [EI] for the [EI] values below 5 mM. One of these plots, for the peptide Keap-297, is shown in Fig. 1b. Note that instead of plotting $\ln DP_5 - \ln(DP_5 - DP)$ we could have simply plotted $\ln(DP_5 - DP)$ against [EI] to get a line with slope $-tk$. We chose to use $\ln DP_5 - \ln(DP_5 - DP)$ in order to demonstrate how close the intercept is to zero, indicating that the nucleophile is not significantly depleted by side reactions not involving the electrophile. The full set of rate constants is shown in Table 3.

Table 2. Peptide reactions with the electrophile (EI) β -damascone at 30 °C

[EI] (mM)	% Depletion after 24 h of peptide:					
	Cor1-C420	CysReact	Cofill-138	Keap-297	RP17-34	Acetyl-Cys
5	93	80	70	87	85	30
2.5	85	62	55	75	70	16
1.25	70	38	36	56	50	7
0.625	45	17	20	30	29	2
0.3125	25	4	10	13	15	

The % DP values for the peptide Keap-257 are not significantly different from those shown in Table 2 for Keap-297.

Table 3. Rate constants for peptide reactions with β -damascone

Peptide	$10^3 k \text{ (s}^{-1} \text{ M}^{-1}\text{)}$	R^2
Corl-C420	11.3 ± 2.89	0.9931
CysReact	7.65 ± 0.47	0.9996
Cofill-138	7.36 ± 0.45	0.9996
Keap-297	9.65 ± 1.64	0.9969
RP17-34	8.15 ± 0.61	0.9994
Acetyl-Cys	0.887 ± 0.086	0.9990

The peptide Keap-257 is not significantly different in reactivity from Keap-297.

The above data analysis illustrates how rate constants can be determined without sampling over a range of time intervals, and suggests possibilities for developing a high throughput methodology for reaction kinetics.

It is important to stress that the measurement of reactivity belongs to the field of experimental chemistry, and criteria for acceptance or validity of *in chemico* data should not be confused with criteria that may have been requested historically of biological assays. For biological assays it is important to adhere to experimental protocols that are defined in fine detail, and to validate them by such measures as inter-laboratory comparisons. Such measures are neither necessary nor desirable for chemical determination of reactivity. Methods for determining rate constants have been available for many decades, and for a given reaction there are usually several methods to choose from. Rate constants are independent of the method by which they have been measured: they do not need to be measured by the same method in order to be comparable against each other. This being the case, it is best to be free to choose the most convenient and appropriate method for the particular reaction to be studied.

Choice of Nucleophile and Reaction Medium

As discussed above, given that the nature and location of the carrier protein is not known, there is no reason to suppose that any particular model nucleophile in any particular solvent is a more realistic model than any other for the *in cutaneo* reaction. That the choice of model nucleophile for determining reactivity is not critical is borne out by numerous successful correlations of skin sensitisation potential using reactivity parameters for a variety of nucleophiles.

Landsteiner and Jacobs (1936) used aniline in ethanol at ca. 100 °C to discriminate between reactive and unreactive aromatic halo and nitro compounds, enabling them to successfully discriminate between sensitising and non-sensitising compounds.

Butylamine kinetics in various organic solvents have been used to obtain correlations based on the relative alkylation index (RAI, a combination of $\log k_{\text{rel}}$ and

$\log P$) with sensitisation data for sultones (Roberts and Williams, 1982), *p*-nitrobenzylhalides (Roberts *et al.*, 1983), α -(X-substituted methyl)- γ,γ -dimethyl- γ -butyrolactones (Franot *et al.*, 1994a, 1994b).

Taft substituent constants σ^* have been used as relative reactivity parameters for an RAI-based quantitative mechanistic model (QMM) for sensitisation potential of Schiff base electrophiles (Aptula *et al.*, 2006b).

Recently peptides containing a nucleophilic amino acid unit, such as GSH which has a nucleophilic ionised SH group as part of a cysteine amino acid unit and a nucleophilic terminal amine, have come into use as simple nucleophiles for reactivity measurements (Schultz *et al.*, 2005; Gerberick *et al.*, 2004).

GSH has several advantages as a model for soft nucleophilic groups of carrier protein — it is readily available, its concentration can be analysed by readily available methods and, unlike simple thiols, it is odourless and non hazardous to work with. However, it is not a chromophore so does not directly lend itself to methods like HPLC-UV without multi-step methods involving conjugation to carrier chromophores (e.g. as GSH-dinitrophenyl adducts), to detect its presence (Farriss and Reed, 1987). Its major limitation is that although soluble in water it has limited solubility in organic solvents. For determining reactivity of water soluble compounds this is not a problem, but many sensitisers are quite hydrophobic and have very limited solubility in water.

The fact that many sensitisers are hydrophobic (e.g. the 3-alkyl- and 3-alkenyl-catechols which are the active constituents of poison ivy, sesquiterpene lactones, hexadecane sultones and hexadecene sultone) indicates that the reaction with carrier protein occurs in some medium other than simple aqueous solution. One possibility is that the reaction occurs in a cell membrane. Another possibility, bearing in mind that the physical chemistry of water based biological fluids is more complex than that of simple aqueous solutions, and is in many respects similar to that of surfactant solutions, with soluble proteins behaving similarly to surfactant micelles, is that hydrophobic sensitisers are solubilised by micelle-like structures and reaction takes place in the interior of these pseudo-micelles. Using aqueous surfactant solutions or artificial membranes could be a way to make reactivity measurements under conditions more similar to those *in cutaneo*, but the evidence summarised above demonstrates that such laborious measures may be unnecessary, depending on what type of kinetic data are needed for predictions. The successful correlations obtained for skin sensitisation potential using kinetic data for reactions of electrophiles with simple model nucleophiles in simple solvents vindicate the reductionist approach of using the simplest and most convenient models to gain data on relative reactivities, which may contribute to modelling the potency determining steps in the skin sensitisation process.

How To Interpret Chemical Reactivity Parameters In The Context Of Skin Sensitisation

Reactivity data alone cannot provide a reliable prediction of whether a compound will be a strong, moderate, weak or non skin sensitizer, neither in mouse nor in man. This is because absolute sensitisation potency *in vivo* is related both to reactivity and to hydrophobicity. Consequently, not all reactive compounds are sensitizers: a compound may be reactive enough but not hydrophobic enough to sensitize *in vivo*. For example this is the case for lower alkyl halides (up to about C9 chain length), which are non-sensitizers, whereas higher chain length homologues, which are no more reactive, are sensitizers. However, quantitative kinetic reactivity data (interpreted within a mechanistic domain) combined with hydrophobicity can be used not only to predict whether or not a compound will be a sensitizer (yes/no call), but can also be used to derive a new scale to predict relative rank order effects. Several examples have been reported (Patlewicz *et al.*, 2004; Barratt *et al.*, 1997) of how this can be achieved by developing QMMs, in some cases using calculated reactivity parameters, for example the QMM for reactive carbonyl compounds in the LLNA (Aptula *et al.*, 2006b):

$$\log(1/EC3) = 1.12 \Sigma\sigma^* + 0.42 \log P - 0.62 \quad (5)$$

The parameter $\Sigma\sigma^*$ is the sum of Taft substituent constants for the two groups bonded to the reactive carbonyl group, and is a calculated value modelling $\log k$, k being the rate constant for reaction of the carbonyl group with a nucleophile.

For mechanistic domains where QMMs have not been developed, prediction is still possible, using mechanistic read-across. This is illustrated below for the Michael acceptor domain, where good progress has been made towards developing a reactivity database using RC_{50} values for reaction with GSH (Yarbrough and Schultz, 2007).

Use of A Small Reactivity Database For Mechanistic Read-Across

The basic principle of mechanistic read-across is that if two compounds in the same mechanistic domain are similar in their toxicity-determining parameters, they should be similar in their toxicity, irrespective of whether or not they are similar in structure. The ideal scenario is to have access to a large database of experimental reactivity data for skin sensitizers such that for a new compound to be evaluated, there are several compounds with similar parameters and with known *in vivo* sensitization outcome. At present the reactivity database of known skin sensitizers is still quite limited, but even in its

present form can still be used to make estimates by linear interpolation.

Suppose we wish to estimate the sensitization potency of 2,4-hexadienal. Its GSH RC_{50} is 1.533 mM (Terry Schultz, personal communication). We can calculate its $\log P$ by the Hansch and Leo method (1979) = 1.15.

Using the published classification rules (Aptula and Roberts, 2006) we can classify this compound in the Michael acceptor mechanistic domain.

In the database we search for two compounds in the Michael acceptor domain whose RC_{50} and $\log P$ values are either side of the 2,4-hexadienal figures and whose LLNA EC3 values are known. This search does not reveal a pair of compounds which fully meet this criterion, but we find a pair which comes close. These compounds are diethyl maleate and methyl 2-nonynoate.

Whilst this is not an ideal pair of compounds, in that both have $\log P$ values higher than that of the compound to be predicted, thus introducing an element of extrapolation, read-across is still possible and relevant.

The structures of 2,4-hexadienal, diethyl maleate and methyl 2-nonynoate are shown in Fig. 2 together with their RC_{50} , $\log P$ and EC3 values. Note that the smaller the RC_{50} is, the more reactive the compound is.

The reactivity of 2,4-hexadienal lies between those of diethyl maleate and methyl 2-nonynoate. Its hydrophobicity is not greatly different to that of diethyl maleate. From this information, we can make an estimate of skin sensitization as follows: Since RC_{50} is inversely proportional to the rate constant, $\log(1/RC_{50})$ can be used to represent $\log k$. By analogy with many other structure-sensitization correlations published for various types of compounds (e.g. sultones, lactones, alkyl alkanesulphonates, Schiff base electrophiles . . .), relative sensitization potency (expressed as a mol% $\log(1/EC3)$ value) can be assumed to be influenced twice as much by reactivity as by $\log P$. Accordingly a Relative Sensitization Parameter (RSP) can be defined as $RSP = \log(1/RC_{50}) + 0.5 \log P$ (Equation 6), and, based on the RAI model, we can write:

$$\log(1/EC3_{\text{mol\%}}) = a \text{ RSP} + b \quad (7)$$

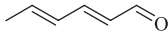
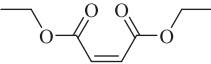
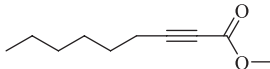
	RC_{50}	$\log P$	EC3
 2,4-Hexadienal	1.533	1.15	To be predicted
 Diethyl maleate	3.28	1.84	5.8
 Methyl 2-nonynoate	0.264	3.25	2.5

Figure 2. Mechanistic read-across: Michael acceptors

Using the RC_{50} and $\log P$ values, an RSP can then be calculated for each of the three compounds. $\log(1/EC3_{\text{mol\%}})$ values can be calculated from the EC3 (wt%) values and molecular weight values for diethyl maleate and methyl 2-nonynoate :

diethyl maleate,	RSP = 0.404; $\log(1/EC3_{\text{mol\%}}) = 1.47$
methyl 2-nonynoate,	RSP = 2.20; $\log(1/EC3_{\text{mol\%}}) = 1.83$
2,4-hexadienal,	RSP = 0.389; $\log(1/EC3_{\text{mol\%}}) = \text{to be estimated}$

Substituting the RSP and $\log(1/EC3_{\text{mol\%}})$ values for diethyl maleate and methyl 2-nonynoate in Equation (7) we can solve for a and b, then we can use Equation (7) to calculate $\log(1/EC3_{\text{mol\%}})$ for 2,4-hexadienal from its RSP value. In this way a $\log(1/EC3_{\text{mol\%}})$ value of 1.47 is derived for 2,4-hexadienal. This corresponds to an estimated EC3 value of 3.25% (since the MW is 96.13). Considering the nature of these calculations is very approximate, we would quote the estimate as between 2.5% and 4% (assuming plus or minus 20% of the calculated value, and rounding to nearest 0.5). The actual observed EC3 for 2,4-hexadienal is 3.5% (Gerberick *et al.*, 2005). It is worth noting that the two compounds used for the read-across prediction are very different in structure from each other and from the compound predicted, as can be seen from Fig. 2. The important point is that although not structurally similar, the three compounds are **mechanistically** similar.

Mechanistic read-across estimates such as the one illustrated here are very dependent on the variability of the EC3 values of the two known compounds either side of the linear interpolation, as well as the accuracy of the reactivity and $\log P$ parameters for all three compounds. Hence, interpretation of such models should be done with care and with knowledge of the inherent biological variability of the bioassay data being used to make such benchmark predictions. With a larger database of experimental reactivity values, more compounds could be predicted and perhaps with greater reliability. What is required and how such a database could be used, are outlined below.

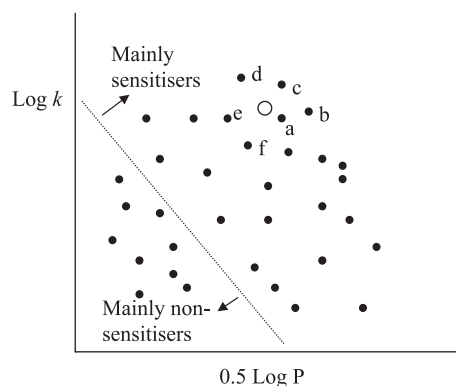
Mechanism-Based Read-Across For Skin Sensitisation

In the situation where new animal testing is not available to us, it is essential to exploit all of the available and existing knowledge learnt from the previous testing of chemicals, in order to make maximum use of these data to evaluate new chemicals. One such resource is the publication by Gerberick *et al.* where high quality LLNA data for over 200 compounds, ranging from

non-sensitisers to extreme ($EC3 < 0.1\%$) sensitisers, were collected (Gerberick *et al.*, 2005). We have since assigned all of these compounds to their appropriate reaction mechanistic domains (Roberts *et al.*, 2007c).

In order to use this LLNA database, or future enlarged versions of it, to predict the relative *in vivo* sensitisation potential of untested chemicals, an urgent requirement is to complement the LLNA database with a large database of experimental kinetic reactivity data (expressed as $\log k$ or mathematically equivalent such as pRC_{50}) and hydrophobicity ($\log P$) for as many of the known sensitisers as possible, for each mechanistic domain. It is important to work within the boundaries of mechanistic domains for many of the reasons stated above, but put simply, one must compare chemical reactivity kinetics of like-with-like. Progress has been made towards establishing such a database (Yarbrough and Schultz, 2007; Aptula *et al.*, 2006a) but more resources need to be devoted to generate new reactivity data and populate this database further.

Figure 3 represents the database visually and conceptually as a graph of $\log k$ against $0.5 \log P^\dagger$. Note that a separate graph would be required for each mechanistic domain. The major mechanistic domains for sensitising electrophiles have been described in detail elsewhere (Aptula and Roberts, 2006; Roberts *et al.*, 2007b; Aptula *et al.*, 2005). They are: S_N2 , S_NAr , Acyl transfer, Michael acceptors, Schiff bases, non reactive. Each point on a graph of this kind will have an associated relative sensitisation potential (expressed as $pEC3$) associated with it. Compounds that are close to each other on the



a, b, c, d, e, f are compounds with known $\log(1/EC3)$ values and are the nearest neighbours of the new compound to be predicted

The $\log(1/EC3)$ value of a is substantially different from those of b...f, so a is considered to be anomalous and is not used in the prediction

○ New compound. Predicted $\log(1/EC3) = \text{average } \log(1/EC3) \text{ for b-f}$

Figure 3. Principle of mechanism-based read-across for skin sensitisation based on a plot of $\log k$ against $0.5 \log P$ for compounds with known $\log(1/EC3)$ values

[†] The factor of 0.5 is used because sensitisation potential tends to be about twice as dependent on reactivity as hydrophobicity.

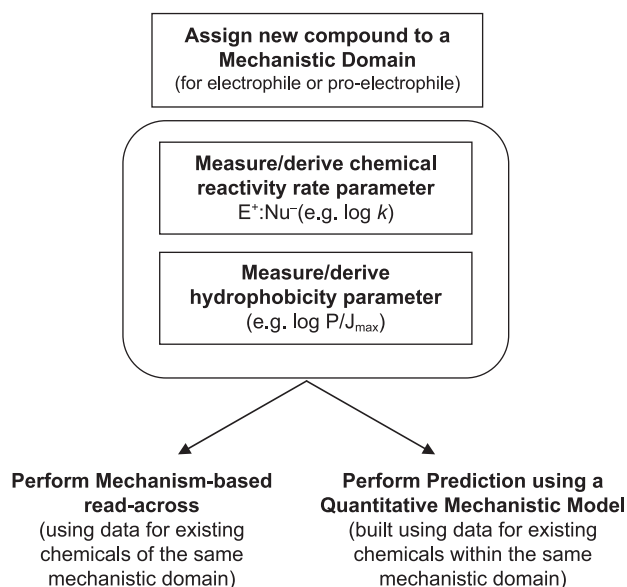


Figure 4. A schema for using chemistry data in predicting relative rank order skin sensitisation potential for a new chemical

graph should in principle be similar in terms of sensitisation potential. As the database is enriched with new data, the graph will become more densely populated and the data gaps will diminish. There will inevitably be some compounds whose relative potency values appear anomalous compared to their neighbours on the graph. These anomalies should be investigated with the aim of understanding the mechanistic subtleties which make them 'special cases'.

For the evaluation of a new compound, either by mechanism-based read-across or by using a QMM the following procedure is proposed (Fig. 4). The reaction mechanistic domain first needs to be identified (either through inspection of the structure or through experimental investigation of its reaction chemistry). Next the appropriate chemical parameters (usually reactivity and hydrophobicity) of the compound need to be quantified. If a QMM has already been developed for the reaction mechanistic domain, it can be used to calculate a potency value for the new compound. If not, the read-across method can be used. The new compound can be plotted on the graph of $\log k$ against $0.5 \log P$ for the appropriate mechanistic domain (Fig. 3). The new compound should have several near neighbours — so long as the underlying reactivity database is sufficiently extensive. The relative rank order sensitisation potential of the compound can then be estimated as the mean of the relative potency values of the near neighbours (as an extra level of sophistication, the near neighbour potencies could be weighted inversely by their distance to the new compound). Any near neighbour whose potency is anomalous compared with its own near neighbours should not be used in the potency estimate. In any case, the chemistry

of the new compound should also be considered carefully, to determine whether it is a potential 'special case'.

Conclusions

In this and our preceding paper (Roberts and Aptula, 2007) we have aimed to highlight the importance of chemical reaction mechanism, hydrophobicity and reactivity as chemistry-based determinants which influence skin sensitisation potency, and how to measure reactivity and express it in a quantitative way which can be used for quantitative mechanistic modelling and mechanism-based read-across. We have illustrated how this chemical data can be integrated into sensitisation potency assessment by means of an example of mechanism-based read-across.

To make these types of mechanism-based read-across assessments viable and practical for routine use in decision making requires the population of a database with empirical chemical kinetics reactivity data for as many known sensitisers as possible. In most cases, adequate estimates of hydrophobicity can be obtained by calculation of $\log P$ from structure. However, if predictions can be improved by measured $\log P$ or even more sophisticated measures of kinetic delivery to the tissue (e.g. measures of the rate ($J_{\max}$) of delivery of epidermal concentrations), methods are technically available. For reactivity data, $\log k$ values from kinetic studies are preferred (as explained earlier), but $\log k$ values from RC_{50} assays could also be used, and could be supplemented by $\log k$ estimates from % depletion assays, but confidence in the latter would be much lower than from properly designed kinetic experiments. Where several estimates of reactivity are available, $\log k$ values from kinetic studies should be preferred over those from RC_{50} determination, which should be preferred over those derived from % depletion data. Chemistry studies on 'special case' compounds should also be carried out in order to facilitate the identification of cases where new compounds are likely to differ significantly in relative potency from their nearest neighbours on the reactivity/hydrophobicity graph.

We envisage the development and population of this reactivity database for skin sensitisation to be part of a wider-ranging database being developed for reactive toxicity in general in the longer term. Reactivity of electrophiles is known to play a part in human health effects other than sensitisation (e.g. liver toxicity) and in environmental toxicity (Lipnick *et al.*, 1986; Lipnick, 1988, 1991; Yarbrough and Schultz, 2007; Schultz *et al.*, 2007).

So far this paper has focused on describing the reactivity of directly acting electrophiles. A number of skin sensitisers are pro-electrophiles, i.e. they are not

themselves electrophilic but sensitise via *in cutaneo* conversion (which may be abiotic or metabolic) to electrophilic derivatives. In many cases these derived electrophiles are unstable and difficult to study in isolation. More work needs to be done on developing new methods to assess whether a new unreactive compound could be a pro-electrophile, and hence likely to sensitise via metabolic activation. Ideally an experimental method for identifying potentially sensitising pro-electrophiles would include appropriate metabolic and abiotic activating capability reflective of that in skin. However, so little is known about skin metabolism to date (recently reviewed in Gibbs *et al.*, 2007), that finding a suitable metabolising system could present a significant challenge in assay development.

Currently the most pragmatic way forward is to assume that all the major pro-electrophilic classes of compounds have been identified and that if a compound cannot be assigned to one of these classes and is not directly electrophilic it is unlikely to be a sensitizer. Prediction of potency for compounds which can be assigned to a pro-electrophile class is a more difficult problem. It is encouraging that several pro-electrophile sensitizers (e.g. lauryl gallate, p-N-methylaminophenol [Metol]), have been found to give significant depletion values in peptide binding assays (Gerberick *et al.*, 2007) indicating that the parent non-reactive chemicals are converted to reactive species (presumably by air oxidation) under the assay conditions. However, peptide depletion under such oxidative conditions may not always be due to covalent adduction between a newly generated electrophilic product of oxidation and the assayed peptide; oxidative conditions may produce an apparent peptide depletion via other mechanisms.

To conclude, chemical reactivity indices based on kinetic measurements, and application of them in mechanism-based read-across are vital areas for further development and show great promise in being able to contribute towards novel non-animal approaches for the prediction of skin sensitisation in the future.

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