Critical Reviews in Toxicology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/itxc20

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To cite this article: David W. Roberts (2021) A critical review of the kinetic direct peptide reactivity assay (kDPRA) for skin sensitizer potency assessment – taking it forward, Critical Reviews in Toxicology, 51:10, 805-819, DOI: 10.1080/10408444.2021.2020212

To link to this article: https://doi.org/10.1080/10408444.2021.2020212

Published online: 15 Feb 2022.

CRITICAL REVIEWS IN TOXICOLOGY
2021, VOL. 51, NO. 10, 805–819
https://doi.org/10.1080/10408444.2021.2020212

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REVIEW ARTICLE

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A critical review of the kinetic direct peptide reactivity assay (kDPRA) for skin sensitizer potency assessment – taking it forward

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ABSTRACT

It is widely recognized that the ability of chemicals to sensitize, and the potency of those chemicals that are sensitizers, is related to their ability to covalently modify protein in the skin. With the object of putting non-animal-based prediction of skin sensitization on a more quantitative footing, a recent paper describes the development of the kinetic Direct Protein Reactivity Assay (kDPRA), in which a matrix of peptide depletion values for different reaction times and test chemical concentrations is generated and analyzed so as to derive a reactivity parameter, logk_{max}, which is used to classify chemicals into one of two potency categories. The present paper demonstrates that the reaction chemistry is not always consistent with the mathematical analysis of the data matrix and the kDPRA protocol does not identify such cases. Consequently the derived logk_{max} value is not always mechanistically meaningful and its application to predict potency can lead to misleading conclusions. It is shown that by adopting a data analysis protocol based on conventional kinetics practice, the kDPRA can be made to provide more reliably meaningful and more extensive information that can be used for purposes such as potency estimation for deriving No Expected Sensitization Induction Level (NESILs) required for quantitative risk assessment (QRA), deriving quality specifications in terms of acceptable impurity levels, and development of structure-activity relationships. Secondly, the paper addresses applicability domain issues, in particular the problem of deciding whether or not the kDPRA is applicable for a given chemical.

ARTICLE HISTORY
Received 19 November 2021
Revised 6 December 2021
Accepted 9 December 2021

KEYWORDS
Reaction kinetics; applicability domains; chemical reaction mechanisms; non-animal methods; structure–activity relationships

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1. Introduction

It is widely acknowledged that covalent binding to carrier protein in the skin is the molecular initiating event and the potency-determining step in the skin sensitization process (e.g. Roberts and Aptula 2008; Patlewicz et al. 2016; Natsch

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et al. 2020). The ability of chemicals to sensitize, and the potency of those chemicals that are sensitizers, is related to their ability to covalently modify protein in skin. Although the precise nature, number and location of the skin proteins involved in the covalent binding are not known, the reductionist approach of studying reactions with simpler nucleophilic compounds *in vitro* has been highly successful in developing structure–activity relationships for skin sensitization, in some cases on a quantitative basis. Some examples are: Landsteiner and Jacobs (1936) using aniline as the nucleophile; Roberts and Williams (1982) using butylamine as the nucleophile; Roberts and Natsch (2009), Natsch et al. (2011), and Roberts et al. (2017) all using a cysteine-based peptide as the nucleophile. It follows that determining reactivity and measuring it on a quantitative basis can play a major role in non-animal methods for skin sensitization.

The direct peptide reactivity assay (DPRA) (Gerberick et al. 2004) is an OECD-adopted method to assess whether or not a chemical is reactive enough to sensitize. In the DPRA an aqueous solution of test chemical is incubated with a peptide whose structure contains either a nucleophilic cysteine unit or a nucleophilic lysine unit and the extent of peptide depletion is determined. A program aimed at putting it on a more quantitative basis (Wareing et al. 2017) has led to the development of the kDPRA (k for kinetic), in which a matrix of peptide depletion values for different reaction times and test chemical concentrations is generated and analyzed so as to derive a reactivity parameter, $\log k_{max}$ (Natsch et al. 2020). The kDPRA has been proposed as a standalone assay to form the basis of an OECD guideline for identification of GHS 1A sensitizers among chemicals identified as sensitizers by other tests or defined approaches, chemicals with $\log k_{max} > -2.0$ being classified as 1A (Natsch et al. 2020).

This paper considers how, mainly by refining the way the data matrix is analyzed, the kDPRA can be made to provide more accurate and more extensive information that can be used for purposes such as potency estimation for deriving No Expected Sensitization Induction Level (NESIL) required for quantitative risk assessment (QRA) (Api et al. 2008, 2020; Basketter and Safford 2016), deriving quality specifications in terms of acceptable impurity levels, and development of structure–activity relationships. Second, the paper addresses applicability domain issues, in particular the problem of deciding whether or not the kDPRA is applicable for a given chemical.

2. Reaction kinetics and rate constants

The rate constant k is not, as is sometimes assumed, the same as the reaction rate or speed of the reaction. It is the proportionality constant relating the rate (speed) of reaction to the concentrations of the reacting chemicals, so for a second order reaction of A with B the rate equation is:

$$\text{Rate} = k[\text{A}][\text{B}]$$

For the kDPRA one of A and B is the peptide and the other is the test chemical. The concentrations of the reacting chemicals decrease as the reaction proceeds, and consequently the rate of the reaction decreases too. To calculate the extent of reaction occurring in a given time period, the above rate equation is integrated to give a function relating extent of reaction (in the case of the kDPRA, expressed as depletion of the peptide) to a combination of k, time and the initial concentrations. It is common practice to do kinetic experiments under pseudo first order conditions, in which one of the reacting chemicals, say A, is in substantial excess over the other, so that its concentration can be treated as constant. In this situation, the rate equation can be modified to:

$$\text{Rate} = k_{obs}[\text{B}]$$

in which k_{obs}, the pseudo-first order rate constant, $= k[\text{A}]$.

3. The kDPRA protocol and comparison with conventional kinetic practice in chemistry

In the kDPRA an aqueous solution of a cysteine-base peptide at 0.5 mM is incubated with the test compound at a series of concentrations (5, 2.5, 1.25, 0.625, and 0.3125 mM) and for a range of reaction times (30, 90, 150, 210, and 1440 min). The reaction mixtures are analyzed to determine the levels of unreacted peptide, producing a matrix of percentage peptide depletion values (DP) for each time and concentration. The data are then processed as follows, with the underlying assumptions that the reaction is second order (first order in peptide and first order in test compound) and that the concentration of the test compound, being in excess over the peptide, can be treated as being constant throughout the reaction (i.e. the reaction can be treated as pseudo first order). Although the second assumption does not apply at the 0.3125 mM concentration, if the extent of reaction is quite small the error may not be significant. The natural logarithm (ln) of the non-depleted peptide concentration is plotted against the concentration of the test chemical at each time point and these plots are tested for linearity ($R > 0.9$). The slope of each plot, if it meets the linearity criterion, is divided by the incubation time to give estimated values of the second order rate constant. The largest of these estimates, in units of l mol^{-1} s^{-1}, is referred to as k_{max} and its logarithm (to base 10), $\log k_{max}$, is used as the parameter quantifying the reactivity of the test compound.

Table 1 compares this protocol with the conventional kinetics approach.

Comment 1. The reason arithmetic progression of concentrations (and time intervals) is preferred is that it gives even spacing of points, whereas with geometric progression the point corresponding to the highest concentration or time value is more distant from the other points than the other points are from each other and consequently has greater leverage (i.e. makes a larger contribution than the other points to determining the slope of the plot of ln(100-DP) versus [E] or t). This is not a major problem if the plot shows a high degree of linearity; geometric progression is often used for initial range-finding and often the range-finding plot may be good enough to be used in deriving the rate constant.

Comment 2. Although it is not usual, there is nothing wrong in principle with doing log plots against concentration then plotting the slopes of these plots against time to find

Table 1. kDPRA protocol compared with conventional kinetics practice.

Feature	kDPRA	Conventional kinetics	Comment
Time point series	Arithmetic progression	Arithmetic progression	
Concentration series	Geometric progression	Arithmetic progression	1
First plots	Ln(100-DP) versus [E]	Ln(100-DP) versus *t*	2
Estimation of rate constant	Each slope of ln(100-DP) versus [E] divided by *t* and largest value taken as k_{max}	Slopes of ln(100-DP) versus *t* plotted against [E] and slope taken as *k*. Intercept gives the first order rate constant for [E]-independent peptide depletion	3

DP = % depletion of peptide; [E] = initial concentration of test compound.

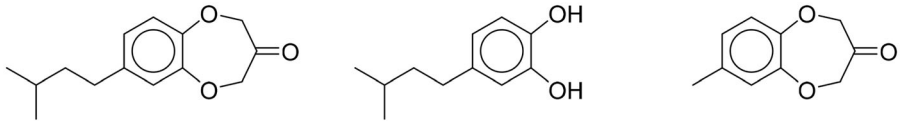


Figure 1. Azurone and related compounds.

the rate constant. However, doing it in the conventional way makes the plots easier to interpret if the reaction deviates from ideal behavior (e.g. if there is an initial lag at the start of the reaction; if there is a more reactive impurity present; if the test compound separates out of solution; if the reaction stops short of 100% completion) or if it is not a second order reaction. There seems no reason why the kDPRA data matrix should not be analyzed by the conventional method of first plotting ln(100-DP) versus *t*, then plotting the slopes against [E].

Comment 3. For situations where the reaction is second order and behaves ideally, the k_{max} value determined by the kDPRA protocol should be identical, within experimental error limits, to the true second-order rate constant. In the kDPRA protocol the point ($t = 0$, DP = 0) plays a key role in determining k_{max}, which is in effect the slope of the two-point line between this point and one of the ln(100-DP) versus [E] slopes. This contrasts with conventional kinetics, where it is often found that the $t = 0$ point is off-line and it is acceptable to ignore it. The rationale for ignoring the $t = 0$ point is that the presence of a reactive impurity, or a short induction period (e.g. corresponding to mixing of the reactant solutions), can affect the extent of reaction in the initial time period.

The next section discusses two illustrative examples where data analysis according to conventional kinetics practice would give different conclusions from the kDPRA protocol.

4. Illustrative examples

4.1. Azurone

Azurone has the structure shown in Figure 1, which also shows the related compound Calone. Azurone, as supplied and considered to be of high purity based on GC analysis, was found to have unexpectedly high sensitization potency (LLNA EC3, 1.4%) and was positive in the original non-kinetic version of the DPRA (Natsch et al. 2010). In contrast Calone, differing structurally from Azurone only in the size of an alkyl group remote from any potential reaction center, had only low reactivity in the original DPRA and was non-sensitizing in the LLNA (tested at concentrations up to 30%). Suspecting that the positive LLNA result observed with Azurone might be due to the presence of a highly potent impurity, Natsch et al. (2010) carried out further analytical investigations and found isoamyl catechol (Figure 1) to be present as an impurity at about 2.5%. Isoamyl catechol is similar to the poison ivy allergens – it is not directly reactive but is easily and rapidly oxidized to a highly reactive quinone. Removal of this impurity reduced the DPRA reactivity of Azurone to that of Calone, and changed the LLNA EC3 from 1.4% (1A) to about 14% (1B).

From the 24 h depletion value observed in the DPRA with purified Azurone at 5 mM, the rate constant for peptide depletion by Azurone *per se* can be estimated as 3.2×10^{-4} l mol^{-1} s^{-1}. Using this rate constant value, and assuming that the isoamyl catechol impurity has reacted completely within the first time interval (30 min), it is straightforward to calculate the shapes of the plots of ln(100-DP) versus [E] that would be obtained for Azurone (containing 2.5% impurity) by the kDPRA protocol.[1] Figure 2 shows the plot that would be obtained for the $t = 210$ min. data; the plots for the other *t* values are very similar and almost indistinguishable to the naked eye. This is because the depletion values are mainly due to the fast-reacting impurity with only a relatively small contribution from the main component. All of these plots would be expected to pass the linearity test, and by the kDPRA protocol the slope of the 30 min plot would be used to give the k_{max} value as shown in Table 2. At this point, it may be noted that although Azurone and Calone show some reactivity due to the main component, this may indicate peptide oxidation rather than adduct formation. Azurone and Calone have a Schiff base electrophile

[1]The assumption that the isoamyl catechol impurity reacts completely over the first 30 min. may or may not be true. The impurity per se is not reactive but is easily oxidised in air to a highly reactive species (ortho-quinone). Consequently, it is possible that the extent of peptide binding may depend on the availability of oxygen. However, based on 0.26 mM/l as the aqueous solubility of oxygen in water at 25 °C https://www.engineeringtoolbox.com/oxygen-solubility-water-d_841.html there should be enough oxygen present under kDPRA conditions to oxidise all of the impurity and it is therefore considered likely that the assumption is correct. Azurone is used here simply to illustrate the general case of a non-reactive or low-reactive chemical containing a reactive impurity, so the general argument is not affected by whether the assumption of rapid and complete reaction of the impurity is correct or not in this particular case.

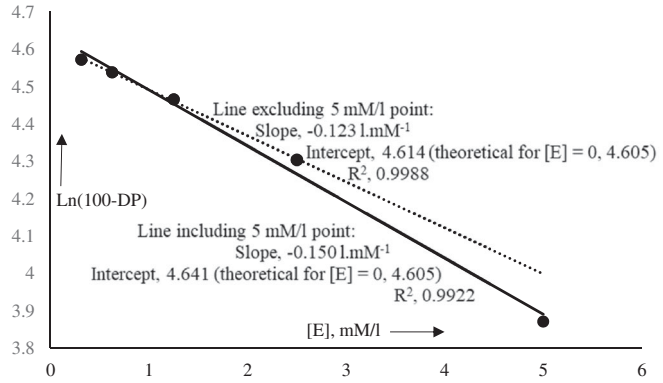


Figure 2. Azurone with 2.5% highly reactive impurity: reconstructed kDPRA plot of ln(100-DP) versus [E] at 210 min.

Table 2. Azurone with 2.5% highly reactive impurity: analysis of reconstructed kinetic data by the kDPRA protocol.

Time, t (min)	Slope (l mmol^{-1})	Slope/t (l mmol^{-1} min $^{-1}$)	k_{max} (l mol^{-1} s^{-1})
30	−0.143	-4.76×10^{-3}	7.9×10^{-2}
90	−0.145	-1.61×10^{-3}	
150	−0.148	-9.83×10^{-4}	
210	−0.150	-7.14×10^{-4}	
			Logk_{max} −1.10

Table 3. Calculations for Azurone with various levels of reactive impurity.

% Impurity	Calculated logk_{max}	Potency class	Potency class and EC3 based on experimental EC3 values[a]	
2.5	−1.10	1A	1A	1.4%
1.5	−1.39	1A	1B	2.2%
1.0	−1.60	1A	1B	3.0%
0.5	−1.92	1A	1B	5.0%
0.4	−2.01	1B	1B	5.7%

Slopes of 30 min plot divided by 30 and converted to l mol^{-1} s^{-1} units before taking logs to give logk_{max} values.

[a]Experimental EC3 values of pure Azurone (14%) and Azurone with 2.5% impurity (1.4%), are used to calculate EC3 of the reactive impurity (0.04%) using the mixture potency equation: $1/EC3_{mixt} = f_1/EC3_1 + f_2/EC3_2$, where f_1 and f_2 are the fractions in the mixture of components 1 and 2 respectively. This equation is then used to calculate EC3 for other impurity levels.

alert (ketone with electronegative substituents), and Schiff base electrophiles have been found to promote oxidation, but not to form stable adducts, in the DPRA (Natsch and Gfeller 2008).

The logk_{max} value would assign this material as 1A, and the analysis by the kDPRA protocol would not reveal that the potency could be reduced to 1B by removing an impurity.

Knowing that pure Azurone has an EC3 value of 14% and that the impure material with an EC3 value of 1.4% contains 2.5% of the reactive impurity, it is straightforward to calculate that if the impurity level is reduced to 1.5% the EC3 would be slightly above 2%, i.e. potency class 1B. How does this compare with what would be predicted by the kDPRA protocol?

For different levels of reactive impurity, the calculated plots are similar to Figure 2: good linearity, slopes only slightly different from each other but with significantly different intercepts. The 30 min plots give the k_{max} figures, as shown in Table 3.

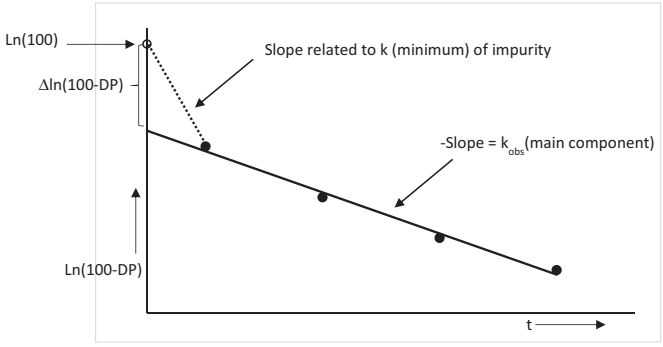


Figure 3. General plot of ln(100-DP) versus t for a chemical with a highly reactive impurity.

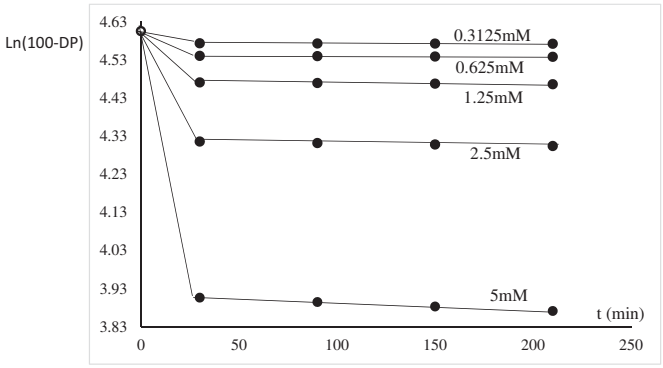


Figure 4. Azurone with 2.5% impurity. Ln(100-DP) versus t for reconstructed kinetic data.

It can be seen from Table 3 that Azurone with an impurity below 1.5% would in reality meet the 1B criterion (EC3 > 2%) but would be classed by the kDPRA protocol as 1A with any impurity level above 0.4%.

The general conclusion from the analysis so far is that the kDPRA data analysis protocol does not distinguish between strongly reactive chemicals and non-reactive or weakly reactive chemicals containing a highly reactive impurity, and can overestimate the potency class of the latter.

The conventional kinetics approach, in which analysis of the data matrix begins with plots of ln(100-DP) versus t, is more informative. Figure 3 shows the general shape of the plot that would be obtained and the information that could be derived from it.

As Figure 3 shows, the ln(100-DP) versus t plot can provide further information in addition to the rate constant for the main component. If the initial negative slope between the ($t = 0$, DP = 0) point and the first measured point is steeper than the slope for the other points, the likely presence of a reactive impurity is indicated. From the initial slope (dotted line in Figure 3) an estimate of the minimum reactivity of the impurity can be made, and the Δln(100-DP) value gives an indication of the level of the reactive impurity.[2]

[2]The percentage molar equivalents of reactive impurity (%ME_{RI}) in the test chemical can be calculated by: $\%ME_{RI} = 100(1-e^{-\Delta ln(100-DP)})/C$ where C is the initial concentration of test chemical. For an impurity that reacts with the peptide on a 1:1 basis, the mole percentage of reactive impurity is equal to %ME_{RI}. For an impurity that reacts on a 1:2 impurity:peptide basis (as is the case with Azurone), the mole percentage of reactive impurity is equal to %ME_{RI}/2.

Although it is convenient to consider plots of this type in terms of two distinct linear portions, this is really a simplifying approximation. The true function is a continuous curve in which the degree of curvature is low except for a narrow region (in Figure 3 this is near the intersection of the full and dotted lines) where the slope changes rapidly.

Figure 4 shows reconstructed ln(100-DP) versus *t* plots for Azurone containing 2.5% highly reactive impurity, for the various initial concentrations [E] that would be used in the *kDPRA* protocol of Natsch et al. (2020).

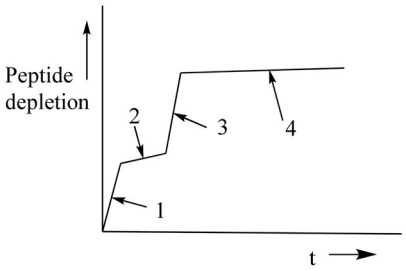
These reconstructed plots conform to the general plot of Figure 3. The effect of the impurity is more obvious at higher concentrations of the test chemical. The higher the concentration of test chemical, the higher the concentration of impurity and the greater the contribution of the impurity to the total peptide depletion. It should be noted that the slope of the steep part of the plot gives a minimum limit for the rate constant for the reactive impurity, not the rate constant *per se*. The plots do not indicate whether the reactive impurity has already reacted completely within a shorter time period than 30 min. A useful modification of the experimental *kDPRA* protocol would be to add an extra time point, say $t = 5$ min, aiming for the shortest reaction time possible.

Summarizing for Azurone (unpurified). If it had been tested in the kDPRA with data analysis as per the kDPRA protocol, it would have been assigned 1A with no obvious indication that the potency was mainly due to an impurity. With analysis of the kDPRA data according to conventional kinetics practice, it would have been obvious that Azurone itself was either 1B or NS and that a reactive impurity was present at a single figure percentage level.

4.2. Paraphenylenediamine (PPD)

PPD is an example of a skin sensitizer that is not directly reactive but is readily converted by oxidation to a short-lived highly reactive species. Skin exposure provides a high surface to volume ratio and ready access of air, conditions conducive to oxidation. The behavior of such compounds in the kDPRA will depend on the availability of oxygen. PPD is one of the compounds listed by Natsch et al. (2020) as a 1A sensitizer incorrectly classified by the kDPRA protocol as 1B. Test details for PPD are given in a paper by Wareing et al. (2017), presenting results from an earlier version of the kDPRA in which the time intervals are different; in particular data are measured for $t = 5$ and 10 min as well as 30 min and above, and the $\log k_{max}$ threshold for 1A is > -1.73. On this basis, PPD is classed as 1A based on the $t = 5$ and $t = 10$ points, but it would be 1B based on the 30 min point ($\log k = -1.77$). The kDPRA results pattern for PPD seems very similar to what we would expect for Azurone – see earlier – and indeed if nothing were already known about PPD the results might be interpreted in terms of PPD being a non-sensitizer but the sample containing a reactive impurity. Based on what is already known about the chemistry of PPD, the pattern of kDPRA results can be rationalized as follows.

At the start of the assay when the reaction solutions are mixed, the oxygen present in solution reacts with PPD to form a short lived highly reactive species (probably a di-imine) which rapidly reacts with the peptide. The reaction with oxygen is likely to be the rate-determining step. When most of the oxygen originally in the solution has reacted, mass transfer of oxygen from the atmosphere into the reaction medium becomes the rate-determining step and the peptide depletion becomes much slower. The course of the reaction can be depicted as proceeding in up to four stages, as shown in Figure 5, which applies generally for a test chemical that is activated by oxidation during the assay.



1. Reaction of highly reactive impurity, if present
2. Induction period (might not be detectable)
3. Rate determining step:oxidation by O_2 in solution
4. Rate determining step: mass transfer of O_2 from atmosphere

Figure 5. Generic plot of peptide depletion versus *t* for a chemical activated by oxidation.

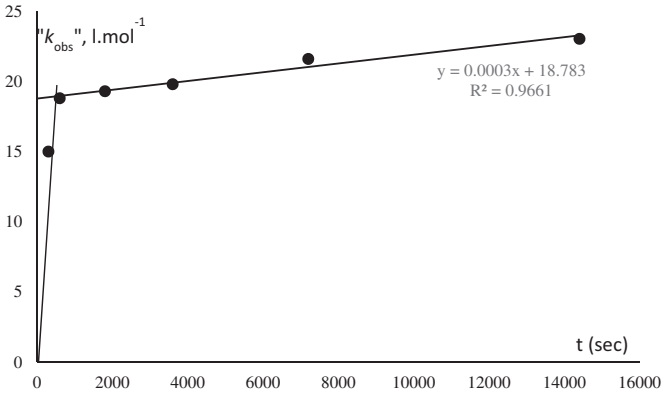


Figure 6. PPD: "k_{obs}" (slope of ln(100-DP) versus [E]) against t (data from Wareing et al. 2017).

1. The test material may already contain a small amount of highly reactive oxidation product(s) before the start of the assay. In the specific case of PPD, oxidation in the absence of a nucleophile gives an oxidized trimer, Bandrowski's Base, via initial formation of the short-lived di-imine and a sequence of further reactions with PPD (Corbett 1973). Bandrowski's base is a strong sensitizer, even more potent than PPD, but it is not generally considered to play a major role in sensitization by PPD, since PPD and Bandrowski's base do not exhibit much cross-reactivity (White et al. 2006). The presence of impurities of this type may lead to a very rapid initial peptide depletion reaction.
2. There may be a short induction period during which the short lived highly reactive species builds up to a steady state concentration. However, in many cases, the induction period may be too short to be detected under kDPRA protocol conditions.

3. After 1 and 2 above, if applicable, oxidation of the test chemical by the oxygen present in solution becomes rate determining. The rate of peptide depletion is dependent on the concentration of test material (which, being in excess, does not change significantly) and on the concentration of oxygen in the reaction medium.
4. After most of the oxygen in solution has become depleted, mass transfer of oxygen from the atmosphere to the reaction solution becomes the rate-determining step.

In Figure 6, the slopes of ln(100-DP) versus [E] are plotted against t for PPD (data taken from Wareing et al. 2017). Stages 1 and 2 above are not detectable (this does not mean that they do not occur, but in this case they are not distinguishable from stage 3). It is clear that the apparent k value derived by the kDPRA protocol differs widely depending on the first time point – in the Wareing et al. (2017) paper the first time point is 5 min and gives a k value 5 times greater than the k value from the 30 min point.

Without the full kDPRA data matrix for PPD, Figure 6 cannot be interpreted unambiguously. For ideal second order kinetics, the plot should be linear and the intercept (the "k_{obs}" value corresponding to $t = 0$) should not be significantly different from zero. This is clearly not the case in Figure 6. One possibility is that the initial steep part of the plot corresponds to reaction of peptide with a reactive impurity such as Bandrowski's base as the rate-determining step and the later part of the graph corresponds to reaction of PPD with dissolved oxygen as the rate-determining step. Another possibility is that the initial steep part of the graph corresponds to reaction of PPD with dissolved oxygen as the rate-determining step and the later portion corresponds to mass transfer of oxygen from the atmosphere as the rate-determining step. Since the peptide is not involved in the rate-determining step, the slope of the later part of the graph does not represent a true rate constant.

There are numerous other chemicals that, similarly to PPD, are not directly reactive but can react readily with oxygen to form peptide-reactive species and which can sensitize via oxidation to electrophilic derivatives. In some cases, like PPD and similar chemicals, the oxidation is likely to be the rate-determining step. The kDPRA protocol does not specify an initial oxygen concentration in the test solutions, so the extent to which oxidation can occur may vary. Consequently, the apparent k_{max} value cannot be a reliable predictor of sensitization potency for such chemicals.

5. Applicability domain issues

Discussing non-animal testing strategies for skin sensitization, Basketter et al. (2013) state "critical to data acceptance, particularly to support a negative outcome, is demonstration that the substance under investigation falls within the 'applicability domain' of the assay, i.e., that the substance is known to be capable of being tested meaningfully."

Whether a given chemical is within or outside of an applicability domain depends on its physicochemical properties, which in turn depend on its structure.

In the present context, there are two applicability domain issues to consider:

1. Is the kDPRA protocol able to generate the true rate constant for reaction of the kDPRA peptide with the test chemical? This is the kinetics applicability domain issue.
2. Is the above true rate constant, whether determined by the kDPRA protocol or by any other method, able to correctly classify the chemical as 1A or 1B depending whether log$k$ is greater or less than −2? More broadly, can this rate constant alone predict the potency? This is the chemistry-potency applicability domain issue.

5.1. The kinetics measurement applicability domain issue

The kDPRA protocol as originally published (Natsch et al. 2020) is applicable if all of the following four conditions are met:

1. The chemical is either (a) a directly-reacting electrophile or (b) it is neither reactive nor able to be activated under the assay conditions. For (b), the kDPRA would correctly assign the chemical as non-reactive and non-1A.
2. It is soluble in the aqueous reaction medium or is able to remain in supersaturated solution long enough for peptide depletion measurements to be made.
3. Its reaction product with the peptide is hydrolytically stable, at least over the first time period of the assay.
4. The test chemical does not contain a reactive impurity at a level sufficient to give a significant overestimate of the rate constant. For a modified version of the kDPRA with data analysis by a conventional kinetics protocol, this condition does not apply, since the effect of the impurity can be easily detected and taken into account as illustrated in Section 4.1 for azurone.

If these conditions are met, then:

> The plots of ln(100-DP) versus [E] should be linear and their intercepts should be close to ln(100), i.e. 4.605
>
> Dividing the negative slopes of ln(100-DP) versus [E] by t should give similar values for all t values, i.e. if the negative slopes of ln(100-DP) versus [E] are plotted against t the plots should be linear and pass through the origin

The k_{max} database provided by Natsch et al. (2020) does not give the data matrices, so it is not possible to assess the extent to which these conditions are met in the database. However, a partial assessment can be made by considering the data in the paper by Wareing et al. (2017) presenting an earlier version of the kDPRA. Although the data matrices are not given, Table 7 of that paper gives k values, calculated as the slope of ln(100-DP) versus [E] divided by t, corresponding to up to six t values (5, 10, 30, 60, 120, and 140 min) for each chemical. Considering only those chemicals for which k is given for at least 4 values:

> Four chemicals give the same calculated k value, within a factor of 2, for all t values.
>
> Thirteen chemicals show a clear trend with calculated k values decreasing with increasing t.

For seven chemicals, the calculated k values vary, showing no clear correlation with t.

Clearly, many of the chemicals do not meet all four of the necessary conditions for the kinetics measurement applicability domain. Without the full data matrices, it is not possible to fully assess for each chemical which conditions fail to be met. However, it is clear that a logk_{max} value derived by the kDPRA protocol cannot automatically be taken as a reliable index of a chemical's true reactivity toward the peptide or its true relative reactivity toward other nucleophiles.

Bearing in mind the conditions for applicability, chemicals that are outside the kinetics measurement applicability domain of the kDPRA are:

1. Many hydrophobic chemicals. If tested they would tend be classed as non-reactive, irrespective of whether they are reactive or not. For reactive chemicals that can form a supersaturated solution when the reaction mixture is made up but that come out of solution during the assay, it may not be obvious whether it is a case of a reactive impurity being consumed or the test compound coming out of solution during the course of the assay.
2. Many chemicals belonging to the acyl transfer mechanistic domain. These are either hydrolyzed directly or their initial peptide adducts are hydrolyzed, resulting in them being classed as non-reactive or as having low reactivity.
3. Chemicals belonging to the Schiff base mechanistic domain. In most cases these do not give stable adducts with the aqueous cysteine-based peptide. However, in many cases, they do give peptide depletion by oxidation of the peptide -SH group to an -S–S– linkage.
4. Chemicals that are not directly reactive but can sensitize as a result of oxidation to reactive species under exposure conditions. These are likely to give erratic and unreliable results in the kDPRA – there may or may not be sufficient dissolved oxygen in the reaction medium to oxidize the test chemical to a significant extent and the kDPRA rate equation is inappropriate. PPD (Section 4.2) is an example of such such chemicals.
5. Chemicals that are not directly reactive but can sensitize as a result of metabolic activation in the skin. Eugenol would fall into this category. Many aliphatic amines may also fall into either this category or category 4 above.

5.2. The chemistry-potency applicability domain issue

Assignment of chemicals with logk_{max} >-2 to the 1A potency class and chemicals with logk_{max} <-2 as either 1B or NS is based on two implicit assumptions:

1. Molecular weight is not substantially different from 185, the average of the molecular weights of the chemicals in the Natsch et al. (2020) dataset. This is because the rate constant is defined in molar units and potency is defined in weight units.
2. Electrophilic reactivity is the only significant determinant of potency. This applies for all chemicals.

Regarding implicit Assumption 1, the rate constant k is expressed in molar units but the EC3 is defined as a weight percentage. Consequently, two chemicals with the same molecular potency will have different EC3 values if their molecular weights, M, are different. For a set of chemicals with a narrow molecular weight distribution, as seems to be the case with the Natsch et al. (2020) dataset, a threshold k value that works quite well may be derivable, but since industrial and biological chemicals span a wide range of molecular weights no threshold k value can be universally applicable. Low molecular weight chemicals will tend to be underestimated, high molecular weight chemicals will tend to be overestimated. The obvious conclusion is that using a k value in molar units as the threshold for 1A/1B classification is not a scientifically valid concept. This issue is most simply addressed, without need to redefine the 1A/1B threshold, by adding log(185/M) to the logk_{max} value.

Implicit Assumption 2, that electrophilic reactivity is the only significant determinant of potency, is not supported by the evidence from numerous structure–activity studies. For some frequently encountered types of chemicals, potency is not solely related to reactivity but to a combination of reactivity and hydrophobicity. The role of hydrophobicity, usually represented quantitatively by logP (octanol/water), in determining potency for some classes of chemicals is considered to reflect partitioning between lipid and aqueous environments in the skin, as discussed by Roberts and Aptula (2008). Hydrophobicity dependence applies to chemicals that react by the S_N2 mechanism, such as epoxides, sulfonate esters, and aliphatic halides, and to acyl transfer agents such as anhydrides. LLNA potency of aldehydes and ketones that can react as Schiff base electrophiles is also correlated with a combination of reactivity and hydrophobicity parameters (Aptula et al. 2006) although this has recently been challenged by Böhme et al. (2021) based on kinetic studies of Schiff base formation from a series of aliphatic aldehydes reacting with glycine-*para*-nitroanilide, in which not only the forward rate constants were determined but also the rate constants for the reverse reaction, and hence the equilibrium constant. It was found that the equilibrium constant alone, but not the forward rate constant alone, gives a good correlation with LLNA potency. This raises the question of whether logP in the original QSAR for Schiff base electrophiles serves as a surrogate for the reverse reaction, or whether the reverse reaction rate constant serves as a surrogate for logP. Whichever is the case, the experimental evidence indicates that implicit Assumption 2 does not apply for Schiff base electrophiles.

The hydrophobicity effect can be illustrated by two examples based on the kDPRA dataset of Natsch et al. (2020).

Methyl methanesulfonate has an EC3 of 8.1% and is correctly assigned 1B based on its logk_{max} value of -2.15. The homologue methyl dodecanesulfonate has not been tested in the kDPRA (it is likely that it would separate out of the reaction solution, but not necessarily before the 30 min peptide depletion measurement point) but its reactivity would not be greater than that of methyl methanesulfonate. Therefore, it would not be predicted 1A. However, it is a

strong sensitizer with an LLNA EC3 value of 0.39%, corresponding to the 1A classification.

Trimellitic anhydride and phthalic anhydride are acyl transfer agents. They give very similar results in the kDPRA: logk_{max} values are −0.13 and −0.07, respectively.[3] Based on these logk_{max} values they would both be classified as 1A. However, the EC3 values in the LLNA are very different: 9.2% for trimellitic anhydride and 0.16% for phthalic anhydride (Natsch et al. 2020). This large difference in potency is consistent with the difference in hydrophobicity, which corresponds to replacement of a hydrogen atom of phthalic anhydride by an ionized aromatic carboxylate group. The calculated difference in logP values is 4.36 (from the -H fragment value of 0.23 and $-CO_2^-$ fragment value of −4.13 given by Hansch and Leo 1979). In QSARs correlating pEC3 as a function of reactivity and logP, the logP coefficient is usually about 0.4 (Roberts et al. 2017 and references therein). Using this value, the difference in pEC3 between phthalic anhydride and trimellitic anhydride is estimated at about 1.7, corresponding to the EC3 values differing by a factor of about 70. This agrees quite well with the observed EC3 difference, a factor of 58.

5.3. Why are the performance statistics for the kDPRA as good as they are?

Based on the above considerations, it seems likely that many of the logk_{max} values in the Natsch et al. (2020) database do not truly represent the reactivity of the chemical and are not well correlated with sensitization potency. Nevertheless, for the database of 180 chemicals, the logk_{max} values show good performance in discriminating between the 1A and non-1A categories. Among the reasons why this is the case are:

1. Overlap of the two applicability domains means that many chemicals that owe their 1A potency partly to their hydrophobicity, and are thus outside the chemistry-potency applicability domain, do not get tested because they are outside the kinetics measurement applicability domain. If their logk_{max} values were to be determined (e.g. by working at lower concentrations, possibly with a different analytical method) or by using read-across from a different reaction system, these chemicals would be incorrectly classified as non-1A.
2. The performance statistics are based on a dataset that includes a substantial number of Schiff base electrophiles, most of which have 1B potency but do not react significantly in the kDPRA. Since the assay is only assessed for its performance in distinguishing between 1A sensitizers and compounds that are not 1A (i.e. either 1B or NS), it correctly predicts these as non-1A. Some Schiff base electrophiles have been found to give peptide depletion with cysteine peptide, but by oxidation of the peptide -SH groups rather than forming a stable adduct. The nature of this reaction does not appear to have been investigated. It appears to be very sensitive to minor fluctuations in the assay conditions, based on the quite large standard deviations on k_{max} with aldehydes in the ring trials and repeatability studies (Wareing et al. 2020).

For a more realistic assessment of the extent to which the kDPRA would be generally applicable, it would be useful to test its performance against a wider ranging dataset.

5.4. Preliminary assessment of applicability against a more diverse dataset

Gerberick et al. (2005) published details of LLNA studies, with full dose–response data, for a diverse range of over 200 chemicals, with the intention that, each study having been carefully evaluated by a panel of experts, it could serve as a Gold Standard database for, inter alia, developing and evaluating alternative approaches. This was followed up by a classification of the chemicals into their chemical reaction mechanistic domains (Roberts et al. 2007).

5.4.1. Michael acceptor and S_NAr domains

A total of 30 chemicals were assigned to the Michael acceptor or S_NAr reaction mechanistic domains. For these mechanistic domains, potency in the LLNA is dependent only on reactivity, so for these chemicals logk_{max} should be a reliable discriminator between 1A and 1B/NS and could be used for more precise prediction of potency, provided condition 2 (solubility) for the measurement applicability domain is met.

In total, 28 chemicals were assigned as pro- or pre-Michael acceptors. These are outside the applicability domains of the kDPRA. In many cases the kDPRA protocol would produce a logk_{max} value, but it would not represent the true reactivity of the test compound. A logk_{max} value greater than −2 would predict 1A potency with similar high confidence as for direct Michael acceptors, but a logk_{max} value below −2 would not be a reliable predictor of 1B.

5.4.2. S_N2 domain

In total, 42 chemicals were assigned to the S_N2 reaction mechanistic domain. All of these are outside the chemistry-potency applicability domain, since potency of S_N2 electrophiles is dependent on both reactivity and hydrophobicity. More than half of these chemicals would also be outside the kinetics measurement applicability domain due to their low solubility. For the S_N2 domain, a logk_{max} value greater than −2 would in most cases predict 1A potency with reasonable confidence (exceptions being hydrophilic chemicals), but a logk_{max} value below −2 would not be a reliable predictor of 1B/NS. In this dataset, eight of the S_N2 chemicals have EC3 < 2%, i.e. classified as 1A; these are summarized in Table 4. Four of these have been evaluated in the kDPRA with three correctly assigned as 1A and one incorrectly assigned as 1B. Three of the chemicals are, based on their high logP values, likely to be outside the kDPRA kinetics

[3]Although this is not stated in Natsch et al. (2020), the logk_{max} figure of -0.07 for phthalic anhydride is derived from peptide depletion values at 5 minutes (Wareing et al. 2017). The 30 minute depletion data as per the kDPRA protocol give a logk_{max} value of -0.86.

measurement applicability domain: if logk_{max} values could be determined for these compounds one (1-chloromethyl-pyrene) would be expected to be >-2, correctly predicting 1A, and two (methyl dodecanesulfonate and methyl hexadec-3-enesulfonate) would incorrectly be predicted 1B based on logk_{max} values <-2.

Although the S_N2 electrophiles are outside the chemistry-potency applicability domain, this does not mean that logk_{max} values cannot be used to predict potency for these compounds. The final four entries in Table 4 show the S_N2 electrophiles that are classified as 1B (i.e. EC3 > 2%) and have measured logk_{max} values. Thus, Table 4 contains all seven of the S_N2 electrophiles for which Natsch et al. (2020) list logk_{max} values. Figure 7(a) shows a plot of pEC3 against logk_{max} values. The correlation is rather poor, with an R^2 value of 0.768 and the regression equation:

$$\text{pEC3} = 0.76(\pm 0.19)\log k_{max} + 3.40(\pm 0.42) \quad (1)$$

$n = 7$, $R^2 = 0.768$, $s = 0.55$, $F = 16.6$

To take the contribution of hydrophobicity into account, Relative Alkylation Index (RAI) values derived by combining logk_{max} with logP can be used. RAI is calculated as logk_{max} + 0.4logP, in accordance with the relative dependence of sensitization potency on reactivity and logP (Roberts et al. 2016 and references therein). Figure 7(b) shows a plot of pEC3 against RAI values. The correlation is better than for Equation (1), with an R^2 value of 0.905, and the regression equation:

$$\text{pEC3} = 0.69(\pm 0.10)\text{RAI} + 2.69(\pm 0.17) \quad (2)$$

$n = 7$, $R^2 = 0.905$, $s = 0.35$, $F = 47.8$

Although the RAI-based Equation (2) clearly fits the data better than the logk_{max}-based Equation (1), there are some deviations from the line. This may reflect the fact that the dataset contains examples from three S_N2 sub-domains: H-polar, non-H-polar and epoxides. Nevertheless, from Equation (2), a threshold to discriminate 1A sensitizers can be derived, based on logk_{max}, logP and equivalent weight (EW; in most cases this is equal to the molecular weight, M, but in cases such as bisphenol A-digylcidyl ether, with n identical reactive groups, EW = M/n):

$$\text{Predicted 1A if } \log k_{max} + 0.4\ \text{logP} - 1.45\text{logEW} > -4.35$$

The expression logk_{max}+ 0.4 logP −1.45logEW is an RAI-based potency index (RAI-PI) specific to the S_N2 reaction mechanistic domain. As shown in the right-hand column of Table 4, the RAI-PI function correctly assigns all of the S_N2 sensitizers with logk_{max} values and correctly predicts the 1A potency of methyl dodecanesulfonate and methyl hexadec-3-enesulfonate.

A further four compounds from the Gerberick et al. (2005) database, assigned as special cases with the comment "… S_N2 reaction at the S-atom can be proposed" have been tested in the kDPRA. These are shown in Table 5. There seems to be no evidence of correlation between potency and logk_{max}, which suggests that possibly some of the compounds react by mechanisms other than S_N2 and/or give rise to more reactive species by oxidation. The high logk_{max} values observed in all four cases suggest that the cysteine-based peptide nucleophile used in the kDPRA is particularly highly reactive toward sulfur-based electrophilic centers.

5.4.3. Schiff base (SB) domain

In total, 40 chemicals in the Gerberick et al. (2005) database were assigned to the SB domain. These chemicals are aldehydes, activated ketones or (four cases) considered to be precursors of aldehydes. Although it is convenient to refer to this as the Schiff base domain, it is not known whether chemicals of this type sensitize via Schiff base formation (reacting with amino groups of lysine units) or via another mechanism such as reaction with ionized thiol groups of cysteine units to form hemithioacetal groups. Aldehydes tend to be more reactive with thiol nucleophiles than with amine nucleophiles (Lienhard and Jencks 1966), but in both cases, the reaction products are readily hydrolyzed under the dilute aqueous conditions of the DPRA and the kDPRA.

Table 4. S_N2 domain chemicals with 1A potency in Gerberick et al. (2005) database.

Name	logP	EC3 (%)	kDPRA assignment and logk_{max}	Comment	RAI-PI[a] value and assignment
1-Chloromethyl-pyrene	5.4	0.005	ND	Probably too insoluble for kDPRA; if testable, would give 1A	
4-Nitrobenzyl bromide	2.7	0.05	1A, −0.01		−2.33, 1A
Propiolactone	−0.2	0.15	ND	kDPRA would give 1A	
Dimethyl sulfate	−0.3	0.19	ND	kDPRA would give 1A	
Benzyl bromide	2.9	0.20	1A, −0.43		−2.51, 1A
Methyl dodecanesulfonate	5.2	0.39	ND	Probably too insoluble for kDPRA; if testable, would give 1B[a]	−3.46[b], 1A
Methyl hexadec-3-enesulfonate[c]	6.8	0.80	ND	Probably too insoluble for kDPRA; if testable, would give 1B[a]	−2.95, 1A
Bisphenol A-digylcidyl ether	4.1	1.5	1B, −2.53	Incorrect assignment by kDPRA due to neglect of logP	−4.19, 1 A
1B chemicals in Gerberick et al. (2005) with logk_{max} values in Natsch et al. (2020)					
Diethyl sulfate	1.1	3.3	−2.60		−5.34, 1B
(2-Bromoethyl)benzene	3.1	6.2	−2.53		−5.12, 1B
Methyl methanesulfonate	−0.40	8.1	−2.15		−5.30, 1B
Butyl glycidyl ether	−0.49	31	−2.73		−5.30, 1B

[a]Relative Alkylation Index based Potency Index (see text).
[b]Based on the kDPRA result with the less hydrophobic homologue methyl methanesulfonate (see above, Section 5.2).
[c]Incorrectly named as methyl hexadec-1-ene sulfonate in Gerberick et al. (2005).

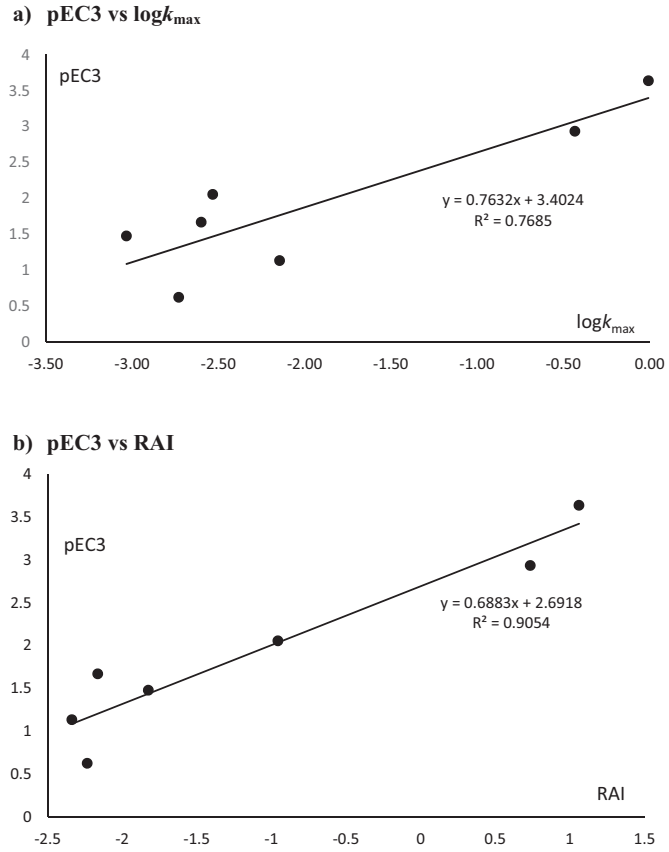


Figure 7. Regression plots for S_N2 electrophiles. (a) pEC3 versus logk_{max}. (b) pEC3 versus RAI.

Consequently, as stated in Section 5.1, chemicals in the SB mechanistic domain are outside the kDPRA kinetics measurement applicability domain. They are also outside the chemistry-potency applicability domain, since for SB electrophiles potency is related not solely to reactivity but also to hydrophobicity. Nevertheless, the DPRA performs reasonably well, although with several false predictions, in distinguishing between sensitizing and non-sensitizing aldehydes and ketones on the basis of cysteine peptide depletion by oxidation.

In the Gerberick et al. (2005) database, 4 of the 40 SB domain chemicals have EC3 values below 2%, corresponding to the 1A classification. These four chemicals are shown in Table 6.

It may be noted that although formaldehyde is classed as a strong (1A) sensitizer, this is more because of its low molecular weight than because of its chemistry. On a molar basis (EC3 = 0.20 M), it is slightly less potent than anethole (EC3 = 2.3%, 0.16 M) and 2-decenal (EC3 = 2.5%, 0.16 M) which are both classed as 1B sensitizers (data from Natsch et al. 2020). Glutaraldehyde, although it is the strongest sensitizer of the 4, shows only marginal peptide depletion in the original DPRA and no significant reaction in the kDPRA. There is evidence that its action as a protein fixative agent involves cross-linking via further reaction of initial lysine adducts forming pyridinium units (Hardy et al. 1979), and the same mechanism probably applies to its action as a skin sensitizer. Similarly glyoxal, although 1A based on its weight percent EC3, has the same molar potency as methyl pyruvate, a 1B sensitizer with EC3 = 2.4%, 0.24 M (data from Natsch et al. 2020).

Overall, for the Schiff base domain, a logk_{max} value greater than −2 would in most cases predict 1A potency with reasonable confidence, but a logk_{max} value below −2 would not be a reliable predictor of 1B/NS.

5.4.4. Acyl transfer domain

In total, 26 chemicals in the Gerberick et al. (2005) database were assigned to the acyl transfer domain. However, some of these assignments are questionable and in light of subsequent findings should be classified differently. For example, 2-mercaptobenzothiazole has, on paper, at least three possibilities for protein binding (Figure 8).

Although it was originally assumed that the (thio)acyl pathway applies, the pro-/pre-S_N2 pathway now seems more likely. Chipinda et al. (2007) synthesized the dimeric oxidation product and found it to be cross-reactive with 2-mercaptobenzothiazole. The observed LLNA potency of 2-mercaptobenzothiazole shows unusually large vehicle effects, possibly reflecting differences in the proportions of the two tautomers. The NICEATM database of LLNA studies indicates that 2-mercaptobenzothiazole has a higher EC3 value when tested in AOO (mean EC3 = 9.8%) compared with DMF (mean EC3 = 2.5%) (ICCVAM 2008). Its reported logk_{max} value as determined by the kDPRA protocol is −0.15 (Natsch et al. 2020), which would suggest a higher potency than the LLNA data indicate. This adds weight to the suggestion in Section 5.4.2 that the cysteine-based peptide nucleophile used in the kDPRA is particularly highly reactive toward sulfur-based electrophilic centers.

Apart from 2-mercaptobenzothiazole, eight of the chemicals assigned to the acyl transfer domain had EC3 values ≤ 2, corresponding to 1A. Only one of these appear with a logk_{max} value in Natsch et al. (2020): this is oxazolone, with a logk_{max} value of −0.14, which correctly predicts it as a 1A sensitizer. The reaction chemistry of oxazolone is quite complex, and although its ultimate reaction products are acyl transfer derivatives, the initial reactions are probably by Michael addition (Natsch et al. 2010).

Overall, for the acyl transfer domain, a logk_{max} value greater than −2 would in most cases predict 1A potency with reasonable confidence (exceptions being hydrophilic chemicals such as trimellitic anhydride), but a logk_{max} value below −2 would not be a reliable predictor of 1B/NS.

5.4.5. Special cases

In total, 12 chemicals were assigned as special cases, on the basis that they were considered likely to be reactive or likely to be converted to reactive derivatives but could not be assigned confidently to one of the major reaction mechanistic domains. Four of these 12 are now recognized as likely to act by S_N2 attack at sulfur and are already listed as such in Table 5, together with their logk_{max} values reported by Natsch et al. (2020). No logk_{max} values have been reported for the other eight chemicals. Seven of these eight chemicals

Table 5. Sensitizers classified as acting by S_N2 at sulfur.

Name	logP	EC3 (%)	Experimental kDPRA assignment	Comment
5-Chloro-2-methyl-4-isothiaolin-3-one	0.9	0.009	1A (correct)	$\log k_{max} = 0.60$
2-Methyl-2*H*-isothiazolin-3-one	0	0.4[a]	1A (correct)	$\log k_{max} = -0.25$
1,2-Benziso-thiazolin-3-one	1.3	2.3	1A (incorrect, marginally)	$\log k_{max} = -0.12$
Tetramethylthiuram disulfide	1.7	5.2	1A (incorrect)	$\log k_{max} = 0.74$

[a]EC3 incorrectly given as 1.9% in Gerberick et al. (2005). This error resulted from it not being recognized that the material tested was a 20% solution (Roberts 2013).

Table 6. Schiff base domain sensitizers with 1A potency.

Chemical	MW	% Depletion in original DPRA (24 h)	$\text{Log}k_{max}$, (l. mol^{-1} s^{-1})	$\text{Log}k_{max}$, weight units (l. g^{-1} s^{-1})	EC3 (weight %)
Formaldehyde	30	41.3	−0.67	−2.15	0.61
Glutaraldehyde	100	13.8	Not reactive		0.10
1-Phenyl-1-2-propanedione	148	61.0	−1.18	−3.35	1.3
Glyoxal	58	70.1	−1.97	−3.81	1.4

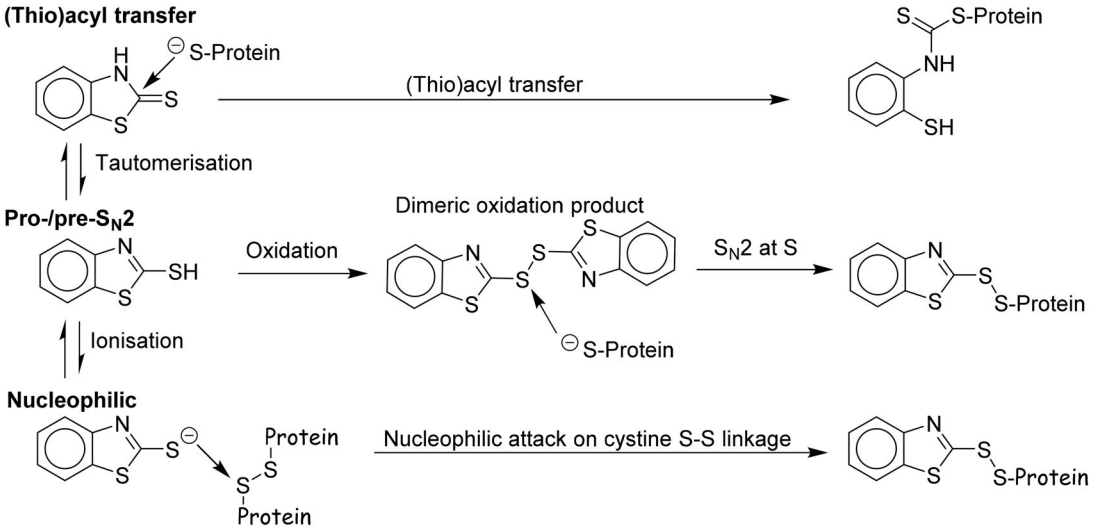


Figure 8. Possible protein binding pathways for 2-mercaptobenzothiazole.

have EC3 values < 2 and would be classed as 1A sensitizers, the exception being clotrimazole with an EC3 value of 4.8%. Three of these chemicals with EC3 < 2 are N-nitroso derivatives and would be expected to show high reactivity in the kDPRA. The remaining four would probably not show high reactivity in the kDPRA: two are polycyclic aromatic hydrocarbons, one is inorganic (potassium dichromate) and one (1-naphthol) has only low reactivity in the conventional DPRA (Gerberick et al. 2009).

5.4.6. Neither reactive nor pro-reactive domain

In total, 32 chemicals were classified as neither reactive nor pro-reactive. Strictly, "non-reactive" in this context means "not considered to be reactive enough to sensitize." For example sodium lauryl sulfate (SLS), although it has a history of use in the chemical industry as an S_N2 electrophile in reactions carried out at $>100\,^{\circ}C$, is considered to be non-reactive in the context of skin sensitization. Included in this list are "slow prehaptens" such as limonene and linalool, that are regarded as non-sensitizing but liable, depending on their history of air exposure, to contain varying amounts of oxidation-derived allergenic impurities. Two of the 32 chemicals deserve further comment:

Abietic acid has an EC3 value of 15%. It has slow pre-hapten alerts (tertiary allylic hydrogen potentially able to be oxidized to tertiary allylic hydroperoxide), but unlike other slow prehaptens its EC3 values from multiple testing do not show wide variation (Roberts et al. 2016). Tested in the kDPRA, its $\log k_{max}$ value of −0.55 is unexpectedly high and on that basis it would be predicted incorrectly as a 1A sensitizer (Natsch et al. 2020). In addition to the slow pre-hapten alerts mentioned above abietic acid also has an alert – conjugated diene with one or both of the double bonds in a ring (Bergström et al. 2006) – for activation to a reactive epoxide. It seems possible that rapid formation of this epoxide by oxidation under kDPRA conditions could account for the high $\log k_{max}$ value, and its formation under LLNA conditions could account for the EC3 value. Both abietic acid and its epoxide would have low hydrophobicity, because of the carboxyl group that is ionized at skin pH, so the potency would be lower than the $\log k_{max}$ value alone would predict.

Table 7. kDPRA-based predictions for 1A sensitizers in Gerberick et al. (2005).

Reaction mechanistic domain and number of 1A in that domain	kDPRA data available, prediction based on $\log k_{max}$		kDPRA data not available, prediction based on inferred $\log k_{max}$	
	Correct, 1A	Incorrect, non-1A	Correct, 1A	Incorrect, non-1A
Direct MA, 5 and S_NAr, 2	4		3[a]	
Pre- or pro-MA, 14	7	2[b]		
S_N2, 8	2	1[c]	2[d]	3[e]
SB, 4	3	1[f]		
Acyl, 9[g]	2[h]	0		5[i]
Special cases, 9	2	0	4[j]	3[k]
Totals	20	4	9	11

[a]Two of these compounds are Michael acceptors: 3-methyl-4-phenyl-1,2,5-thiadiazole-1,1-dioxide (CAS 3775-21-1) and 5,5-dimethyl-3-methylene-dihydro-2(3*H*)furanone (CAS 29043-97-8). There is nothing to suggest that either of these compounds would be outside the kDPRA kinetics measurement applicability domain. The third compound is 2,4,6-trichloro-1,3,5-triazine (CAS 107-77-0) an S_NAr electrophile, also predicted to be within the kDPRA kinetics measurement applicability domain.

[b]These two compounds are paraphenylenediamine (CAS 106-50-3) and 2-aminophenol (CAS 95-55-6). See discussion in Section 4.2.

[c]This compound is bisphenol A-digylcidyl ether, outside the chemistry-potency applicability domain and incorrectly predicted non-1A. See section 5.4.2.

[d]These two compounds are propiolactone and dimethyl sulfate which would both be within the kDPRA kinetics measurement applicability domain and can confidently be predicted to be reactive enough to give $\log k_{max} > -2.0$.

[e]These three compounds are: 1-chloromethylpyrene – although highly reactive this compound is very hydrophobic (logP, 4.89) and would probably not remain in solution sufficiently long for its high reactivity to be detected under kDPRA conditions; methyl dodecanesulfonate and methyl hexadecenesulfonate – these compounds have 1A potency, despite their predicted $\log k_{max}$ values (if measurable under kDPRA conditions) being < -2.0, because of the contribution from their hydrophobicity. See Section 5.4.2.

[f]This compound is glutaraldehyde. See Section 5.4.3.

[g]For some of these compounds, it is not possible to make any confident inference about $\log k_{max}$ values.

[h]These two compounds are oxazolone (CAS 15646-46-5) and 2-mercaptobenzothiazole (CAS 149-30-4). Although originally classed as acyl transfer agents, other mechanisms are now thought to apply. See Section 5.4.4.

[i]Four of the 1A compounds in this domain would be expected to react rapidly with the kDPRA nucleophile to give thioesters which would be hydrolyzed with regeneration of the nucleophile. These compounds are: 2-methyl-4*H*,3,1-benzoxazin-1-one (CAS 525-76-8); C6-azlactone (CAS 176665-02-4) nonanoyl chloride (CAS 764-85-2) and C6-azlactone (CAS 176664-99-6). A fifth compound, listed under the name methyl 2-sulfophenyl octadecenoate was classified as an acyl transfer agent on the basis of an incorrect name and structure in Gerberick et al. (2005). The correct structure is $C_{16}H_{33}CH(CO_2Me)SO_3Me$ (dimethyl sulfostearate), and it is an S_N2 electrophile. It may be too insoluble to give a $\log k_{max}$ value in the kDPRA, but its predicted $\log k_{max}$ value of -2.15 (see Footnote e and Section 5.4.2) would classify it as non-1A.

[j]One of these four compounds is potassium dichromate, an inorganic powerful oxidizing agent that would deplete the peptide by oxidation. The other three compounds are highly electrophilic N-nitroso derivatives.

[k]These three compounds are benz[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene and 1-naphthol, which are commonly believed to be non-reactive, requiring metabolic activation to produce toxic effects.

Oxalic acid has an EC3 value of 15% and its $\log k_{max}$ value was found to be -1.01, predicting it as a 1A sensitizer (Natsch et al. 2020). It has no alerts for reactivity and it is regarded as a false positive in the LLNA (Montelius et al. 1998). However, there seems to be no obvious explanation for the high $\log k_{max}$ value in the kDPRA.

The remaining 30 chemicals are all either negative or weakly positive (EC3 > 10%) in the LLNA. With the probable exception of isopropyl myristate, all of these chemicals should be sufficiently soluble to be tested in the kDPRA and would be expected to be correctly assigned as non-1A.

5.4.7. Overall assessment of the kDPRA against 1A sensitizers in Gerberick et al. (2005)

The Gerberick et al. (2005) dataset contains a total of 51 chemicals with EC3 values of 2% or less. These would now be classed as 1A sensitizers. For 24 of these 51 1A chemicals, $\log k_{max}$ values determined by the kDPRA method are reported by Natsch et al. (2020). Table 7 summarizes, for each reaction mechanistic domain, the predictive performance of the $\log k_{max}$ values for the 24 1A sensitizers with kDPRA data. For those 1A chemicals that do not have experimental $\log k_{max}$ values the hypothetical performance, based on physico-chemical considerations, is given and the underlying reasoning is summarized in the footnotes.

From the totals in Table 7, of the 24 1A chemicals in the Gerberick et al. (2005) dataset for which $\log k_{max}$ values have been reported, 20 are correctly predicted, i.e. 83%. For the full total of 51 1A chemicals, in the dataset, it is estimated that 29 would be correctly predicted, i.e. 57%.

6. Conclusions and suggestions for further refinement of the kDPRA

The kDPRA represents a major advance toward predicting skin sensitization potency without animal testing. However, there is scope for improvement. As it currently stands its performance in assigning chemicals to the 1A or non-1A potency classes is quite good, although there are some groups of chemicals for which it is not applicable. For example, many chemicals encountered in surfactant and oleochemical technology, because they are likely to be too hydrophobic to be testable in the aqueous conditions of the kDPRA; many chemicals useful in hair coloring products, because they are not directly electrophilic and cannot be assumed to be activated under kDPRA conditions in the same way and to the same degree as they are activated in the skin sensitization process.

There is also the danger that, particularly since the kDPRA has been adopted as an OECD test guideline, $\log k_{max}$ values determined by the current kDPRA protocol will come to be seen as "gold standard" reactivity parameters in preference to true rate constants (or their logarithms) determined by more rigorous conventional kinetic studies. The issue is not that kDPRA $\log k_{max}$ values are less accurate than they could

be, but rather that in some cases the reaction chemistry is not consistent with the mathematical analysis of the data matrix and consequently the derived k_{max} value is not truly a rate constant at all.

A major limitation of the current kDPRA protocol is that it does not indicate whether the k_{max} value truly represents the reactivity of the chemical *per se* or whether it reflects the presence of a reactive impurity. As discussed by Roberts and Basketter (2009), substances are not always pure, and the same substance (nominally) may vary in its levels of impurities. Consequently a chemical that *per se* is only a weak sensitizer and is only weakly reactive, may, depending on the manufacturing conditions, contain enough of a reactive impurity to classify as 1A by the present kDPRA protocol even though the impurity would not in reality lead to an EC3 value <2% if the material were to be tested in the LLNA (as demonstrated by the illustrated example with Azurone in Section 4.1). The kDPRA protocol as it stands could lead to situations where the regulatory status of substance X depends on how pure or impure the first tested sample was. Modifying the protocol for analysis of the data matrix by adopting the conventional kinetics approach of plotting ln(100-DP) versus *t* would enable the true rate constant of the chemical *per se* to be determined and would reveal the presence of reactive impurities that could be dealt with and controlled by appropriate manufacturing and quality control specifications.

As a general rule, one can have more confidence in a 1A prediction than in a non-1A prediction. False 1A predictions can arise due to reactive impurities (even if these are not present at levels sufficient to make the sample have an EC3 < 2%), but false predictions of this type can be avoided if the modified version of the kDPRA with data analysis by a conventional kinetics protocol is adopted. At this point, it is appropriate to state precisely what is meant by "false 1A prediction" in this context. Consider a substance that registers 1A in the kDPRA by giving a logk_{max} value > -2, where most of the potency comes from a highly reactive and highly potent impurity. The kDPRA result is false 1A if it would give an LLNA EC3 > 2% if tested, and true 1A if it would give an LLNA EC3 < 2%. The LLNA result that the substance would give, if tested, is determined by the potency of the impurity and how much is present. The logk_{max} value in such cases does not model either of these. As illustrated with azurone, a reactive impurity at levels too low to give a 1A LLNA result can give a 1A kDPRA result.

Another potential source of false 1A predictions is high reactivity together with strong hydrophilicity for a chemical belonging to a reaction mechanistic domain where potency is partly dependent on hydrophobicity. Trimellitic anhydride is the only example encountered in the Natsch et al. (2020) database.

Although the logk_{max} values are not always an accurate representation of the true reactivity, partly as a result of impurity effects not being detected by the existing protocol, the KDPRA is in many cases capable of giving reactivity values good enough to enable quantitative modeling of potency, as illustrated by the QSAR for S_N2 chemicals developed in Section 5.4.2.

In determining properties of chemicals, it is important to know whether the property that is being measured is that of the chemical *per se* or that of impurities that it may contain. As it stands, the kDPRA does not distinguish between these two possibilities.

To address some of the limitations and extend the scope of the kDPRA some simple refinements to the protocols are suggested.

6.1. The experimental protocol

6.1.1. Replace the current geometric progression of five concentrations ranging from 5 mM down to 0.3125 mM by an arithmetic, or approximately arithmetic, progression of four concentrations: 5, 3.5, 2, and 0.5 mM. This modification gives equal weight to the data from all concentrations rather the highest concentration data having greater leverage in regression equations as under the current protocol. See comment 1 to Table 1. This does not invalidate the logk_{max} values that have already been determined using geometric progression.

6.1.2. Add an extra time point, as short as practicable, before the 30 min. This enables more information to be gathered on reactive impurities, if present.

An incidental benefit of modifications 6.1.1 and 6.1.2 together is that the number of analyses per assay is reduced by 4% (from 25 to 24).

6.2. The data analysis protocol to derive the rate constant

First plot ln(100-DP) versus *t* for each initial concentration [E] of test chemical then, if these plots meet linearity criteria, plot the slopes against [E]. The slope of this plot is the second order rate constant *k*. This is more in line with conventional practice in chemical kinetics and has the benefit of more easily detecting and interpreting deviations from ideal second-order behavior. In particular, involvement of reactive impurities is more straightforward to detect and interpret.

A simpler, though less accurate, version of this modification would be:

Plot ln(100-DP) versus *t* (measured points only, not $t = 0$) for each [E] and find slopes. Divide each slope by its corresponding [E] and take the largest value as k_{max}. This is the rate constant for the compound per se, and within normal error limits the estimate is independent of whether or not a more reactive impurity is present. In this way, *k*(compound X per se) can be determined even if the chemical contains a more reactive impurity.

Taking it further, it can be checked whether the intercept of the 5 mM plot is < ln(100), and if it is an estimate can be made as to how much reactive impurity is present and a minimum value for its rate constant can be derived.

6.3. Predicting potency

For assignment of 1A potency, the current protocol uses $\log k_{max} > -2$ as the criterion for 1A. $\log k_{max}$ is currently defined as the largest value obtained when slopes of ln(100-DP) versus [E] are divided by their corresponding t values. There is no practical reason why this should not continue, although it should be born in mind that the $\log k_{max}$ value is a property of the sample tested rather than an intrinsic property of the chemical per se. It is recommended that if use of $\log k_{max}$ is to be continued the criterion for 1A should be $\log k_{max} - \log(M/185) > -2$. This corrects the anomaly that the k_{max} value is a molar quantity whereas the EC3 threshold of <2% for 1A is based on weight percentage, and extends the applicability of $\log k_{max}$ to a wider range of molecular weights.

For deriving NESILs required for QRA (Api et al. 2008; Basketter and Safford 2016) and development of structure–activity relationships prediction of potency on a continuous scale is required. For this the second order rate constant k, determined as summarized in Section 6.2 above, is the more appropriate parameter. However, confident prediction of potency based on kDPRA data also requires that the chemical be assigned to its reaction mechanistic domain. In many cases, this is obvious (to an organic chemist or to an expert system trained on mechanistic organic chemistry input), but not always. With the reaction mechanistic domain assigned, it can be judged whether the potency can be predicted based on k alone (e.g. using the quantitative mechanistic models (QMMs) of Roberts and Natsch (2009) and Natsch et al. (2011) for the Michael acceptor and S_NAr domains respectively), whether k needs to be used in combination with another parameter, such as logP in a QMM of the type derived in Section 5.4.2 for the S_N2 domain, or whether potency cannot be predicted from kDPRA data.

6.4. Extending the applicability domain of the kDPRA

Two of the main limitations of the kDPRA protocol, as with the original DPRA, are that being based on reaction in aqueous solution, it is unsuitable for many hydrophobic chemicals that are not sufficiently soluble to be tested in the kDPRA and it is unsuitable for chemicals that are hydrolytically unstable or whose peptide adducts are hydrolytically unstable. This illustrates a fundamental dissonance between, on the one hand, the perceived need for assays with rigorously defined protocols and, on the other hand, the range and diversity of the chemistry underlying skin sensitization, which requires flexibility in how chemical properties are determined to enable the best estimates of skin sensitization potency. Allowing this flexibility, a well-established physical organic chemistry approach can be applied to deal with chemicals that are outside the kinetics measurement applicability domain for reasons of low aqueous solubility or hydrolytic instability of either the chemical or its peptide adduct: choose an alternative system with a sulfur-based nucleophile soluble in an organic solvent in which the test compound is soluble; determine the rate constants for several chemicals in the same reaction mechanistic domain that are soluble both in the kDPRA system and in the organic solvent system, and plot $\log k$(kDPRA) versus logk(alternative nucleophile/organic solvent) to establish the A and B values for a linear free-energy relationship of the form:

$$\log k(\text{kDPRA}) = \text{A } \log k(\text{alternative system}) + \text{B}$$

This equation can then be used to convert the measured $\log k$(alternative system) value for the water-insoluble or hydrolytically unstable chemical to a $\log k$(kDPRA) value.

A cruder version of this approach would be to determine rate constants in the alternative system (AS) for only two compounds: the test chemical X and a chemical Y from the same reaction mechanistic domain that has a known rate constant in the kDPRA and to calculate:

$$\log k(\text{X, kDPRA}) = \log\{k(\text{X, AS}) \times k(\text{Y, kDPRA}) / k(\text{Y, AS})\}$$

In this way, chemicals that are incompatible with an aqueous reaction medium can be brought into the applicability domain of the kDPRA. This still leaves outside the applicability domain chemicals that are not electrophilic but can sensitize either by being activated to electrophilic derivatives or by a non-electrophilic mechanism. In general for such compounds, a 1A result in the kDPRA could be accepted with high confidence but a non-1A result could not be taken as reliable. Structural alerts for non-electrophilic sensitizers can be applied to assess whether a given chemical is of this type.

6.5. A final recommendation

Although the kDPRA experimental protocol can be improved as outlined in Section 6.1 above, the data already generated by the original protocol can provide a rich source of information beyond simply providing the $\log k_{max}$ value. However, the $\log k_{max}$ values listed in the database provided by Natsch et al. (2020) are in many cases not truly representative of the chemical's reactivity and these cases are not immediately identifiable from the database. It would be inadvisable to use them as they stand for any deeper analysis such as structure-potency modeling, or correlation with results from other non-animal assays. It is therefore recommended that the existing full data matrices be made available, enabling more detailed kinetic analysis as discussed in Section 6.2. which can, as well as providing reliable reactivity information, lead to further insights into mechanisms and structure–activity relationships.

Acknowledgments

The author is grateful to two anonymous reviewers selected by the Editor-in-Chief. Their comments were helpful and constructive and improved the quality of this paper.

Declaration of interest

The author reports no conflict of interest and has not during the past 5 years been involved in any regulatory, legal or advocacy activities. This review is the outcome of the author's longstanding interests in chemical reaction kinetics and toxicological chemistry. Its writing was not part of any formal project and received no funding from any entity.

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