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Principles for identification of High Potency Category Chemicals for which the Dermal Sensitisation Threshold (DST) approach should not be applied

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ABSTRACT

An essential step in ensuring the toxicological safety of chemicals used in consumer products is the evaluation of their skin sensitising potential. The sensitising potency, coupled with information on exposure levels, can be used in a Quantitative Risk Assessment (QRA) to determine an acceptable level of a given chemical in a given product. Where consumer skin exposure is low, a risk assessment can be conducted using the Dermal Sensitisation Threshold (DST) approach, avoiding the need to determine potency experimentally. Since skin sensitisation involves chemical reaction with skin proteins, the first step in the DST approach is to assess, on the basis of the chemical structure, whether the chemical is expected to be reactive or not. Our accompanying publication describes the probabilistic derivation of a DST of 64 μg/cm^2 for chemicals assessed as reactive. This would protect against 95% of chemicals assessed as reactive, but the remaining 5% would include chemicals with very high potency. Here we discuss the chemical properties and structural features of high potency sensitisers, and derive an approach whereby they can be identified and consequently excluded from application of the DST.

1. Introduction

An essential step in ensuring the toxicological safety of chemicals used in consumer products is the evaluation of their skin sensitising potential. Where a chemical is shown to be a contact allergen, knowledge of sensitising potency and exposure can be utilised to restrict its level in consumer products based on Quantitative Risk Assessment approaches to protect human health (Api et al., 2008).

Similar to the Threshold of Toxicological Concern (TTC), the Dermal Sensitisation Threshold (DST) has been demonstrated to provide effective risk assessments for skin sensitisation in cases where human exposure to a material is sufficiently low (Safford, 2008). The DST was originally developed based on a probabilistic assessment of Local Lymph Node Assay (LLNA) data and subsequently refined for application with chemicals that were not considered to be reactive to skin proteins, and thus unlikely to initiate the first mechanistic steps leading to the induction of skin sensitisation (Safford et al., 2011). In our publication accompanying this one (Safford et al., 2014), we describe the derivation of a DST for chemicals classified as protein reactive. In the present context we use the term "reactive" to include chemicals that are not themselves directly reactive but are readily converted (either metabolically or abiotically) to reactive derivatives under skin exposure. Following a similar probabilistic approach, we arrived at a DST of 64 μg/cm^2 for reactive chemicals. This DST for reactive materials would be protective against 95% of chemicals. However, the 5% against which the reactive DST would not be protective would include highly potent skin sensitisers.

The application of TTC concepts routinely utilises structural based filters to group materials into appropriate threshold categories, such as Cramer decision tree classifications (Cramer et al., 1978; Munro et al., 1996), or Cohort of Concern classification to exclude specific chemical classes from TTC approaches (Kroes et al., 2004). Analogously, in order to increase the conservatism for risk assessment and establish safe concentration limits for formulations containing reactive chemicals, an approach is needed to proactively identify highly reactive and potentially highly potent materials for which the reactive DST should not be applied. We will refer to such chemicals as High Potency Category (HPC) chemicals.

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The purpose of the work described here was, therefore, twofold. The first was to develop chemical structure based rules to identify HPC chemicals. To this end, the DST dataset was evaluated and chemicals with known potency below the reactive DST were taken as the starting point to develop HPC structural alerts. The second goal was to benchmark the alerts against a test set of LLNA data not included in the dataset used to develop the DST. The results suggest that by combining the reactive DST with knowledge of chemistry a threshold can be established below which there is no appreciable risk of sensitisation for a protein-reactive chemical.

2. The approach – chemistry-based identification of High Potency Category (HPC) Chemicals

The chemical principles of skin sensitisation have been, and continue to be, quite extensively investigated. The fundamental mechanistic basis of structure activity relationships for sensitisation is that for a chemical to sensitise it must be reactive, i.e. able, either as such or after *in cutaneo* activation, to covalently modify the structure of cutaneous proteins or peptides (Landsteiner and Jacobs, 1936; Roberts et al., 2006, 2007a; Roberts and Aptula, 2008).

Reactive chemicals can be classified into reaction mechanistic domains, according to the organic reaction mechanism by which they can react with proteins (Aptula and Roberts, 2006). In the vast majority of cases the reaction involves the chemical or its activated derivative acting as an electrophile and reacting with a nucleophilic group, usually a thiol or an amino group, of the peptide or protein. The five major reaction mechanistic domains are the Michael acceptor domain, the Schiff base domain, the Acyl transfer domain, the S_N2 domain and the S_NAr domain. A set of rules for assigning chemicals to these reaction mechanistic domains, based on an original compilation by Aptula and Roberts (2006) for use in reactive toxicity Structure–Activity Relationships (SAR) work in general, not solely for skin sensitisation, is shown in Fig. 1. For present purposes we need to extend this by two further domains. These are: (a) *organic peroxides*, i.e. any compound with the substructure C–O–O, and (b) *structurally complex compounds* that, because of the presence of unfamiliar substituents or because of the presence of multiple substituents, have to be classed as complex (i.e. not directly predictable from the chemical structure).

The need for the new *organic peroxide* domain arises as a result of extensive work published since 2000 on the allergenicity of organic peroxides that can be formed, by autoxidation, as allergenic contaminants of various organic compounds (e.g. Brared Christensson et al., 2006; Karlberg et al., 2008). Most of the organic peroxides that have been investigated have been found to be strong sensitisers, although the chemical mechanism is still not completely understood. These autoxidation-derived peroxides are not formed in sufficient quantities to make the contaminated chemical strongly allergenic, and are unlikely to be used in their own right in consumer products. The parent chemicals that give rise to these peroxides by autoxidation are described as prohaptens, and are covered by the reactive DST. However, the fact that in themselves these hydroperoxides are quite potent suggests that other organic peroxides that may be used in consumer products, such as diacyl peroxides RCO.OOCOR, need to be considered.

The *structurally complex* classification is to cover those compounds that cannot be confidently assigned to any of the other reaction mechanistic domains, and cannot be confidently classified as non-reactive. A compound may need to be assigned to this group if it possess an unfamiliar substituent whose reactivity (or non-reactivity) is uncertain, or if it possesses a number of substituents, that in combination could possibly, but not predictably, give rise to significant reactivity. Much more so than with the other reaction mechanistic domains, assignment to the structurally complex domain is a subjective process, depending on the knowledge/experience of the person making the assignment.

Within reaction mechanistic domains structure-potency trends can be found, and in several cases these can be expressed quantitatively in the form of Quantitative Mechanistic Models (QMMs). A QMM may be regarded as a mechanism-based Quantitative Structure–Activity Relationship (QSAR) model in which the predictive parameters have been derived by mathematical modelling (in the case of skin sensitisation the mathematical basis is the relative alkylation index (RAI) model (Roberts and Williams, 1982)), rather than by statistical trial and error (Roberts et al., 2007b). Skin sensitisation QMMs are based on a reactivity parameter either alone or in combination with a hydrophobicity parameter, depending on the reaction mechanistic domain.

Thus, the chemical mechanism based approach for estimating the potency of a chemical is:

1. Assign it to its reaction mechanistic domain. This can often be done by inspection of the structure, manually (Aptula and Roberts, 2006; Roberts et al., 2007b) or *in silico* (Roberts et al., 2007c) but if not the mechanistic domain can be determined by appropriate chemical experimentation.
2. Quantify its reactivity, relative to other chemicals with known potency in the same domain. This can sometimes be done from structure, for example using substituent constants (e.g. Roberts et al., 2006; Roberts and Aptula, 2014) or computational chemistry indices (e.g. Enoch et al., 2008; Enoch and Roberts, 2013) but if not, experimental chemistry can be done to quantify reactivity in terms of rate constants with model nucleophiles (e.g. Roberts and Natsch, 2009).
3. If necessary, calculate or experimentally determine hydrophobicity (usually expressed as log *P*, *P* being the octanol/water partition coefficient).
4. If a QMM is available, use it to calculate potency (usually as the LLNA EC3) for the target chemical from its reactivity and (if necessary) hydrophobicity parameters. If no QMM is available, a more approximate estimate may still be possible by chemistry-based read-across, using data for similar chemicals with known potency in the same mechanistic domain. This involves comparing the target chemical against chemicals with known potency to assess whether it is likely to be more reactive or less reactive, more hydrophobic or less hydrophobic, than the already known chemicals.

It is not always possible to estimate potency in this way – for some target chemicals there may be a lack of potency data for related chemicals in the same domain, for others an activation step that is currently not well modelled may be potency-determining. However, despite the current existence of such capability gaps, the chemistry-based approach can be used to derive rules for identifying HPC chemicals. We start by using the most potent chemicals in the DST database, based on their experimental EC3 values, to determine how their potency is related to their chemical structure, We then apply these chemistry principles to formulate structure-based rules that could be used, without recourse to experimental chemistry or chemical reaction mechanism expertise, to decide whether a given chemical should be classified as HPC. The rules are based on using established principles of physical organic chemistry to rank, and in some cases quantify, structural alerts in terms of their impact on reactivity and hence on potency. The rules are assessed against the dataset used to establish the reactive DST and against a second dataset of chemicals tested in the LLNA.

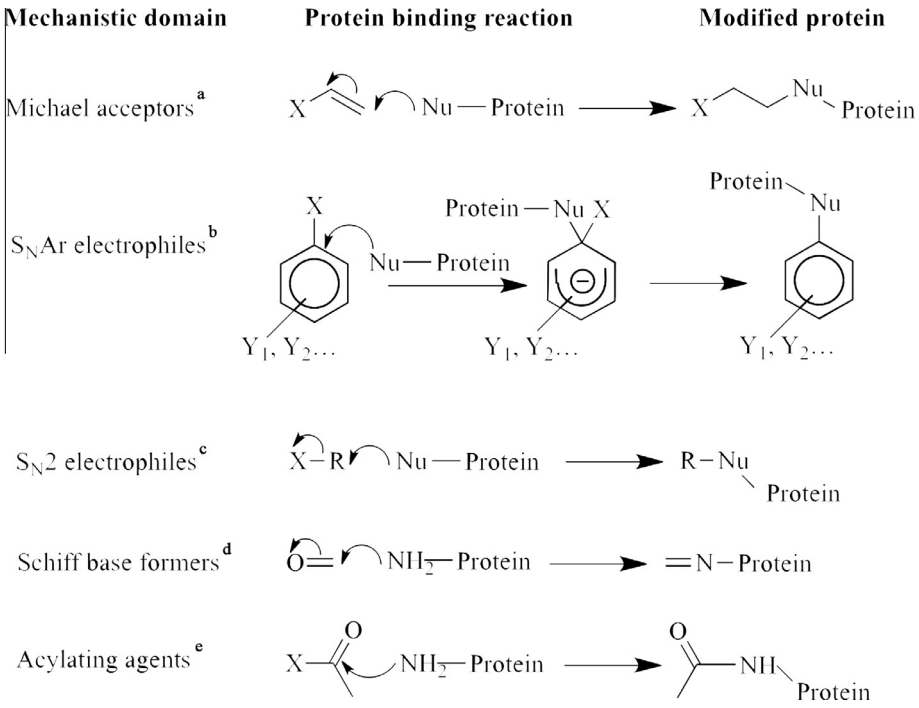

Fig. 1. Reaction mechanistic applicability domains. (a) Double or triple bond with electron-withdrawing substituent X, such as –CHO, –COR, $-CO_2R$, –CN, $-SO_2R$, $-NO_2$. Includes paraquinones and ortho quinones often formed by oxidation of para and ortho di-hydroxy aromatics acting as pro-Michael acceptors. X can also be a heterocyclic group such as 2-pyridino or 4-pyridino. (b) X = halogen or pseudohalogen, Y's are electron withdrawing groups (at least two are necessary for high allergenic potency) such as $-NO_2$, –CN, –CHO, $-CF_3$, $-SO_2Me$, ring fused nitrogen. One halogen is too weak to act as a Y, but several halogens together can activate. (c) X = halogen or other leaving group, e.g. OSO_2(R or Ar), OSO_2O(R or Ar) bonded to primary alkyl, benzylic, or allylic carbon. OR and NHR or NR_2 do not usually act as leaving groups, but can do so if part of a strained 3-member ring (e.g. epoxides, ethylenimine and substituted derivatives). (d) Reactive carbonyl compounds such as aliphatic aldehydes, some α,β- and α,ϒ-diketones, α-ketoesters. Not single monoketones and aromatic aldehydes. Other hetero-unsaturated systems can behave analogously, e.g. C-nitroso compounds, thiocarbonyl compounds (C=S), cyanates and isocyanates, thiocyanates and isothiocyanates. (e) X = halogen, or other group (e.g. $-OC_6H_5$) such that XH is acidic enough for X to act as a good leaving group. Includes anhydrides, cyclic or non-cyclic. X = -OAlkyl does not qualify, except when part of a strained lactone ring, e.g. β-propiolactone (but not ϒ-butyrolactone). Analogous reactions can occur with attack at sulphonyl S, phosforyl P and thioacyl C.

3. Development of HPC rules

The aim here is to define structure-based rules that can be applied without the need for experimental work or specialist expertise in physical organic chemistry and QSAR. However, to develop these rules we apply the experimental data and chemistry/QSAR expertise available to us.

The underlying principle of this approach is that there is a relationship between the potency of a chemical and its ability to react covalently with relevant skin proteins, this relationship taking the general form shown in Fig. 2, in which individual chemicals lie along the diagonal line. This principle was originally proposed in 1936 (Landsteiner and Jacobs, 1936) and has been confirmed by many subsequent studies. The equation of this line is not known – not only can the ability of a chemical to react covalently with the specific relevant protein or proteins not currently be measured, but the exact nature of the relevant protein or proteins is not fully understood. However, depending on the nature of the chemical:

1. There may be a QMM for this type of chemical. A QMM may be considered to be the equation of the line relating potency to one or more physico-chemical parameters modelling protein reactivity. In such cases the potency can be calculated from physico-chemical parameters (these may have to be measured, or they may be able to be calculated from structure), and assigned as HPC or non-HPC accordingly. Putting it another way, knowing the potency of chemical A or chemical B, we can estimate the lengths of the arrows A or B, in units of physico-chemical parameters, that would take a modified version of A out of the HPC region or would take a modified version of B into the

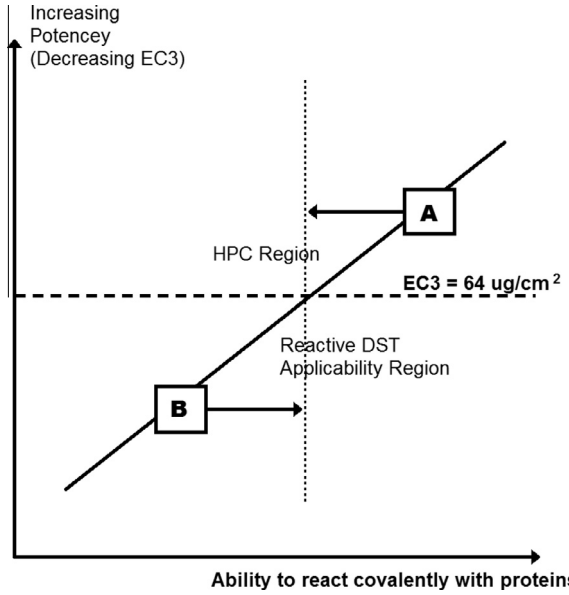

Fig. 2. Schematic representation of the relationship between potency and ability to react covalently with skin proteins relevant to sensitisation. The length of the arrow A represents the extent to which modification to the structure A would need to reduce protein binding ability in order to take the modified chemical into the reactive DST applicability region. The length of the arrow B represents the extent to which the protein binding ability of structure B could be increased by structural modification before taking the modified chemical out of the reactive DST applicability region. *Abbreviations:* DST, Dermal Sensitisation Threshold; HPC, High Potency Category Chemicals.

HPC region. For such chemicals, our challenge for present purposes is to represent the QMM equation in terms of structure-based rules that will discriminate between EC3 < 64 μg/cm^2 (HPC) and EC3 > 64 g/cm^2 (reactive DST applicable). Michael acceptors, Schiff base electrophiles and S_NAr electrophiles fall into this category.

2. There may be a qualitative mechanistic understanding of how the chemical reacts, but not a quantitative model. For such cases, a read-across approach may be used to compare a new chemical against A and B in Fig. 2, to assess whether the structural differences will make the new chemical more able or less able to react with protein. If there are known chemicals A and B with EC3 values close to 64 μg/cm^2, knowing whether a structural modification will make the chemical more reactive or less reactive (and for some reaction mechanistic domains more hydrophobic or less hydrophobic) may enable a confident assessment of the HPC or non-HPC status to be made. Again, our challenge for present purposes is to represent the read-across principles in terms of structure-based rules that will discriminate between EC3 < 64 μg/cm^2 (HPC) and EC3 > 64 μg/cm^2 (reactive DST applicable). The acyl transfer agent domain falls into this category.
3. There may not be an adequate understanding of how the chemicals of this type react. For such chemicals, if one is known to have an EC3 < 64 μg/cm^2, all have to be assigned HPC, accepting that some may in reality be false positives. Fortunately we have found few chemicals to which this degree of uncertainty applies. Organic peroxides fall into this category.

Based on these principles, the approach for deriving HPC rules is as follows. Chemicals with an EC3 < 64 μg/cm^2 are identified, and classified into one of the five reaction mechanistic domains where possible and for each of these chemicals, the reactive substructure (reactivity alert) is identified.

For each chemical, the effects of substituting the reactive substructure for different reactive substructures for the same reaction mechanism are considered, applying well-established principles of physical organic chemistry to evaluate which of these substitutions will increase chemical reactivity and which will decrease it. Likewise, for each chemical, the effects of modifying the overall structure while retaining the same reactive substructure are considered, applying well established principles of physical organic chemistry to evaluate which of these substitutions will increase chemical reactivity and which will decrease it. In addition, the effects of such changes on partitioning behaviour are considered, and QMM insights are applied to assess how these changes in reactivity and hydrophobicity will impact sensitisation potency.

Comparing chemicals within the same reaction mechanistic domain with EC3 values <64 μg/cm^2, ranges of structural variation (both of the reactive substructure and of substituents modifying reactivity of the same substructure) that are expected to keep the potency in the HPC range (i.e. EC3 < 64 μg/cm^2) are estimated. For several domains, because QMMs have been developed and/or there are good quantitative insights into structure-chemical reactivity based on physical organic chemistry, it is possible to extend these rules to chemicals with reactive substructures and substituent patterns that are not represented in the DST dataset.

4. HPC chemicals in the DST database

Table 1 lists the most potent chemicals in the DST database (i.e. EC3 < 64 μg/cm^2), with their reaction mechanistic domains and EC3 values. The final column gives the HPC alert for each compound. In the Reaction Mechanistic Domain column some entries are noted as (pro/pre), for example Benzo[a]pyrene, S_N2 (pro-/pre- hapten). This indicates chemicals that are not themselves reactive but are converted (either metabolically or abiotically) under test or exposure conditions, to a reproducible extent, to reactive "ultimate haptens". Such chemicals have reproducible EC3 values (within LLNA variability limits). Since in many cases it is not known whether the conversion is metabolic (pro-haptens) or abiotic (pre-haptens), and the question is not relevant for present purposes, we refer to these chemicals as pro/pre. We reserve the term "pre-haptens" for chemicals that are not reactive per se but are susceptible to formation of reactive impurities (usually by air oxidation) under conditions of storage and handling. For these chemicals the EC3 is highly dependent on the history of the sample, limonene being a classic example (Karlberg et al., 2008). Although in some cases the pure oxidation products have been found to be highly potent, there have been no reported cases where the oxidised material (i.e. a mixture of the original pre-hapten plus oxidation products) has a potency approaching the DST value of 64 μg/cm^2.

Overall, apart from the anomalous hexyl salicylate (see Table 1), tetrachlorosalicylanilide (structurally complex) and glutaraldehyde, the high potency of all the chemicals in Table 1 is easily rationalised in terms of established structure activity principles, according to the alerts shown in the right hand column. The reason for classifying tetrachlorosalicylanilide as structurally complex are as follows. The structure does not indicate direct reactivity, but one of the rings contains a phenolic hydroxyl group and two chlorine substituents (see Fig. 3).

The phenolic hydroxyl group should be significantly ionised at physiological pH (the electronegative CONHAr and Cl groups enhance its acidity, and its estimated pK_a is 6.8, corresponding to being about 50% ionised at neutral pH (NLM Toxnet HSDB, 0000)) and the ionised phenol should be relatively easily oxidised, since the ortho and para Cl substituents can stabilise the resulting phenoxy radical. Several reaction pathways can then be envisaged, some of which would give highly reactive species such as quinones, but without experimental data it is not possible to decide which, if any, oxidation pathway would prevail and whether or not highly potent derivatives would be formed. Because of this uncertainty, tetrachlorosalicylanilide has to be classified as structurally complex, and if nothing were known about its LLNA potency it would have to be classified as a HPC chemical.

Glutaraldehyde is easily identified from its structure as a Schiff base (SB) electrophile, but as a Schiff base domain sensitiser it would not be expected to be very potent. Its assignment to the HPC classification is based on the knowledge that it is used as a protein derivatisation agent, for embalming and preservation purposes (Aptula et al., 2005). The underlying chemistry for this use is based on the fact that glutaraldehyde in solution in solution exists as mixture of dimeric and trimeric self-condensation products, with Michael acceptor properties, that act as cross-linking agents.

We next considered how the insights gained so far could be converted into a predictive strategy.

5. Principles for predicting HPC chemicals

Since sensitisation potency is dependent on ability to covalently modify protein, it should be possible to base prediction of HPC chemicals on reaction chemistry information. Here we suggest a set of chemistry-based rules to identify HPC chemicals. Criteria for success of such rules are correct assignment of a high proportion of the known high potency chemicals (having EC3 < 64 μg/cm^2, and a low number of chemicals with EC3 substantially above 64 μg/cm^2 incorrectly assigned as HPC.

The rules are grouped mainly on the chemical reaction mechanism. For each mechanism, we propose simple rules based only on the chemical structure. These rules are based on established SAR

Table 1
High Potency Category Chemicals (HPC) Chemicals from the Dermal Sensitisation Threshold (DST) dataset.

Chemical name	CAS number	Reaction mechanistic domain	EC3 (μg/cm^2)	HPC alert
Benzo[a]pyrene	50-32-8	S_N2 (pro/pre)	0.225	Bay region epoxide
4′-Hydroxy chalcone	Not known	MA	0.5	QM tautomer
Oxazolone	15646-46-5	MA[a]	0.75	Doubly activated MA
Diphenylcyclopropenone	886-38-4	MA[a]	0.75	Doubly activated MA
Chlorothalonil	1897-45-6	S_NAr	1.0	Multi-activated S_NAr
1-Chloromethylpyrene	1086-00-6	S_N2	2.5	Benzylic, Ar-CH_2-LG
7,12-Dimethylbenz[a]anthracene	57-97-6	S_N2 (pro/pre)	2.5	Bay region epoxide
5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	MA[b]	2.5	>Doubly activated MA
p-Benzoquinone	106-51-4	MA	2.5	Quinone
1-Methyl-3-nitro-1-nitrosoguanidine	70-25-7	S_N2 (pro/pre)	7.5	MeN(NO)–C=X (X = O or NH)
Tetrachlorosalicylanilide	1154-59-2	Complex	10	See text
Bandrowski's base	20048-27-5	MA	10	Di-imine analogue of quinone
4-Nitro-benzene-1,2-diamine	99-56-9	MA (pro/pre)	12.5	Di-imine analogue of quinone
4-Nitrobenzyl bromide	100-11-8	S_N2	12.5	Benzylic, Ar-CH_2-LG
N-Methyl-N-nitrosourea, toxic	684-93-5	S_N2 (pro/pre)	12.5	MeN(NO)–C=X (X = O or NH)
1-Chloro-2,4-dinitrobenzene	97-00-7	S_NAr	15	Doubly activated S_NAr
4-((2-Hydroxyethyl)amino)-3-nitrophenol	65235-31-6	MA (pro/pre)	17.5	Quinone-imine
2,4,6-Trichloro-1,3,5-triazine (cyanuric chloride)	108-77-0	S_NAr	22.5	Multi-activated S_NAr
Toluene 2,4-diisocyanate	584-84-9	Acyl	25	–N=C=O
Glutaraldehyde	111-30-8	SB	25	Protein derivatisation agent (see text)
2′,3′,4′-Trihydroxy chalcone	1482-74-2	MA	27.5	QM tautomer; poison ivy analogue
1,4-Dihydroquinone	123-31-9	MA (pro/pre)	27.5	Quinone
Fluorescein-5-isothiocyanate	3326-32-7	Acyl	35	N=C=S
β-Propiolactone	57-57-8	S_N2	37.5	Primary carbon reaction centre in strained ring
1,4-Phenylenediamine	106-50-3	MA (pro/pre)	40	Di-imine analogue of quinone
Hexyl salicylate	6259-76-3	Anomalous[c]	45	Anomalous
Dimethyl sulphate	77-78-1	S_N2	47.5	$MeOSO_2O$–
2,5-Diamino-toluene	95-70-5	MA (pro/pre)	50	Di-imine analogue of quinone
4-Amino-3-nitrophenol	610-81-1	MA (pro/pre)	50	Quinone-imine
Benzyl bromide	100-39-0	S_N2	50	Benzylic, Ar-CH_2-LG
Benzoyl peroxide	94-36-0	Peroxide	50	Peroxide

Abbreviations: HPC, High Potency Category Chemicals; QM, quinonemethide; LG, leaving group.

[a] Oxazolone (Natsch et al., 2010) and diphenylcyclopropenone both have structural alerts for highly reactive Michael acceptors. The initial Michael adducts are also reactive and the ultimate reaction products with protein nucleophiles are unlikely to be simple Michael adducts.

[b] 5-Chloro-2-methyl-4-isothiazolin-3-one has the structural features of a highly reactive Michael acceptor, and in the absence of any experimental evidence of its reaction chemistry it would be expected to be a strong sensitiser on the basis of its Michael reactivity. In fact, however, it has been found to be more reactive as an S_N2 electrophile, with the sulphur atom as the reaction centre (Alvarez-Sánchez et al., 2003).

[c] Hexyl salicylate appears anomalous. Although salicylate esters can act as acyl transfer electrophiles, they are not highly reactive and would not be expected to be highly potent sensitisers. LLNA data are available for several salicylate esters, and apart from hexyl salicylate none has an EC3 value lower than 250 μg/cm^2. Furthermore, several of these salicylates, including hexyl salicylate, have been found to be non-sensitising in human assays. Thus it appears that the reported EC3 value of 45 μg/cm^2 for hexyl salicylate may not be well representative of the true potency.

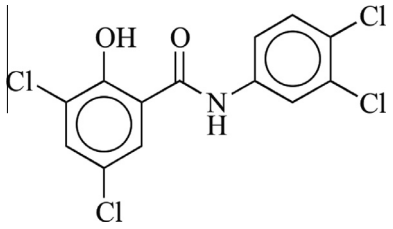

Fig. 3. Chemical structure of tetrachlorosalicylanilide.

and QMM principles relating chemistry to potency, and on well-established physical organic chemistry principles relating chemical properties to structure. (e.g. Isaacs, 1995).

The first step is to assign the chemical to its reaction mechanistic applicability domain. This can be done applying the rules given by Aptula and Roberts (2006), reproduced in slightly modified form in Fig. 1. For each reaction mechanistic domain, we have developed rules assigning compounds to the high potency category.

The HPC rules are:

1. *Compounds used as protein derivatisation agents*, are automatically assigned as potential HPC.
2. *Direct acting Michael acceptors*. For direct Michael acceptors, EC3 can be predicted from a QMM based on experimental kinetic data (Roberts and Natsch, 2009; Natsch et al., 2011).

For DST purposes structure-based rules, based on well established chemical relationships linking structural changes to kinetic changes, may be applied as follows when deciding whether a Michael acceptor should be classified as HPC:

2a. Quinones, di-imines and quinone-imines should be automatically assigned as potential HPC. Compounds that can have tautomeric forms with a quinone-type (*ortho* or *para*) or quinone-methide-type (*para* only; *ortho* quinone-methides are highly unstable) structure should also be included, e.g. 4′-hydroxychalcone (see Fig. 4).

2b. Michael acceptors with a single activating group selected from –CHO, –COR, –CO_2R, –$CONR_2$ can be assumed to be less potent than the DST. However, electronegative substituents on the double bond, such as halogen, alkoxide –OR, thio –S–, increase reactivity and such compounds with *CHO*, *–COR*, *–CO_2R*, *–$CONR_2$* activating groups should be assigned HPC. Michael acceptors of general structure C=C–X, where X is a stronger activating group than carbonyl (e.g. NO, –NO_2, SO_2R, SOR, SO_3R, 2,4-dinitrophenyl. . .) should be treated as HPC. Alk-1-ene-1,3-sultones (Fig. 5) are examples with SO_3R as the activating group (Roberts and Williams, 1982; Roberts et al., 2007d). The C16 homologue (R=$C_{13}H_{27}$ in Fig. 5) is positive in the LLNA (Haneke et al., 2001) and the C10–C16 homologues are all highly potent in humans and in guinea pigs (Ritz et al., 1975).

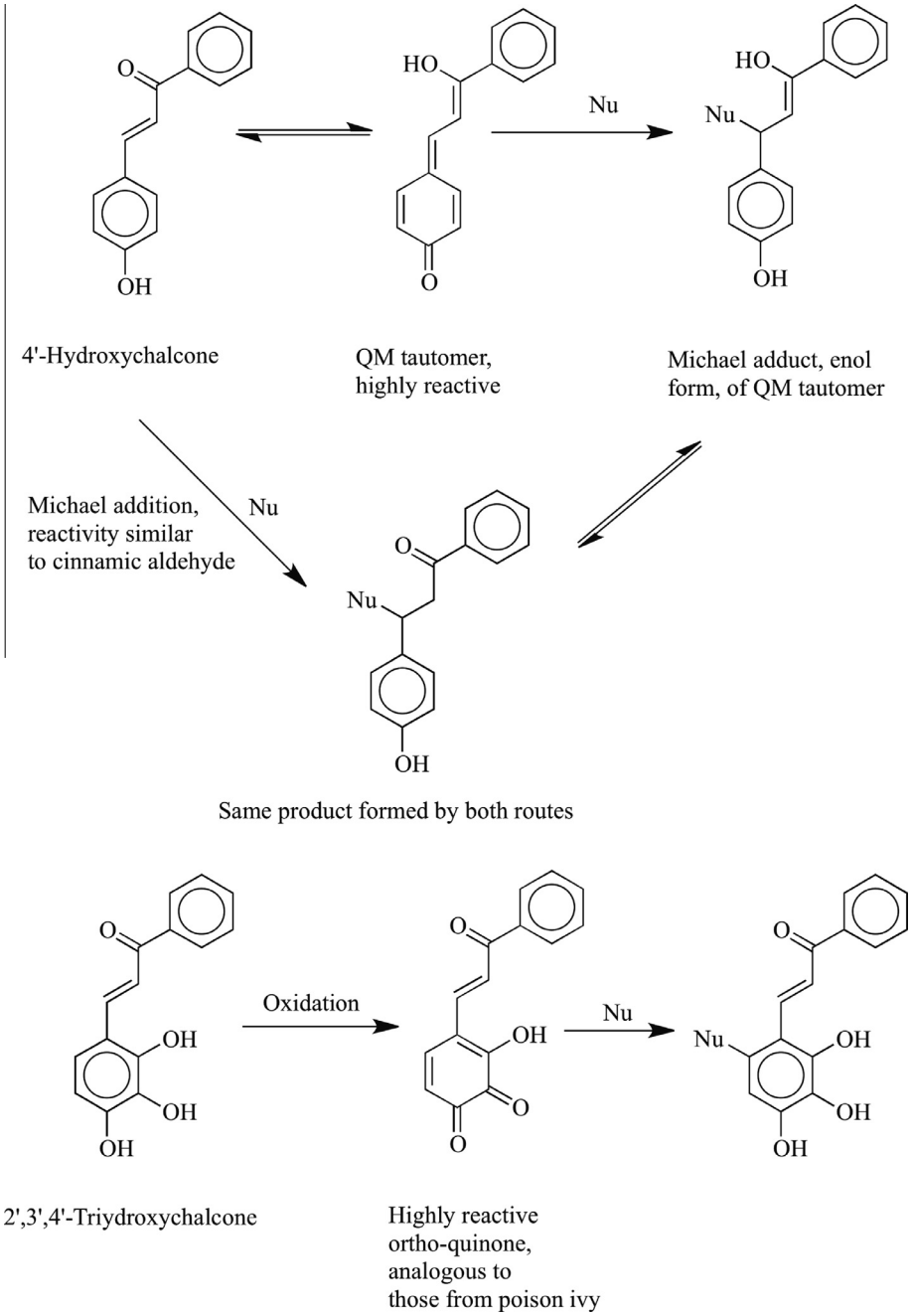

Fig. 4. Michael addition reactivity of 4′-hydroxychalcone.

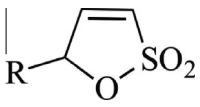

Fig. 5. Chemical structure of alk-1-ene-1,3-sultones.

Compounds that can be formally regarded as hydrogen halide adducts of Michael acceptors, i.e. with the sub-structure X–C–CH–M where X is halogen (or pseudohalogen) and M is a strong Michael activating group should be treated as HPC, since they can be expected to lose HX *in cutaneo* with formation of the reactive Michael acceptor –C=C–M. Examples are 2-bromo- and 2-chloroalkane-1,3-sultones (Fig. 6), which are similarly potent to the corresponding alk-1-ene -1,3-sultones (Ritz et al., 1975).

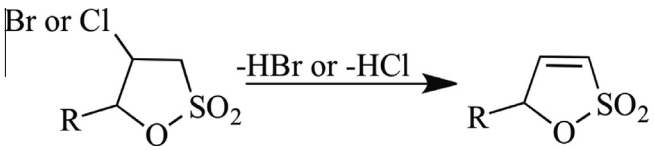

Fig. 6. Chemical structure and schematic of 2-haloalkane-1,3-sultones.

2c. Michael acceptors with more than one activating group on the double bond should be assigned as HPC. Dimethyl fumarate (trans MeOCOCH=CHCOOMe) and maleic anhydride are examples whose EC3 values (87.5 and 42.5 μg/cm^2 respectively) are very close to the HPC threshold. There are likely to be some exceptions to this rule, for example if one or both

of the activating groups is/are relatively weak (e.g. $-CONH_2$). Another example of an exception is octocrylene, $Ph_2C{=}C(CN)CO_2R$ (R = 2-ethylhexyl), whose EC3 is 7.7% (Karlsson et al., 2011). In this case the two activating groups (CN and CO_2R) are on the same carbon atom, and this should give rise to high reactivity as a Michael acceptor. However, the two groups also facilitate the reverse reaction of the Michael adduct. The net result is that the compound does not form a stable Michael adduct with cysteine peptide, but reacts with amino groups RNH_2 to form Schiff base adducts $Ph_2C{=}NR$. In effect the $=C(CN)CO_2R$ group behaves as a pseudo =O group (Karlsson et al., 2011). This is a case where the structure-based rule would wrongly assign as HPC, but an experimental kinetic study would correctly assign as non-HPC. It is possible that many other Michael acceptors with two activating groups on the same carbon atom may react similarly to octocrylene, and would be mis-classified as HPC by this structure-based rule. Since we cannot at present confidently predict such cases, in the absence of experimental data all doubly activated Michael acceptors should be classified as HPC.

3. *Pro/pre-Michael acceptors*
   3a. 1,2- and 1,4- dihydroxy, di-amino, aminohydroxy benzenes (including heteroaromatics where one or more carbon atoms of the benzene is replaced by a nitrogen atom, and applying where the aromatic ring is fused to other rings, e.g. as part of a naphthalene structure) are automatically assigned as potential HPC. Many, but not all compounds of this type have HPC potency (Aptula et al., 2009), so some compounds will be overpredicted – at present there is insufficient mechanistic understanding to discriminate within this group of chemicals. 1,3-Dihydroxy, di-amino and aminohydroxy compounds can also be oxidised to Michael acceptors, but they are less potent than HPC (Aptula et al., 2009) and are not included in this rule.
   Other pro/pre-Michael acceptors that are activated by oxidation of allylic alcohol groups to carbonyl groups so as to form an activating group are not treated as HPC – these are usually weak or at worst moderate sensitisers.
4. *Schiff base electrophiles*. Apart from formaldehyde and glutaraldehyde, which both have special chemistry and fall into class 1 above, none of the SB electrophiles that have been tested in the LLNA fall into the HPC range. Potency for the SB domain is well modelled by a QMM based on reactivity and hydrophobicity parameters (Roberts et al., 2006).
   On this basis we can formulate simplified rules based on structural alerts:
   Simple mono-aldehydes, with the sub-structure $-CR_2CHO$, where R is H, alkyl or aryl, are not reactive enough to be HPC. Compounds with a keto or aldehyde group bonded directly to a second carbonyl group (–COR or CO_2R) are more reactive, and if sufficiently hydrophobic they may fall into the HPC range. For example, $CH_3COCO_2CH_3$ (methyl pyruvate) has EC3 = 600 μg/cm^2, so is not HPC, but $CH_3COCO_2C_6H_{13}$ (hexyl pyruvate) is predicted to have EC3 = 75 μg/cm^2, i.e. close to the HPC borderline. PhCOCOMe has EC3 = 1.55%, so is not HPC, but introduction of two methylene groups, i.e. $C_2H_5C_6H_4COCOMe$, or $MeC_6H_4COCOC_2H_5$ or $PhCOCOC_3H_7$ would lead to calculated EC3 values of 75 μg/cm^2, i.e. near-borderline HPC. A carbonyl group bonded to two other carbonyl groups, for example the central CO group (bold highlighted) in dimethyl ketomalonate, MeO.CO**CO**.CO.OMe is even more reactive, and compounds with this sub-structure should be assigned as HPC. Dimethyl ketomalonate is predicted to have an EC3 value of 7.5 μg/cm^2. To apply these rules it may be convenient to read across from reference chemicals with predicted EC3 values close to the HPC borderline (Table 2). So rule 4a is:
   4a. Schiff base electrophiles meeting the HPC criteria. Reference SB domain compounds predicted to have EC3 values close to the HPC borderline value of 64 μg/cm^2 are presented in Table 2. Homologues of these reference compounds should be classified HPC if more hydrophobic and non-HPC if less hydrophobic.
5. *Acyl transfer agents*. The acyl transfer domain includes several sub-groups of chemicals – activated esters (i.e. esters of alcohols with relatively low pKa, such as phenols), anhydrides, acid chlorides, isocyanates, that can react with amines RNH_2 to produce amide linkages RNH–CO–. There are no QMMs for this domain, mainly because in no single sub-group are there sufficient compounds with EC3 data to develop a QMM, but there are clear indications that potency depends both on reactivity and on hydrophobicity. On that basis we define the following rules:
   5a. Isocyanates and isothiocyanates, i.e. compounds with an –N=C=O group or an –N=C=S bonded to carbon, should automatically be assigned HPC. These groups are known to be highly electrophilic, the nucleophile attacking the central carbon atom (Patai, 1977).
   5b. Anhydrides, i.e. compounds with the substructure –CO.O.CO–, should be assigned HPC if the log *P* value is greater than 1 (this is based on an experimental EC3 value of 90 μg/cm^2 and log *P* value of 1.19 for phthalic anhydride). Activated esters and acid chlorides, on the basis of available LLNA data, are not HPC. Presumably activated esters are not sufficiently reactive (with the possible exception of β-propiolactone, which can also react as an S_N2 nucleophile and is considered under that classification). Since acid chlorides are more reactive than anhydrides (Wade, 2012), it might be expected that they would fall into the HPC category, but the LLNA evidence does not support this. Possibly this reflects their hydrolytic instability.
6. *S_N2 electrophiles*. Although there are QMMs for S_N2 electrophiles, relating potency to a combination of reactivity and hydrophobicity (log *P*), the reactivity parameters are quite complex, based on using the Swain–Scott relationship to estimate relative rate constants for a hypothetical nucleophile with a Swain–Scott *n* value of 6.0, corresponding roughly to a cysteine unit (Roberts et al., 2007a). This is an area where an experimental programme of measuring rate constants for compounds with known EC3 values would pay dividends. Based on current insights, structure-based rules may be applied as follows:
   6a. Benzylic halides and pseudohalides, $ArCH_2X$ should be assigned HPC. The known examples that have been tested in the LLNA all have EC3 values below the DST (Roberts et al., 2007a). Allylic primary halides and pseudohalides $-C{=}C-CH_2-X$, and halides and pseudohalides with substructures $-COCH_2X$, $-OCH_2X$ should also be considered likely HPC, on the basis of their known high reactivity comparable to that of benzylic halides (Hine, 1962). Pseudohalide should

Table 2
Reference Schiff base domain compounds predicted to have EC3 values close to the High Potency Category Chemicals (HPC) borderline value of 64 μg/cm^2.

Compound	Calculated EC3 (μg/cm^2)
n-$C_4H_9OCO.CHO$ butyl glyoxylate	65
$CH_3COCO_2C_6H_{13}$ hexyl pyruvate	75
$C_2H_5C_6H_4COCOCH_3$	75
$CH_3C_6H_4COCOC_2H_5$	75
$PhCOCOC_3H_7$	0.3
Any with –COCOCO–	<64

be interpreted as a leaving group –X for which the corresponding XH is strongly acidic. –SCN, $-OSO_2R$ and $-OSO_20R$ are examples. –OCOR and $-N^+R_3$ groups are not reactive enough to trigger this rule.

6b. Methylating agents, CH_3-X, should be assigned HPC for X=OSO_2OR (any R) and for X = OSO_2R (R = aryl, R = ⩾C12 alkyl or alkenyl). Also N-methyl nitroso amido compounds, Me–N(N=O)–[C=O/N–]– should be assigned HPC. This rule is based on the known high reactivity and high LLNA potency of methylating agents of these types (Roberts et al., 2007a).

6c. Special cases include β-propiolactone and the diol epoxides derived from PAHs (Polycyclic Aromatic Hydrocarbons). PAHs with carcinogenicity alerts are assigned HPC. β-Propiolactone is a unique case, and for PAHs a general rule – any compound with 4 or more aromatic rings fused together – is appropriate for present purposes. Saturated alkyl halides are not sufficiently reactive, and are non-HPC.

7. *S_NAr electrophiles*. The EC3 values of S_NAr electrophiles can be predicted from substituent constants of the leaving group X and of the activating groups Y, and also from rate constants for reaction with cys-peptide. (Roberts and Aptula, 2014; Natsch et al., 2011). A more simple structure-based rule, derived from the QMM reported by Roberts and Aptula (2014), which will identify HPC S_NAr compounds, although some non-HPC compounds may also be mis-assigned as HPC, is:

   7a. S_NAr electrophiles with more than one activating group. This covers any aromatic compound with a leaving group and two or more activating groups selected from nitro, nitroso, ring nitrogen (e.g. as in 4-chloro-3-nitropyridine) or groups more electronegative than nitro, in ortho + ortho or ortho + para positions. For HPC purposes, the leaving group X may be considered as any covalently bonded group other than hydrocarbon, H, OH, amino (NH_2, NHR, NR_2), $CONH_2$, CONHR, $CONR_2$, CO_2R (R = H, alkyl or aryl), CO_2H.
8. *Organic peroxides (sub-structure C–O–O–)*. Most of the organic peroxides that have been investigated, mainly in the context of research on allergenic autoxidation-derived contaminants of pre-haptens, have been found to be strong sensitisers, although the chemical mechanism is still not completely understood (Karlberg et al., 2013). Compounds with this substructure should therefore be classified as HPC. However, the corresponding pre-haptens from which autoxidation-derived hydroperoxides can be formed should not be classed as HPC. Although many of the organic peroxides that have been tested are strong sensitisers, many have EC3 values above 64 μg/cm^2, so this rule is somewhat over-protective. Compounds with O-halogen (organic hypohalites) or N-halogen bonds should be considered as peroxide analogues and also classified as HPC.
9. *Structurally complex chemicals*. This classification is to cover those compounds that cannot be confidently assigned to any of the other reaction mechanistic domains, and cannot be confidently classified as non-reactive. A compound may need to be assigned to this group if it possess an unfamiliar substituent whose reactivity (or non-reactivity) is uncertain, or if it possesses a number of substituents, that in combination could possibly, but not predictably, give rise to significant reactivity. Note that in the context of this paragraph, the term "reactivity" applies not just to the ability of the compound itself to react with protein, but also to its ability to be converted under test or exposure conditions to protein reactive species (i.e. its ability to behave as a pro/pre-hapten). Much more so than with the other reaction mechanistic domains, assignment to this classification is a subjective process, depending on the knowledge/experience of the person making the assignment. Chemicals for which this rule applies should be treated as HPC and the DST should not be applied.
10. *Pre-electrophiles*. These are defined for present purposes as chemicals that when pure are non-sensitisers or only weak sensitisers, but which become more potent when allowed to undergo autoxidation. Although autoxidation products such as allylic hydroperoxides (which are assigned HPC by rule 8) can themselves be strong sensitisers (Karlberg et al., 2013), they do not reach sufficiently high levels in the oxidised material to make any known pre-electrophile sufficiently potent to be classified as HPC.

6. Test of the HPC rules against the DST dataset

Since the HPC rules given above were developed from consideration of the chemicals with EC3 < 64 μg/cm^2 in the DST dataset, all of the chemicals (with the exception of hexyl salicylate, which is regarded as anomalous) in that dataset are assigned as HPC by the rules. Table 3 shows for each compound the rule used to assign it as HPC.

Table 4 summarises the performance of the HPC rules for the various potency ranges above the DST of 64 μg/cm^2.

More than half of the compounds with EC3 below 250 but above 64 (all these would be classed as strong sensitisers) are predicted HPC. To that extent the HPC rules are over-protective. For moderate and weak sensitisers (250–2500 and 2500–25,000 μg/cm^2 respectively), only a small proportion are predicted HPC. Many of these are predicted by rule 3 (pre/pro MA, for which there is currently insufficient SAR understanding to be fully predictive).

Table 3
High Potency Category Chemicals (HPC) from the Dermal Sensitisation Threshold (DST) dataset listed in order of the HPC rules.

Chemical name	HPC rule
Glutaraldehyde	1
4′-Hydroxy chalcone	2a
p-Benzoquinone	2a
Bandrowski's base	2a
2′,3′,4′-Trihydroxy chalcone	2a
5-Chloro-2-methyl-4-isothiazolin-3-one	2b
Oxazolone	2c
Diphenylcyclo-propenone	2c
4-Nitro-benzene-1,2-diamine	3
4-((2-Hydroxyethyl)amino)-3-nitrophenol	3
1,4-Dihydroquinone	3
1,4-Phenylenediamine	3
2,5-Diamino-toluene	3
4-Amino-3-nitrophenol	3
Toluene 2,4-diisocyanate	5a
Fluorescein-5-isothiocyanate	5a
1-Chloromethylpyrene	6a
4-Nitrobenzyl bromide	6a
1-Methyl-3-nitro-1-nitrosoguanidine	6b
N-Methyl-N-nitrosourea, toxic	6b
Dimethyl sulphate	6b
Benzyl bromide	6b
Benzo[a]pyrene	6c
7,12-Dimethylbenz[a]anthracene	6c
β-Propiolactone	6c
Chlorothalonil	7
1-Chloro-2,4-dinitrobenzene	7
2,4,6-Trichloro-1,3,5-triazine (cyanuric chloride)	7
Benzoyl peroxide	8
Tetrachlorosalicylanilide	9
Hexyl salicylate	None

Abbreviation: HPC, High Potency Category Chemicals.

Table 4
Summary of predictions for compounds with EC3 > 64 μg/cm^2.

EC3 range (μg/cm^2)	Rules performance
64–128 (up to factor of 2 > DST)	9/13 predicted HPC
128–250	9/13 predicted HPC
250–2500 (1–10%)	6/88 predicted HPC
2500–25,000 (10–100% and NS)	2/75 predicted HPC

Abbreviation: HPC, High Potency Category Chemicals.

Table 5
Summary of predictions for the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) LLNA database.

EC3 range (μg/cm^2)	Rules performance
<64 (below DST)	7/8 predicted HPC
64–128 (up to factor of 2 > DST)	3/6 predicted HPC
128–250	3/6 predicted HPC
250–2500 (1–10%)	5/38 predicted HPC
2500–25,000 (10–100% and NS)	0/14 predicted HPC

Abbreviations: DST, Dermal Sensitisation Threshold; HPC, High Potency Category Chemicals.

Overall the rules correctly predict all the chemicals with EC3 < 64 μg/cm^2 (hexyl salicylate being considered a "false HPC"); about 70% of chemicals that are strong sensitisers but not genuinely HPC are predicted as HPC; about 7% of moderate sensitisers are predicted HPC; about 2.5% of weak sensitisers and non-sensitisers are predicted HPC. On the basis of the DST dataset, the rules are to a large extent conservative.

7. Further test against the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) LLNA database

We next assessed the HPC rules against LLNA data in the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) LLNA database downloaded from http://ntp.niehs.nih.gov/?objectid=40AFDDF1-D2B6-1850-EE321D717F2910 20 in June 2014. After removing multiple entries, inorganic chemicals (not covered by the DST), chemicals already included in the DST database, and entries for which the dose–response pattern does not enable a reliable EC3 value to be estimated, the database contains 72 sensitisers with EC3 values. Several of these are pharmaceutical intermediates. Table 5 summarises the breakdown of EC3 values and HPC predictions.

The breakdown in Table 5 demonstrates the extent to which the rules are conservative. 50% of the chemicals that are strong sensitisers but with EC3 > DST are predicted HPC, 13% of the moderate sensitisers are predicted HPC, none of the weak sensitisers or non-sensitisers are predicted HPC. The highest EC3 value for a chemical predicted HPC was 2.4%, 611 μg/cm^2.

Of the 54 chemicals assigned as non-HPC by the rules, only one has EC3 < 64 μg/cm^2. This is ethyl-2-(hydroxymethyl)-1,3-propanediol triacrylate, EC3 0.13%, i.e. 32 μg/cm^2. This is a factor of 2 below the DST. Based on the electrophilic reactivity of the acrylate ester group, reacting as a Michael acceptor, mono-acrylates would be expected to have EC3 values close to 1% (i.e. above the DST value by a factor of about 4). In practice, most acrylates, hydroxyethyl acrylate being an exception (Roberts and Natsch, 2009), have EC3 values substantially greater than 1%, attributed to their strong tendency to polymerise in exposure to air or moisture. The most likely reason for the atypical high potency of ethyl-2-(hydroxymethyl)-1,3- propanediol triacrylate is that it can act as a protein cross-linking agent, having 3 reactive groups (each alone could correspond to EC3 close to 1% if not lost by polymerisation) that will not lose reactivity after the first one has reacted. So we should suspect higher potency. Since, for this chemical, the difference between the EC3 and the DST is only a factor of 2, we have not modified the HPC rules in light of this result.

Overall the HPC rules perform similarly well against the NICEATM LLNA database as they do against the DST database.

8. Discussion

The fundamental SAR principle that skin sensitisation potency is dependent on the chemical's ability to react covalently with skin

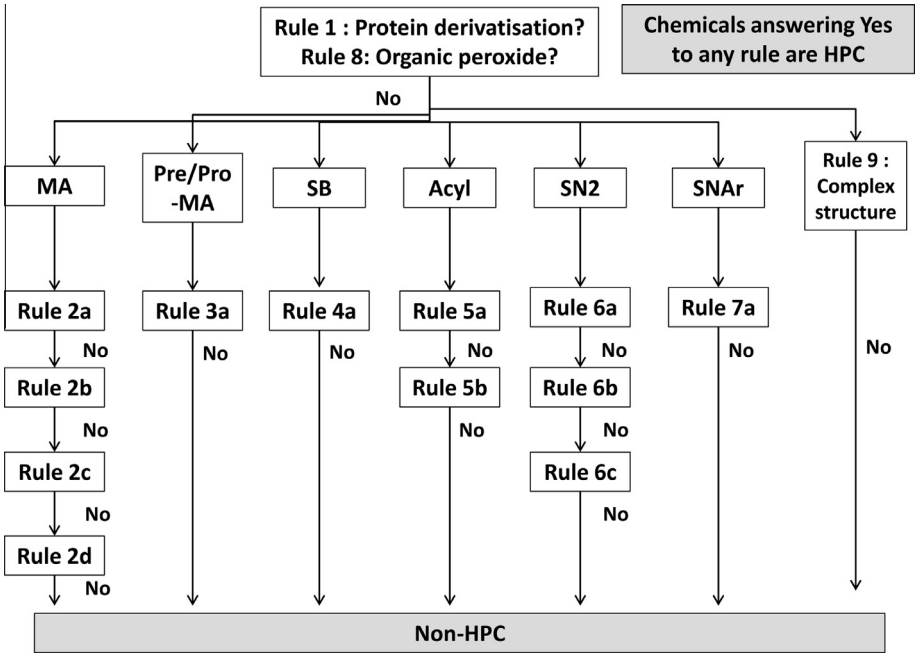

Fig. 7. Workflow for assignment of chemicals as High Potency Category Chemicals (HPC) or non-HPC. Following the workflow, it is not necessary to check against rule 10 (pre-haptens), since any prehapten not triggering Yes to any of rules 1–9 can be regarded as non-HPC. *Abbreviations:* HPC, High Potency Category Chemicals.

proteins, originally proposed by Landsteiner and Jacobs (1936) has been verified by numerous SAR studies on various types of chemicals. This principle, coupled with development of parameters to model protein binding ability, based on well-established physical organic chemistry principles relating chemical properties to structure, has enabled QMMs to be developed for several of the major types of skin sensitisers.

We have applied these principles to the development of a set of rules (the HPC rules) based on structural alerts, enabling chemicals with EC3 values below the DST of 64 μg/cm^2 to be predicted with high reliability. Of the 33 compounds with reported EC3 values <64 μg/cm^2 in the two databases studied, 31 are correctly predicted by the HPC rules. For one of the chemicals not predicted, (hexyl salicylate) the LLNA result is considered to be anomalous, and is not in agreement with human data. The other chemical not predicted (ethyl-2-(hydroxymethyl)-1,3-propanediol triacrylate) has an EC3 value below the DST by a factor of only 2.

Being classified as HPC by these rules does not necessarily mean that a chemical should not be used in consumer products. It simply means that the chemical should only be used, even at levels below the DST, after investigation of its potency. This investigation may simply require application of mechanistic organic chemistry and SAR/QMM expertise, or it may require experimental chemical data to be generated. We intend in a future publication to discuss further how organic chemistry (experimental and theoretical) together with SAR/QMM principles, can be applied to such cases.

In the most difficult cases a confident assessment of its potency may be impossible without an LLNA study. A method for estimating potency from a single dose, in a modified version of the rLLNA, has recently been derived (Roberts, 2015) and would be useful in this situation.

The present HPC rules as they stand can be applied manually without specialist chemistry expertise, to decide whether the DST is applicable to a given chemical. We intend as a next step to formulate them similarly to the Cramer rules (Cramer et al., 1978), in a way that can be encoded *in silico*. For the present, Fig. 7 shows the workflow for assignment of chemicals as HPC or non-HPC. Note that being assigned HPC does not necessarily mean that a chemical's real potency would correspond to an EC3 < DST, although that will often be the case; it simply means that the DST approach is not applicable to that chemical.

This approach for identifying HPC chemicals complements the reactive DST derived in our accompanying paper, and enables the non-animal strategy presented in that paper to be applied for setting maximum use levels of new chemicals. However, it is important to note that the "structural alerts only" rules have been developed with more emphasis on specificity than on selectivity, i.e. preferring to be overprotective than to be underprotective. A chemical that is classified HPC simply on the basis of structural alerts, need not be rejected out of hand, since there may still be a possibility that after further experimental or computational investigation, it may turn out to be usable at the DST level.

Conflict of interest

None declared.

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