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REVIEW ARTICLE

Peptide reactivity assays for skin sensitisation – scope and limitations

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ABSTRACT

The direct peptide reactivity assay (DPRA) is an OECD test guideline method that aims to determine if a chemical is reactive enough to be a skin sensitiser. It involves incubation of the test chemical at 5 mMolar concentration for 24 h with a cysteine-based peptide at 0.5 mMolar concentration and measurement of the percentage depletion (DP) of the peptide. The kinetic direct peptide reactivity assay (kDPRA) is derived from the DPRA and involves incubating the peptide with the test chemical at a range of concentrations and incubation times to produce a data matrix of DP values, which is analysed to give a reactivity parameter $\log k_{max}$ that assigns chemicals to the 1A potency class (high potency) if $\log k_{max}$ reaches the threshold value of −2. Here the DPRA, with a threshold of 47% DP, is compared against the kDPRA for their abilities to distinguish between the 1A and non-1A potency classes. It is found that they perform very similarly against a dataset of 157 chemicals with known potency, with only marginal differences in predictive performance. The thresholds of −2.0 (kDPRA) and 47% DP (DPRA) to distinguish 1A sensitisers are not scientific absolutes but the best compromises for a heterogenous set of data containing classes of chemicals for which different thresholds would be applicable. It is concluded that although the kDPRA represents a major advance towards predicting skin sensitisation potency on a continuous basis without animal testing, it offers no significant advantage over the DPRA for the purpose of 1A classification.

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1. Introduction

The kinetic direct peptide reactivity assay (kDPRA) was adopted in 2021 as an OECD test guideline to decide whether or not a chemical should be assigned to the 1A potency class for skin sensitisation on the basis of its electrophilic reactivity (OECD 2021a). It is derived from the direct peptide reactivity assay (DPRA), which is used, together with other information, to assess whether or not a chemical should be assigned as a skin sensitiser (OECD 2021a). The 1A potency class is defined in terms of the mouse local lymph node assay (LLNA) which was developed in the 1990s as an *in vivo* test for skin sensitisation hazard identification and characterisation (Dearman et al. 1999; Basketter et al. 2007; OECD 2010). Potency is quantified in terms of the EC3 value, this being the concentration of test chemical that, when applied under the LLNA protocol, would give rise to a three-fold increase in thymidine uptake in the local lymph node. Chemicals with an EC3 value of 2% or less are classed as 1A (UNECE 2021).

In the DPRA (OECD 2021a), the test chemical at 5 mMolar concentration is incubated for 24 h with a peptide, containing a cysteine unit with a reactive thiol group, at 0.5 mMolar concentration. The percentage depletion of the peptide at 24 h is determined by HPLC analysis and is designated the DP value. If the DP value is 13.89% or above, the chemical is predicted to be a skin sensitiser (S), otherwise it is predicted to be a non-sensitiser. A similar assay can be carried out with a peptide containing a lysine unit, providing a reactive amino group, and if the average DP value from the cysteine and

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lysine assays is 6.38% or above the chemical is predicted to be a sensitiser. The DPRA prediction is combined with the predictions from two *in vitro* cell-based assays to give an overall sensitiser/non-sensitiser prediction (sensitisation hazard identification) on a majority voting basis in the 2-out-of-3 defined approach (OECD 2021b).

The kDPRA (Natsch et al. 2020; OECD 2021a) is run similarly to the DPRA, but using a different method (fluorescence readout) to determine DP values. Only the cysteine peptide is used, and DP values are determined for 5 different test chemical concentrations and with 5 incubation times. The resulting data matrix is mathematically analysed to give the reactivity parameter $\log k_{max}$. The chemical is then assigned to the 1A or the 1B/NC potency class, depending whether $\log k_{max}$ is greater than or less than, respectively, -2. The 1A classification corresponds to an EC3 value of 2% or less in the murine local lymph node assay (LLNA). Chemicals with an EC3 value above 2% are classified as 1B/NC and chemicals that are not positive in the LLNA are not classified (NC). The kDPRA is not intended to distinguish between 1B and NC. Because of applicability domain issues and because the presence of low levels of reactive impurities can have a disproportionate effect, the $\log k_{max}$ values determined by the current kDPRA protocol do not in all cases truly represent reactivity and cannot always be relied on to predict sensitisation potency (Roberts 2021a). Nevertheless, as it currently stands the reported (Natsch et al. 2020) performance in assigning chemicals to the 1A or non-1A potency classes was good enough for the kDPRA to be adopted as an OECD test guideline, although there are some groups of chemicals for which it is not applicable, as discussed previously (Roberts 2021a) and in Section 5 of this paper. Throughout the rest of this paper, $\log k_{max}$ is used to refer to the kDPRA parameter resulting from the current kDPRA protocol and $\log k$ is used to refer to the logarithm of the true second-order rate constant for reaction of the peptide with the test chemical. Similarly with k_{max} and k. In many cases k_{max} and k are identical, but in many cases they are not.

The kDPRA protocol includes the generation of a data point at 24 h with 5 mM of test compound, so in that respect it differs from the cysteine-based DPRA (DPRA-cys) only in the analytical method. It is therefore relevant to question whether the DPRA-cys could be used rather than the kDPRA for assignment of 1A potency, in addition to its role in sensitisation hazard identification. This possibility is explored in the present paper.

2. Methods

As a supplement to their paper on the kDPRA Natsch et al. (2020) provide a list of 180 chemicals with their LLNA EC3 values, $\log k_{max}$ values and, in most cases, DP values for the DPRA (both cysteine-based and lysine-based). From this dataset all 157 chemicals with LLNA data and both DPRA-cys data and kDPRA data were selected and the data were analysed as follows:

Where the LLNA entry was NC or $>$X (X being a number greater than 2, typically 25 or 50) a logEC3 value of 2, corresponding to EC3 = 100%, was arbitrarily assigned. Where the $\log k_{max}$ entry was given as "not reactive" a $\log k_{max}$ value of -4 was arbitrarily assigned, this being the first negative whole number lower than the lowest $\log k_{max}$ value reported by Natsch et al. (2020).

A plot of logEC3 vs DP (cysteine-peptide) was made for the cysteine data, and based on visual inspection of this plot a DP value of 47% was chosen as the cut-off value to assess the performance of the DPRA-cys on the basis:

$$\text{If } \mathrm{DP(cys)} > 47\%, \text{ predicted 1A.}$$

$$\text{If } \mathrm{DP(cys)} < 47\%, \text{ predicted 1B/NC.}$$

The same procedure was followed with the kDPRA data, on the basis:

$$\text{If } \log k_{max} > -2, \text{ predicted 1A. If } \log k_{max} < -2, \text{ predicted 1B/NC.}$$

Then comparing with the EC3 values the DPRA and kDPRA predictions were categorised as True1A, False 1A, True 1B/NC or False 1B/NC.

3. Results and discussion

Figure 1(a) shows a plot of EC3 (logarithmic) against % depletion (DP) in the cysteine-only DPRA, referred to from here on as DPRA-cys. For comparison Figure 1(b) shows a plot of EC3 (logarithmic) against $\log k_{max}$ values determined for the same set of 157 chemicals in the kDPRA.

It may be noted that there is a discrepancy between the kDPRA threshold of -2.0 for $\log k_{max}$ and the DPRA-cys threshold of 47% DP applied above. It is easily calculated that a $\log k$ value of -2.0 corresponds to a 24 h DP value of 97%, and indeed it can be seen by visual inspection of Figure 1(a) that a cut-off DP value above 90% would give a better separation between the two potency classes, with the number of false 1A that would become true 1B/NC being larger than the number of true 1A that would become false 1B/NC. This is true for this particular set of data, but a cut-off in this range would not be reliable for new data. This is because differences in DP values in the 90–100% range cannot be treated as meaningful. Bearing in mind the error limits of DP measurement and the fact that even a very fast reaction can stop short of 100% DP (e.g. because it reaches an equilibrium position, or because the peptide adduct is hydrolysed) it cannot be confidently assumed that a chemical giving say 92% DP is less reactive than a chemical giving 98% DP.

Although the distributions of datapoints in Figure 1(a,b) are not identical, the two assays appear very similar in the extent to which they discriminate between the potency classes. This is put on a more quantitative basis by the analysis summarised in Table 1, which compares the predictive performance of the DPRA-cys and the kDPRA in potency classification of the same set of LLNA data. For further comparison, Table 1 also shows the performance data for the kDPRA (-2 $\log k_{max}$ cut-off) and the 5 mM 24 h kDPRA DP value against the full data set of 180 chemicals

The differences in terms of predictive performance between the DPRA-cys and the kDPRA are marginal. The

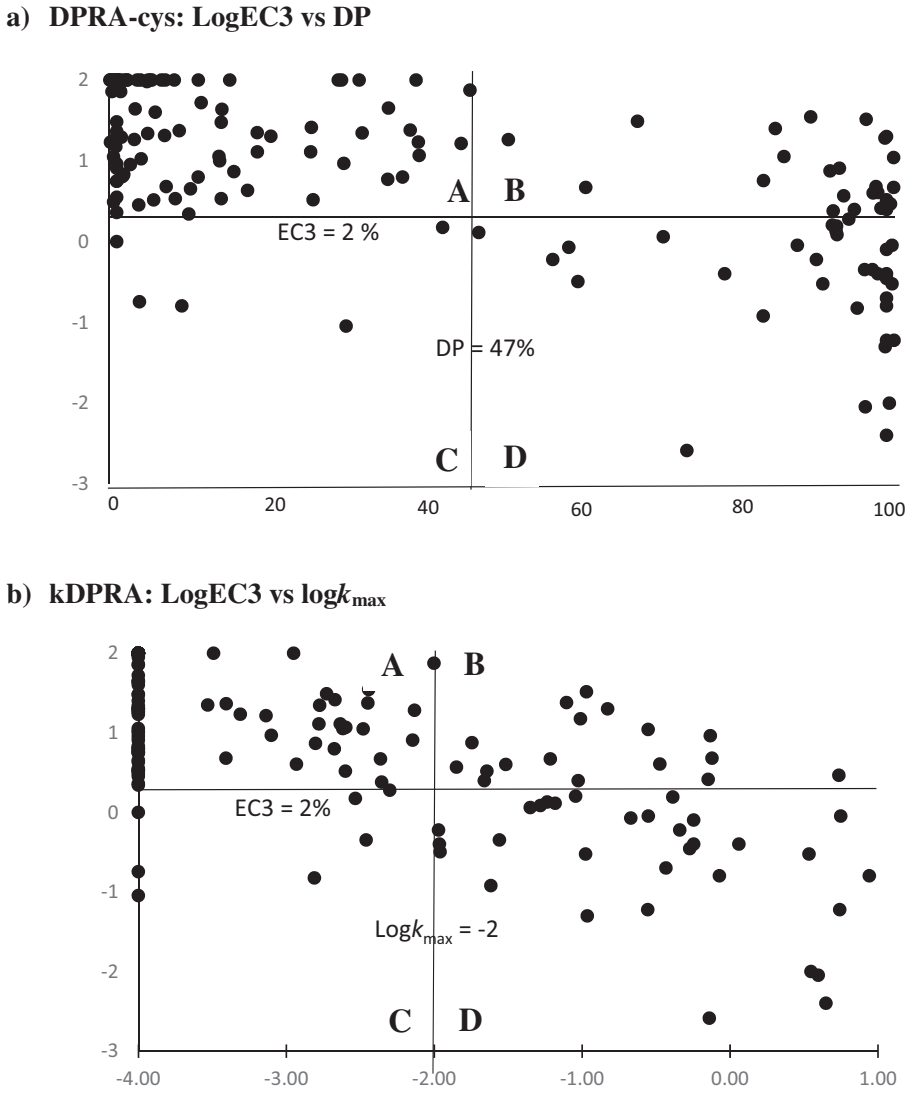

Figure 1. Discrimination between 1A and 1B/NC based on DPRA-cys or on kDPRA. Horizontal axes – %DP (DPRA-cys) or logk_{max} (kDPRA); Vertical axis – logEC3(%). (a) DPRA-cys: LogEC3 vs DP. (b) kDPRA: LogEC3 vs logk_{max}. Points in Areas A – 1B/NC chemicals correctly assigned. Points in Areas B – 1B/NC chemicals incorrectly assigned as 1A. Points in Areas C – 1A chemicals incorrectly assigned as 1B/NC. Points in Areas D – 1A chemicals correctly assigned.

Table 1. Potency classification of LLNA data by DPRA-cys (47% DP cut-off) and by kDPRA (−2 logk_{max} cut-off).

Classifier	DPRA-cys	kDPRA	kDPRA	kDPRA 5 mM, 24 h
Cut-off	47% DP	−2 logk_{max}	−2 logk_{max}	47% DP
Data entries (from Natsch et al. 2020)	157 with both DPRA and kDPRA data		All 180 entries with kDPRA data	
True 1A (quadrants D in Figure 1)	33	31	38	37
False 1A (quadrants B in Figure 1)	24	18	19	27
True 1B/NC (quadrants A in Figure 1)	95	101	116	107
False 1B/NC (quadrants C in Figure 1)	5	7	7	9
Sensitivity (%)	86.8	81.6	84.4	80.4
Specificity (%)	79.8	84.8	85.9	79.9
Accuracy (%)	81.5	84.1	85.5	80.0
Balanced accuracy (%)	83.3	83.2	85.2	80.1
Confidence for 1A prediction (%)	57.9	63.3	66.7	57.8
Confidence for 1B/NC prediction (%)	95	93.5	94.3	92.2

DPRA-cys has a slightly higher sensitivity, while the kDPRA has a slightly higher specificity.

It is important to bear in mind that the performance statistics shown in Table 1 apply to the dataset investigated and do not necessarily indicate the probability that a new chemical will be correctly predicted. For this to be the case requires that the dataset studied here is representative of the world of chemicals in terms of relative frequencies and absences of the various types of structures, corresponding to various reaction mechanistic domains, that can enable chemicals to be skin sensitisers (Aptula and Roberts 2006). The same proviso applies to performance statistics for other non-animal assays and defined approaches that are recognised as OECD test guidelines.

3.1. Variability

It can be seen in Figure 1 that several of the data points are quite close to the cut-off values, so it is appropriate to consider the error limits on these cut-off values and how the performance statistics might be affected if the measured DP or logk_{max} values had differed within their error limits. Referring to Figure 1, quadrants A and D are the ones that contain correct predictions (true 1B/NC in quadrants A, true 1A in quadrants D). In both Figure 1(a,b) it is the A quadrant that contains more data points close to the cut-off value, so for a worst-case scenario quadrants A are the ones to consider.

In their evaluation of DPRA ring trial data, Dimitrov et al. (2016) found that the peak variability of depletion values is at 50% where the 95% confidence interval gets highest (12%). This is close to the 47% threshold applied here to discriminate between 1A and 1B/NC chemicals. It is therefore appropriate to consider the variability about the DPRA-cys DP threshold of 47% in comparison to the kDPRA logk_{max} threshold of −2.

It can be seen from Figure 1(a) that 9 chemicals are correctly assigned 1B/NC in the proposed DPRA-cys but would fall within the lower confidence interval (35–47%). Of these 9 1B/NC chemicals that are within the 95% confidence interval, two are very close to the 47% threshold. For each of these two chemicals the probability of being predicted incorrectly as 1A if the DPRA were repeated is almost 50%. If both were to be re-assayed in the DPRA the probabilities would be:

Both predicted correctly 1B/NC	25%
One predicted correctly and one predicted incorrectly	50%
Both predicted incorrectly 1A	25%

The other 7 chemicals all fall between the 95% and the 67% (1 standard deviation) confidence intervals. To make the mathematics simpler while erring on the side of the worst case scenario, each of these 7 chemicals is considered as having a 33% probability of being incorrectly classified as 1A if the DPRA were repeated. On this basis, using the probability mass function, $\Pr(k) = (n!/(k!(n-k)!))p^k(1-p)^{n-k}$ where in this case $n = 7$, $p = 0.33$ and k is the number falsely predicted as 1A, the probabilities shown in Table 2 can be calculated.

Table 2. Probabilities of false predictions due to variability for the DPRA-cys.

Number of false 1A predictions	Probability (as %) out of the 7 between 1 and 2 SD	Probability (as %) out of the 9 between 0 and 2 SD
0	6	1.5
1	21	5
2	31	28
3	25	27
4	12	23
5	3.7	13
6	0.6	5
7	0.04	1
8		0.2
9		0.01
Aggregate probability of >4	16	19

There is a greater than 80% probability that 5 or more of the 9 chemicals would remain correctly predicted as 1B/NC if the DPRA-cys assays were repeated. Assuming only 5 of these chemicals remain correctly predicted and 4 become incorrectly predicted as 1A, the performance indices for an 81% confident worst case can be calculated (Table 4).

The situation with the kDPRA is quite similar. Wareing et al. (2020), in their report of a ring trial on intra- and inter-lab variability of the kDPRA, give a standard deviation of 0.244 on logk_{max}, so the 95% confidence limits on the cut-off value are −2.488 to −1.512 and the 67% confidence limits are −2.244 to −1.756. From Figure 1(b) we see 4 correctly assigned 1B/NC points between 1 and 2 standard deviations below the cut-off value and 3 correctly assigned 1B/NC points less than 1 SD below the cut-off value. Similar calculations to those above for the DPRA-cys with 47% cut-off indicate a 70% probability that 4 or more of these 7 chemicals would remain correctly predicted as 1B/NC if the kDPRA were to be repeated (Table 3).

Assuming only 4 of these chemicals remain correctly predicted and 3 become incorrectly predicted as 1A, the performance indices for a 70% confident worst case can be calculated.

Table 4 shows the worst case (81% confident) predictive performance for the DPRA-cys and the worst case (70% confident) predictive performances for the kDPRA.

The 81% worst case performance statistics for DPRA-cys are not substantially inferior to the statistics based on the reported DP values, and the 71% worst case statistics for the kDPRA are only slightly worse (to a very similar degree as for DPRA-cys) than the statistics based on the reported logk_{max} values. The predictive performance of the DPRA-cys is not more sensitive to variability in DP measurement than the predictive performance of the kDPRA is sensitive to variability in logk_{max} measurement.

Table 3. Probabilities of false predictions due to variability for the kDPRA.

Number of false 1A predictions	Probability (as %) out of 4 between 1 and 2 SD	Probability (as %) out of 7 between 0 and 1 SD
0	20	2.5
1	40	12.5
2	29	26
3	10	30
4	1	20
5		4
6		4
7		1
Aggregate probability of >3		30

Table 4. Worst case predictive performances for DPRA-cys and kDPRA.

	DPRA-cys, cut-off DP = 47%		kDPRA, cut-off logk_{max} = −2	
Index	81% confident worst case	Original	70% confident worst case	Original
Sensitivity	86.8	86.8	81.6	81.6
Specificity	76.5	79.8	82.4	84.9
Accuracy	79.0	81.5	82.2	84.1
Balanced accuracy	81.7	83.3	82.0	83.2
1A prediction conf	54.1	57.9	59.6	63.3
1B/NC prediction conf	94.8	95.0	93.3	93.5

4. Analysis of false predictions

The sensitivity figures for the 157 chemicals that have both DPRA and kDPRA data are based on only 38 classified as 1A by their LLNA data, so it is not particularly meaningful to attempt to analyse in detail the sensitivity differences between the two assays. However, it is worth noting that paraphenylenediamine (PPD) and 2-aminophenol, both of which are well recognised as strong sensitisers with EC3 values of 0.15% and 0.45% respectively, are correctly classified as 1A by DPRA-cys (DP values of 95.3 and 96.2 respectively) but incorrectly as 1B/N by the kDPRA (logk_{max} values of −2.81 and −2.46, respectively). These chemicals are not directly reactive but can be converted to reactive species by oxidation. The simplest interpretation of these different outcomes between the two assays is that in the DPRA-cys there is a longer time (24 h) for oxidation to occur than in the kDPRA. Although data points are generated at 24 h few if any of the reported logk_{max} values are based on this time point, possibly because at 24 h the dose-response plots do not meet linearity criteria, and in many cases logk_{max} values are based on 30 min data. The longer reaction time in the DPRA-cys can also explain why the marginally 1A compound 2,4-dinitrobenzensulfonic acid (LLNA EC3 = 1.9%) is correctly assigned 1A in the DPRA-cys (DP = 94.2%) but incorrectly as 1B/N in the kDPRA (logk_{max} = −2.30).

Only one of the chemicals is correctly classified as 1A by the kDPRA but incorrectly as 1B/N by DPRA-cys. This is phthalic anhydride, *vide infra*.

Table 5 shows the chemicals that are falsely classified as 1A by both assays. Apart from abietic acid, all of these are either Michael acceptors or sulphur-based electrophiles that react by nucleophilic attack at sulphur. Although the sensitisation potency of Michael acceptors is correlated with reactivity, the dependence on reactivity is recognised as being relatively low compared to other electrophilic sensitisers (Roberts and Natsch 2009; Natsch et al. 2011). For sulphur-based electrophiles it has been noted that reactivity as measured in the kDPRA is high relative to their potency (Roberts 2021a).

The acrylates are well known to be less potent than their reactivity would predict, probably because of a combination of their high volatility and their tendency to polymerise under skin exposure conditions.

Table 6 shows the chemicals that are falsely classified as 1A by DPRA-cys but correctly classified as 1B/NC by the kDPRA.

These differences between the predictions of the two assays reflect the different reaction times used to estimate the reactivity parameter. In the DPRA, reactivity is represented by the peptide depletion after 24 h, whereas in the kDPRA the logk_{max} value is based on data recorded at 30, 90, 150 or 210 min (24 h time points are included in the kDPRA, but few if any of the reported logk_{max} values are based on them). The logk_{max} cut-off value of −2 for the kDPRA corresponds to a half-life of 3.85 h under DPRA-cys conditions. On this basis it is likely that 4 h depletion values measured in the DPRA-cys, with a cut-off value close to 50%, would discriminate better than the 24 h values between the potency classes, but this would need to be confirmed experimentally.

There are only three chemicals that are falsely assigned 1A by the kDPRA but correctly assigned 1B/NC by the DPRA-cys. These are shown in Table 7.

The kDPRA result for oxalic acid is difficult to explain – there is no alert for electrophilic reactivity. Trimellitic anhydride will be discussed later in this paper.

Table 8 shows the five 1A chemicals that are falsely classified as 1B/NC by the DPRA-cys. Four of them are also falsely classified as 1B/NC by the kDPRA, the exception being phthalic anhydride (see below 4.1).

Chlorpromazine is a sensitiser that needs to be activated (possibly acting as a precursor of malondialdehyde by aliphatic amine oxidation) and is therefore outside the applicability domains of the kDPRA (Roberts 2021a). The low DP value in the original DPRA suggests that it does not become significantly activated under DPRA conditions.

Table 5. False 1A in both DPRA-cys (47% cut-off) and kDPRA.

Name	EC3, %	%DP (DPRA)	logk_{max} (kDPRA)	Reaction mechanism
Ethyl acrylate	33	96.4	−0.97	Michael addition
2-Mercaptobenzothiazole	2.6	93.3	−0.15	S_N2 at Sulphur
Tetramethylthiuram disulphide	2.9	99.5	0.74	S_N2 at Sulphur
Benzisothiazolinone	4.8	97.7	−0.12	S_N2 at Sulphur
Benzylidene acetone	3.7	93.5	−1.85	Michael addition
Diethyl maleate	4.7[a]	99.9	−1.21	Michael addition
Trans-2-Hexenal	4.1	97.9	−0.47	Michael addition
Abietic acid	11	99.9	−0.55	Pre- or pro-hapten[b]
Methyl-2-nonynoate	2.5	99.0	−1.66	Michael addition
α-Damascone	3.3	99.0	−1.64	Michael addition
2-Decenal	2.5	94.9	−1.03	Michael addition
Safranal	7.5	91.8	−1.74	Michael addition
Butyl acrylate	20	99.0	−0.83	Michael addition
2,4-Heptadienal	4.0	97.3	−1.52	Michael addition

[a]Diethyl maleate is listed by Natsch et al. (2020) with a consolidated EC3 value of 4.7%. However, this value is largely influenced by the 5.8% value listed by Gerberick et al. (2005) which comes from a dose-response analysis outside the applicability criteria defined by Ryan et al. (2007) for extrapolation (Roberts 2021b). Re-analysis of the dose-response data gives 2.1% (Roberts 2021b), which would make diethyl maleate borderline 1A/1B.

[b]Abietic acid has a pro-hapten alert (conjugated diene with at least one double bond in a ring – in this case both) for sensitisation via metabolic oxidation to a reactive S_N2 electrophilic allylic epoxide (Bergström et al. 2006). It also has pre-hapten alerts for autoxidation to tertiary allyic hydroperoxides.

Table 6. False 1A in DPRA-cys (47% cut-off) but correctly assigned 1B/NC by kDPRA.

Name	EC3, %	%DP (DPRA)	logk_{max} (kDPRA)	Reaction mechanism[a]
Citral	5.7	83.3	Not reactive	SB
Phenyl benzoate	18	50.9	Not reactive	Acyl transfer
Butyl glycidyl ether	31	67.3	−2.73	S_N2
2-Hexylidene cyclopentanone	2.4	92.2	−2.36	MA
Phenylacetaldehyde	4.7	60.7	−2.36	SB
Ethylene glycol dimethacrylate	35	89.3	−2.44	MA
OTNE[b]	25	84.8	Not reactive	SB
2,3-Butanedione	11	85.9	−2.62	SB
2-Ethylhexyl acrylate[c]	19	99.8	−2.13	MA
Methyl methanesulfonate	8.1	93.0	−2.15	S_N2

[a]SB: Schiff base electrophile; S_N2: Bimolecular nucleophilic substitution; MA: Michael acceptor.
[b]1-(Octahydro-2,3,8,8-Tetramethyl-2-Naphthalenyl) Ethanone.
[c]2-Ethylhexyl acrylate has a substantially lower logk_{max} value than two other acrylates (ethyl and butyl) with similarly low LLNA potency (EC values of 33% and 20% respectively) which are both incorrectly assigned 1A in the kDPRA (Table 5), although all three have the same reactive centre and would be expected to be similarly reactive. This may reflect incomplete solubility of 2-ethylhexyl acrylate under the assay conditions.

Table 7. False 1A in kDPRA but correctly assigned 1B/NC by DPRA-cys (47% cut-off).

Name	EC3, %	%DP (DPRA-cys)	logk_{max} (kDPRA)	Reaction mechanism[a]
Imidazolidinyl urea	24	38.4	−1.11	Formaldehyde releaser
Oxalic acid	15	0.9	−1.01	No alerts
Trimellitic anhydride	9.2	1.0	−0.13	Acyl transfer

Table 8. False 1B/NC in the DPRA-cys with 47% cut-off.

Name	EC3, %	%DP (DPRA-cys)	kDPRA assignment	logk_{max} (kDPRA)
Chlorpromazine	1	1	False 1B/NC	Not reactive
Glutaraldehyde	0.09	30.2	False 1B/NC	Not reactive
Hexyl salicylate	0.18	3.9	False 1B/NC	Not reactive
Bisphenol A diglycidyl ether	1.5	42.5	False 1B/NC	−2.53
Phthalic anhydride	0.16	9.3	True 1A	−0.07[a]

[a]Derived from peptide depletion values at 5 min (Wareing et al. 2017). The 30-min depletion data as per the kDPRA protocol give a lower logk_{max} value of −0.86.

Glutaraldehyde is a Schiff base electrophile with special chemistry. It is outside the applicability domains of the kDPRA (Roberts 2021a). Although most aldehydes do not give stable adducts with the cysteine peptide under DPRA conditions, many of them can give significant DP values by conversion of the thiol group to disulphide linkages.

Hexyl salicylate is generally regarded as a false positive in the LLNA, and on that basis the kDPRA and the DPRA predictions would be false predictions of non-potency to stimulate thymidine uptake in the neighbouring lymph nodes but not false predictions of lack of sensitisation potency.

Bisphenyl A diglycidyl ether is an S_N2 electrophile. For the S_N2 reaction mechanistic domain sensitisation is related to a combination of hydrophobicity and reactivity rather than to reactivity alone. Bisphenyl A diglycidyl ether is one of several S_N2 electrophiles whose LLNA potency has been shown to be well correlated in a quantitative mechanistic model (QMM) based on a combination of logk_{max} and logP (Roberts 2021a).

4.1. Trimellitic anhydride and phthalic anhydride

These two chemicals provide a good illustration of the applicability domain issues. One (phthalic anhydride) is a 1A sensitiser (EC3 = 0.16%) and one (trimellitic anhydride) is a 1B sensitiser (EC3 = 9.2%). The DPRA-cys predicts both to be NC, on the basis of low depletion values, whereas the kDPRA predicts both to be 1A, on the basis of high logk_{max} values.

The difference between the results in the two reactivity assays arises because these compounds are outside the kinetics measurement applicability domain. Anhydrides are reactive towards sulphur nucleophiles, but the reaction product, a thioester, is readily hydrolysed with regeneration of the sulphur nucleophile. In a cysteine peptide assay, over a short time period a significant level of peptide depletion may be observed, but over a long time period most or all of the peptide will have been regenerated by hydrolysis of the adduct, as shown in Figure 2.

The number generated by the kDPRA data analysis protocol and reported as logk_{max} does not represent the reactivity of the anhydride towards the peptide but is a negative function of the reactivity of the adduct towards hydrolysis.

The reason why both assays fail to discriminate between the two anhydrides in terms of potency, is that trimellitic anhydride and phthalic anhydride, as acyl transfer agents, are both outside the chemistry-potency applicability domain. For acyl transfer agents, potency is not solely related to reactivity but to a combination of reactivity and hydrophobicity. Although phthalic anhydride and trimellitic anhydride would be expected to be quite similar in reactivity towards the peptide (they are also similar in their logk_{max} values, but as explained above the logk_{max} value does not represent their reactivity), they differ substantially in their hydrophobicity.

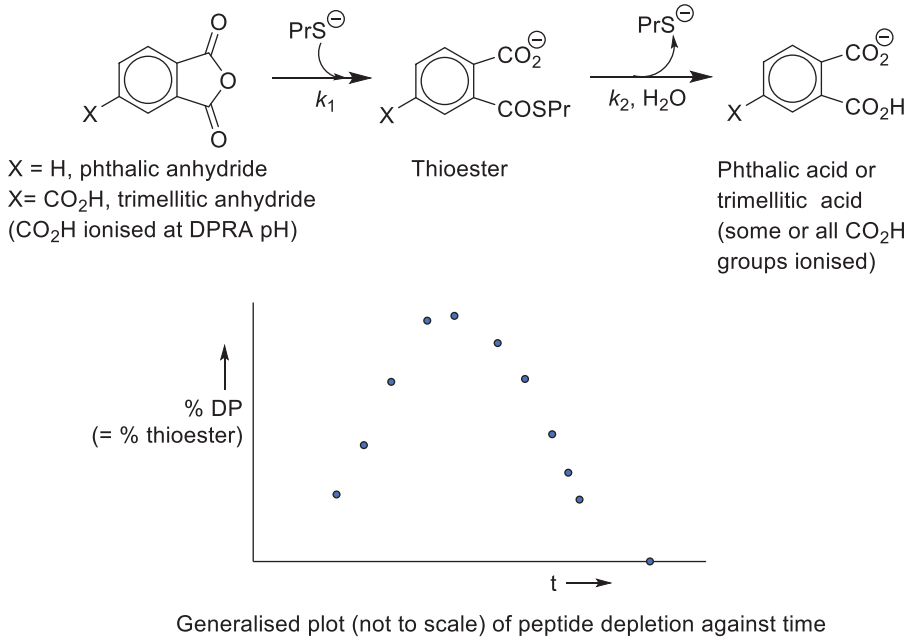

Figure 2. Reaction of phthalic and trimellitic anhydrides with cysteine peptide.

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 to be 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.

These two anhydrides provide a stark illustration of the unreliability of the assumption implicit in the DPRA and the kDPRA, that sensitisation potential and potency can be predicted on the basis of chemical reactivity alone. One of the reasons why these assays give quite good performance statistics is that the datasets against which they have been assessed consist of chemicals that are mostly within a narrow logP range (compounds with high logP values tend to be too insoluble to be assayed and organic compounds with low logP values are relatively infrequent).

5. Applicability domain issues and domain-specific cut-offs

As discussed in an earlier paper (Roberts 2021a), 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 logk_{max} is greater or less than −2? More broadly, can this rate constant alone predict the potency? This is the chemistry-potency applicability domain issue.

Similarly, applicability domain issues also apply to the original DPRA – does the DP value generated by the DPRA protocol represent the chemical's reactivity towards the peptide, and does the chemical's reactivity correctly predict whether the chemical is a sensitiser or not?

For the assignment of chemicals between the potency classes 1A and 1B/NC there is no obvious benefit to using the kDPRA rather than the original DPRA (cysteine-based). Both assays have limitations, as previously discussed in detail for the kDPRA (Roberts 2021a). The DPRA-cys has the same limitations as the kDPRA, with the partial exception of sensitivity to impurities. With the current kDPRA data analysis protocol, low levels of reactive impurities can affect the measured logk_{max} value to a greater extent than they affect the true potency, leading to false 1A assignment. This is less likely with the DPRA-cys: for a reactive impurity to cause a low-reactivity chemical to be classified as 1A it would need to be present at about 5% or higher.

The predictive performance of both assays could be improved by minor amendments to the experimental and data processing protocols. For the DPRA-cys, a shorter reaction time (ca. 4 h) should give a better discrimination, in particular reducing the incidence of false 1A predictions. For the kDPRA, in addition to the experimental procedure modifications proposed by Roberts (2021a), modifying the data analysis protocol in line with conventional kinetics practice would enable reactive impurity effects to be detected and corrected for (Roberts 2021a).

Even when optimised by the amendments above, both the DPRA-cys and the kDPRA have significant limitations resulting from chemistry-potency applicability domain issues. Using a reactivity parameter alone to assign a chemical as a

sensitiser or non-sensitiser (e.g. peptide depletion in the DPRA to assign S or NS), or to assign a potency classification (e.g. logk_{max} in the kDPRA to assign 1A or 1B/NS) is based on the implicit assumption that potency of a chemical is a single increasing function of its reactivity and of nothing else. The predictive performance of a reactivity assay depends on the extent to which this implicit assumption applies. As discussed earlier (Roberts 2021a) and as illustrated here by the example of phthalic and trimellitic anhydrides, for many chemicals potency is related to a combination of reactivity and hydrophobicity rather than reactivity alone. Furthermore, even when potency is dependent on reactivity alone, the degree of dependence varies according to the reaction mechanism.

If the kDRPA is applied on a chemistry-blind and SAR-blind basis, i.e. with no consideration of the chemical properties of the substance being tested and with no consideration of structure-activity relationships, there is a significant probability (based on the performance statistics in Table 1, about 15–20%) that the prediction (1A or 1B/NC) will be incorrect. The same applies to the DPRA-cys.

Applying the reactivity assays on a chemistry-aware and SAR-aware basis the probability of an incorrect prediction should be reduced by addressing the applicability domain issues:

5.1. Kinetics applicability domain issue

Chemicals that are not directly reactive but require activation can usually be identified by chemists and chemistry-based expert systems. For these chemicals a negative result in the DPRA or log$k_{max} < -2$ in the kDPRA cannot be interpreted as a meaningful non-sensitiser prediction or 1B/NC prediction respectively.

Chemicals that although potentially reactive do not give stable adducts with cysteine-based peptides (mainly Schiff-base electrophiles and acyl transfer agents) can be identified as chemicals for which the kDPRA is not applicable. For acyl transfer agents in the DPRA, results with the lysine-based peptide can be used for a sensitiser/non-sensitiser prediction.

DPRA and kDPRA predictions for chemicals outside the kinetics applicability domain may in many cases be correct, but by chance rather than by virtue of the scientific principles underlying the assays. Consequently, the good performance statistics of the assays can lead to overconfidence in their predictive capability.

5.2. Chemistry-potency applicability domain issue

The kDPRA logk_{max} threshold of −2.0 to distinguish 1A sensitisers from 1B/NC is not a scientific absolute but simply the best compromise derived by retrofitting for a heterogenous set of data containing classes of chemicals for which different thresholds would be applicable. The same applies to the DP threshold of 47% derived for the DPRA-cys.

Quantitative models (QMMs) relating sensitisation potency to peptide reactivity have been published for several groups of directly reactive electrophiles. From these, reactivity thresholds for the individual reaction mechanistic domains can be calculated:

S_NAr electrophiles (Natsch et al. 2011):

$$pEC3 = 0.664 \log k + 3.58, \quad \text{for } EC3 = 2\%,\ pEC3 = 1.97,\ \log k = -2.42 \tag{1}$$

Michael acceptors (Natsch et al. 2011):

$$pEC3 = 0.25 \log k + 2.14, \quad \text{for } EC3 = 2\%,\ pEC3 = 1.97,\ \log k = -0.67 \tag{2}$$

The Michael acceptor cut-off value derived from the QMM is substantially larger than the value of −2 specified in the kDPRA protocol. This is consistent with many of the kDPRA false 1A cases corresponding to Michael acceptors (Section 4; Table 5).

S_N2 electrophiles (Roberts 2021a):

$$pEC3 = 0.69\,RAI + 2.69 \quad \text{where } RAI = \log k_{max} + 0.4 \log P. \tag{3}$$

This QMM is based on the only seven S_N2 electrophiles for which kDPRA data are reported by Natsch et al. (2020), and it was assumed without verification that the k_{max} values are true representations of the rate constants for these chemicals. In spite of these reservations, its statistics are good ($R^2 = 0.905$, $s = 0.35$, $F = 47.8$) and, as shown in the following section, its predictive performance has been found to be good.

For the S_N2 mechanistic domain there is no single log$k$ or logk_{max} value that can serve as a kDPRA cut-off to distinguish 1A from 1B/NC, since the potency depends also on logP. For this domain the criterion for classification as 1A can be expressed as:

$$\text{Log } k \geq -1.04 - 0.4 \log P$$

Table 9 shows the domain-specific reactivity cut-offs for these three reaction mechanistic domains. For the S_N2 domain cut-offs for a series of logP values are shown.

The logk and DP cut-off values listed in Table 9 are shown to illustrate how the extent to which the reactivity boundary between 1A and 1B/NC varies depending on the reaction mechanism and, for the S_N2 mechanism, on the chemical's hydrophobicity. It is not suggested that these cut-off values be used for classification purposes – if the reaction mechanistic domain can be identified and log$k$ is determined, the simplest approach is to apply the appropriate QMM equation to predict the EC3 value and to make the classification according to whether the predicted EC3 value is less than or greater than 2%.

6. Assessment of predictive performance of a kDPRA-based QMM

The QMM for S_N2 electrophiles (Equation (3)) was based on the only seven S_N2 electrophiles with logk_{max} values listed by Natsch et al. (2020) and it was assumed without verification

Table 9. Domain specific reactivity cut-offs.

Domain		kDPRA, for 1A	DPRA-cys, 24 h, for 1A	DPRA-cys, 4 h, for 1A
S_NAr		Log$k \geq -2.42$	DP $\geq$ 81%	DP $\geq$ 24%
Michael acceptor		Log$k \geq -0.67$	DP = 100%[a]	DP = 100%[a]
S_N2	LogP	Log$k \geq$:		
	−1	−0.64	DP = 100%[a]	100%[a]
	0	−1.04	DP = 100%[a]	100%[a]
	1	−1.44	DP = 100%[a]	DP $\geq$ 93%[a]
	2	−1.84	DP = 100%[a]	DP $\geq$ 64%
	2.5	−2.04	DP $\geq$ 98%[a]	DP $\geq$ 48%
	3	−2.24	DP $\geq$ 92%[a]	DP $\geq$ 34%
	4[b]	−2.64	DP $\geq$ 63%	DP $\geq$ 15%
	5[b]	−3.04	DP $\geq$ 32%	DP $\geq$ 6%[c]
	5.5[b]	−3.24	DP $\geq$ 22%	DP $\geq$ 4%[c]

[a]Not useable, DP differences in the range 90–100% not being reliable representations of differences in reactivity.
[b]Unlikely to be testable in the kDPRA or DPRA-cys, due to low solubility.
[c]Not useable, DP differences in the range 0–10% not being reliable representations of differences in reactivity.

that the k_{max} values for these seven chemicals are not significantly different from the true rate constants k.

Although there are currently no other S_N2 electrophiles with reported logk_{max} values, the predictive performance of Equation (3) can be evaluated as follows.

Methyl dodecanesulphonate, n-$C_{12}H_{25}SO_3Me$, M = 264

This compound has not been assayed in the kDPRA, and would probably be too insoluble under the test guideline conditions. However, it can confidently be assumed that its k value is not significantly different from that of methyl methanesulphonate, which has a logk_{max} value of −2.15. The logP value for methyl dodecanesulphonate is easily calculated by the Hansch and Leo method (1979) as follows:

$$f_{-SO_3-} + 2f_{-CH_3} + 11f_{-CH_3-} + 12F_b = -2.11 + 2 \times 0.89 + 11 \times 0.66 + 12 \times (-0.12) = 5.49$$

Log$k$ = −2.15 (assumed to be identical to the experimental value for methyl methanesulphonate)

$$RAI = \log k + 0.4\,\log P = -2.15 + 0.4 \times 5.49 = 0.046$$

Applying the S_N2 QMM (Equation (3)):

$$pEC3 = 0.69 \times 0.04 + 2.69 = 2.72$$

$$\text{Predicted EC3} = M \times 10^{-pEC3} = 264 \times 10^{-2.72} = 0.5\%$$

Experimental EC3 = 0.8% (Gerberick et al. 2005)

12-Bromo-1-dodecanol, Br-$(CH_2)_{12}$-OH, M = 266

$$\text{LogP calculated } f_{-Br} + 12f_{-CH_3-} + f_{-OH} + 12F_b = 0.20 + 6.48 - 1.64 - 1.44 = 5.04$$

Since the reaction centre is a substituted primary alkyl bromide, with the substituent (OH) remote from the reaction centre, its reactivity should not differ significantly from that of simple alkyl bromides. No kDPRA data are available for primary alkyl bromides, but n-butyl bromide and n-hexyl bromide have both been assayed in the DPRA (Natsch et al. 2013), giving very similar 24 h DP values with the cysteine peptide: BuBr, DP = 13.8; Hexyl-Br, DP = 14.1. The close agreement between these DP values gives confidence that they are a good representation of primary alkyl bromide reactivity. From the average DP value of 14, the log$k$ value (k in units of $M^{-1}s^{-1}$) can be calculated as:

$$k = [\ln(100/(100 - dp))]/[[E]_o t] = 3.49 \times 10^{-4}$$

$$\log k = -3.46$$

$$RAI = \log k + 0.4\,\log P = -3.46 + 0.4 \times 5.04 = -1.44$$

$$pEC3 = 1.70$$

$$EC3 = 5.4\%$$

Experimental EC3 = 6.9% (Gerberick et al. 2005)

Benzyl benzoate, $PhCO_2CH_2Ph$, M = 212

This compound has an alert for reaction as an S_N2 electrophile but no other alerts. It has an activated reaction centre (benzyl carbon) but a poor leaving group (benzoate). It is listed as unreactive in the kDPRA, so obviously the S_N2 QMM cannot be used to predict its LLNA potency. However, this compound can still be used to assess the S_N2 QMM, by using the listed EC3 value of 17% (Gerberick et al. 2005) to predict the reactivity:

$$pEC3 = \log(M/EC3) = \log(212/17) = 1.10$$

From Equation (3), RAI = −2.32

LogP = 4 (Computed by XLogP3 3.0, PubChem release 2021.05.07)

$$\text{Log}\,k(\text{calc}) = RAI - 0.4\,\log P = -3.92$$

This calculated log$k$ value is below the maximum value of −3.5 for classification as not reactive in the kDPRA (Natsch et al. 2020), so Equation (3) correctly predicts, from the reported EC3 value, the kDPRA result for benzyl benzoate.

Figure 3 shows the original QMM plot of pEC3 vs RAI for the S_N2 sensitisers, with methyl dodecanesulphonate and 12-bromododecanol added. It can be seen that these two compounds fit the regression line very closely.

The good agreement between the experimental data and the predictions of Equation (3), based on a combination of kDPRA data and logP, demonstrates the potential value of the kDPRA for building mechanism-specific models that can predict potency on a continuous basis. Also, Equation (3) and Figure 3 demonstrate clearly the role of hydrophobicity as a determinant of potency for the S_N2 reaction mechanistic domain.

7. Conclusions

For assignment of chemicals between the potency classes 1A and 1B/NC there is no obvious benefit to using the kDPRA rather than the original DPRA (cysteine-based). Of the two, the DPRA-cys is clearly simpler to run. Both assays have limitations, as previously discussed in detail for the kDPRA (Roberts 2021a) and further discussed here. The DPRA-cys has the same limitations as the kDPRA, with the partial exception of sensitivity to impurities. With the current kDPRA data analysis protocol, low levels of highly reactive impurities can affect the measured logk_{max} value to a greater extent than they affect the true potency, leading to false 1A assignment.

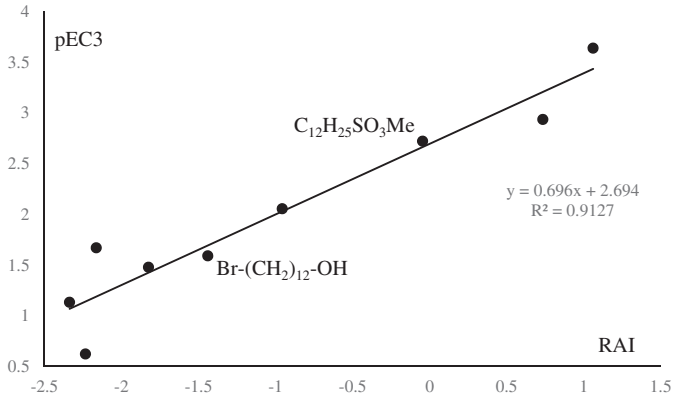

Figure 3. QMM for the S_N2 reaction mechanistic domain.

The predictive performance of both assays could be improved to some extent by minor amendments to the experimental and data processing protocols. For the DPRA-cys, a shorter reaction time (ca. 4 h) should give a better discrimination, in particular reducing the incidence of false 1A predictions. For the kDPRA, in addition to the experimental procedure modifications recommended by Roberts (2021a), modifying the data analysis protocol in line with conventional kinetics practice would enable reactive impurity effects to be detected and corrected for (Roberts 2021a).

Using a single reactivity assay (here, kDPRA or DPRA-cys, but the argument would apply to other assays) on a chemistry-blind basis as a standalone method to identify 1A skin sensitisers involves the implicit assumptions that:

- For all sensitisers, potency depends only on reactivity
- The assay can measure reactivity for all sensitisers
- The dependence of potency on reactivity is quantitatively the same for all sensitisers

None of these implicit assumptions is correct. For the kDPRA and the DPRA-cys:

- Potency depends on reactivity alone for Michael acceptors and S_NAr electrophiles, but not for other reaction mechanistic domains.
- The kDPRA and the DPRA-cys cannot measure reactivity for Schiff base electrophiles, acyl transfer agents and unreactive chemicals that can sensitise via metabolic or abiotic activation. Many of these compounds can still give rise to peptide depletion, from which logk_{max} values can be derived by the current kDPRA data analysis protocol, but these k_{max} values cannot with any confidence be assumed to represent true rate constants. Likewise, for these compounds DP values in the DPRA-cys cannot be assumed to be reliable indices of reactivity.
- Different reaction mechanistic domains have different dependencies of potency on reactivity, as is shown by the log*k* coefficients of Equations (1)–(3):

Equation 1 (S_NAr)	log*k* coefficient = 0.66
Equation 2 (Michael acceptors)	log*k* coefficient = 0.25
Equation 3 (S_N2)	log*k* coefficient = 0.69

Despite these deficiencies the kDPRA and DPRA-cys have been shown to give quite good predictive performance, with balanced accuracy values in the 80–85% range and about 95% probability that a 1B/NC prediction is correct.

Because of the above deficiencies there is limited scope for major improvement in the predictive performance of the assays for use on a chemistry-blind basis. Using a reactivity assay (here, kDPRA or DPRA-cys, but the argument would apply to other assays) as a standalone method to identify 1A sensitisers is based on a compromise derived by retrofitting for a heterogenous set of data containing classes of chemicals for which different thresholds would be applicable.

If used on a chemistry-aware basis (in which the chemistry awareness can come from human chemists or from expert systems), there is rather more scope. A chemical can be assigned to its reaction mechanistic domain, and on that basis it can be decided whether or not it is in the kinetics applicability domain and if so the appropriate QMM can be applied to predict the EC3 value and hence the 1A or 1B/NC category. For out of domain chemicals, kDPRA or DPRA predictions cannot be considered to be reliable.

The kDPRA appears to offer no substantial advantage over the DPRA-cys for the purpose of assignment of potency class. However, by enabling rate constants to be generated under a uniform set of conditions it has the potential to provide a reliable self-consistent set of reactivity indices, although this only applies to chemicals within the applicability domains. This represents a major advance towards predicting skin sensitisation potency without animal testing. For deriving NESILs (No Expected Sensitisation Induction Level) required for quantitative risk assessment (QRA) and for development of structure-activity relationships, prediction of potency on a continuous scale is required (Api et al. 2008, 2020). If the refinements recommended previously (Roberts 2021a) are implemented, in particular with analysis of the data matrices according to conventional kinetic practice, the kDPRA can provide good quality rate constants that can be used, either alone or in combination with hydrophobicity, in mechanism-based models to predict potency values on a continuous scale.

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Declaration of interest

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