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Determinants of skin sensitisation potential

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ABSTRACT: Skin sensitisation is an important toxicological endpoint. The possibility that chemicals used in the workplace and in consumer products might cause skin sensitisation is a major concern for individuals, for employers and for marketing. In European REACH (Registration, Evaluation, and Authorisation of Chemicals) legislation, the sensitising potential should therefore be assessed for chemicals below the 10 ton threshold. Development of methods for prediction of skin sensitisation potential without animal testing has been an active research area for some time, but has received further impetus with the advent of REACH and the EU Cosmetics Directive (EU 2003). This paper addresses the issue of non-animal based prediction of sensitisation by a mechanistic approach. It is known that the sequence of molecular, biomolecular and cellular events between exposure to a skin sensitiser and development of the sensitised state involves several stages, in particular penetration through the stratum corneum, covalent binding to carrier protein, migration of Langerhans cells, presentation of the antigen to naïve T-cells. In this paper each of these stages is considered with respect to the extent to which it is dependent on the chemical properties of the sensitiser. The evidence suggests that, although penetration of the *stratum corneum*, stimulation of migration and maturation of Langerhans cells, and antigen recognition are important events in the induction of sensitisation, except in certain specific circumstances they can be taken for granted. They are not important factors in determining whether a compound will be a sensitiser or not, nor are they important factors in determining how potent one sensitiser will be relative to another. The ability to bind covalently to carrier protein is the major structure-dependent determinant of skin sensitisation potential. A chemistry-based prediction strategy is proposed involving reaction mechanistic domain assignment, reactivity and hydrophobicity determination, and application of quantitative mechanistic modelling (QMM) or read-across. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS: skin sensitiser; penetration; protein binding; migration; antigen recognition

Introduction

Skin sensitisation is an important toxicological endpoint. The possibility that chemicals used in the workplace and in consumer products might cause skin sensitisation is a major concern for individuals, for employers and for marketing. In European REACH (Registration, Evaluation, and Authorisation of Chemicals) legislation (EU, 2006), the sensitising potential should therefore be assessed for chemicals below the 10 ton threshold (Annex V). According to an ECB assessment of additional testing needs under REACH, the highest number of tests is required for this endpoint (EU, 2003).

Skin sensitisation is a T-cell mediated immune response. Research dating back more than 7 decades has established a very strong, although still incomplete, mechanistic understanding of the chemical and biological basis of skin sensitisation (Lepoittevin *et al*., 1997; Roberts *et al*., 2007a, 2007b; Rustenmeyer *et al*., 2006; Ryan *et al*., 2005) which can be summarised briefly as follows.

The sensitising chemical reacts with skin protein in the epidermis so as to make it antigenic. The antigenic protein is processed by Langerhans cells in the epidermis and these Langerhans cells are consequently stimulated to migrate to a lymph node where they present the antigen to naïve T-cells. T-cells with receptors able to specifically recognise the antigen are stimulated to proliferate and circulate throughout the body. Sensitisation has now been induced, i.e. the subject is now sensitised. These events take place during the induction stage of a sensitisation test, and are collectively referred to as the induction phase or afferent phase.

On subsequent exposure to the same sensitiser, or a second sensitiser cross-reactive with the first, reaction with protein and processing of the resulting antigenic protein by Langerhans cells again occurs, after which the antigen presented by the Langerhans cells is recognised by the circulating T-cells, triggering a cascade of biochemical and cellular processes that produce the clinical sensitisation response. These events take place at the challenge stage of a sensitisation test and are collectively referred to as the elicitation phase or efferent phase.

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Guinea-pig testing was widely used for the identification of skin sensitising chemicals up to the mid 1990s. These tests involve an induction stage in which the animals are treated with the test compound, followed by a challenge stage where the animals are again treated with the test compound and examined to determine whether a sensitisation response has occurred (Maurer *et al*., 1994). More recently the murine local lymph node assay (LLNA) has tended increasingly to supplant guinea-pig testing. In the LLNA mice are treated with the test compound applied topically, and the stimulation of proliferative responses by draining lymph node cells is measured and compared with the response in control animals. A test/control ratio (referred to as the stimulation index, SI) of 3 or more is taken as an indication of sensitisation, and potency is quantified as EC3, (EC for Effect Concentration), the test concentration which gives a stimulation index of 3 (Kimber *et al*., 2002). This method has been evaluated extensively and validated internationally, and is now embraced widely as a guideline method for skin sensitisation hazard identification (Basketter *et al*., 2002).

Development of methods for prediction of skin sensitisation potential without animal testing has been an active research area for some time, but has received further impetus with the advent of REACH and the EU Cosmetics Directive (EU, 2003).

The sequence of molecular, biomolecular and cellular events between exposure to an allergen and development of the sensitised state involves several stages, including penetration through the stratum corneum, covalent binding to carrier protein, migration of Langerhans cells, presentation of the antigen to naïve T-cells (Kimber and Dearman, 2003; Jowsey *et al*., 2006). In attempting to predict sensitisation potential from chemical data, it is necessary to consider which of these stages are dependent on the chemical properties of the allergen, and which are not.

It is convenient, following the precedent of Jowsey *et al*. (2006), to consider the above stages of the skin sensitisation process in terms of an analogy with a set of hurdles which a chemical must negotiate successfully in order to cause skin sensitisation. A dose of chemical applied to the skin in a sensitisation test or a human exposure situation consists of many millions of molecules (for dinitrochlorobenzene at its LLNA EC3 a single dose, 25 µl at 0.6% by weight, consists of about 4.5 million million million molecules). Some of these will fail at one hurdle, some at another, but some will negotiate all hurdles and these will be the ones which contribute to sensitisation. Of the molecules of the chemical that come into contact with the skin, only those which negotiate all the hurdles can succeed in contributing to sensitisation. The more molecules which fail at these hurdles, the less potent the sensitiser will be.

It is therefore appropriate to consider the nature of these cutaneous hurdles and to consider the chemical properties which determine how well or how badly the molecules can negotiate them. This is the purpose of the present paper.

Penetration of the Stratum Corneum

It seems logical to assume that this first hurdle should be an important one. It seems an obvious argument that if a compound cannot penetrate the stratum corneum (SC) then it should not be able to sensitise and that, other things being equal, a compound with greater ability than another to penetrate the SC will be the stronger sensitiser of the two. However, there are several pieces of evidence against SC penetration being a determining factor for sensitisation potential, and very little evidence in favour.

Role of Partition Coefficient in Structure-Sensitisation Correlations

As pointed out by Basketter (1998), quantitative structure activity relationships for skin sensitisers always tend to include a hydrophobicity parameter (usually log P, P being the octanol–water partition coefficient), as well as a reactivity parameter (typically log k, or an equivalent calculated parameter, k being the rate constant for reaction with a model nucelophile). Several of these are reviewed by Barratt *et al*. (1997), and a more recent example is reported by Aptula *et al*. (2006). At first sight it may seem reasonable to assume that the hydrophobicity term models the penetration of the sensitiser through the stratum corneum and the reactivity term models covalent binding to the carrier protein in the epidermis. This assumption is often made (e.g. de Silva *et al*., 1996; Ashby *et al*., 1995; Miller *et al*., 2005), and the model which appears to be implicitly assumed may be summarised:

Sensitiser → Penetrated sensitiser Rate constant ∝ P

Penetrated sensitiser → Carrier protein binding Rate constant ∝ k

Thus, it is implied, overall rate of carrier protein binding ∝ Pk

However, it is easily demonstrated that this model does not lead to dependence on both reactivity and hydrophobicity.

If k_p and k_r are the rate constants for penetration and reaction with carrier protein respectively, and D is the concentration of sensitiser applied at the skin surface, then the extent of carrier protein binding PB after time t is given by:

$$PB = D\{1 + [k_r\exp(-k_p t) - k_p\exp(-k_r t)]/(k_p - k_r)\} \quad (1)$$

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This is the integrated kinetic expression for two consecutive first order or pseudo-first order processes (Frost and Pearson, 1961). Since both penetration rates and reaction rates for sensitisers can range over several orders of magnitude, in the vast majority of cases either $k_p >> k_r$ or $k_p << k_r$ will apply. For the situation where penetration is faster than reaction, i.e. $k_p >> k_r$, the expression is simplified to:

$$PB = D[1 - \exp(-k_r t)] \quad (2)$$

i.e. the extent of protein binding is dependent on the reaction rate but not on the penetration rate. For the situation where the reaction is faster than penetration, i.e. $k_p << k_r$, the expression is simplified to:

$$PB = D[1 - \exp(-k_p t)] \quad (3)$$

i.e. the extent of protein binding is dependent on the penetration rate but not on the reaction rate.

Thus, if carrier protein binding is simply a consecutive process of penetration followed by reaction, structure-activity relationships based on both reactivity and hydrophobicity would not be expected.

The foregoing is a mathematically formalised presentation of the principle of the rate determining step: the overall rate of a process consisting of a sequence of steps is equal to the rate of the slowest step.

In fact the RAI (relative alkylation index) model relating sensitisation potency to a combination of reactivity and hydrophobicity was originally derived (Roberts and Williams, 1982) without taking penetration into account. The role of the hydrophobicity term was to model the partitioning of the sensitiser into aqueous biological fluid (leading to elimination) in competition with reaction with lipid-bound nucleophiles (leading to sensitisation).

In summary, the observation that skin sensitisation potency depends both on reactivity and on hydrophobicity is not evidence for dependence of potency on SC penetration ability. It is, however, evidence that the reaction with carrier protein occurs in a hydrophobic environment.

If it is accepted that the epidermal reaction of the sensitiser with carrier protein (contributing to sensitisation) occurs in competition with an inversely log P-dependent epidermal process (leading to loss of sensitiser), then it remains conceivable that SC penetration ability may contribute to potency, if it is slower than the epidermal processes, by affecting the bioavailability of the sensitiser. At this point it is appropriate to define rigorously what we mean by bioavailability in the context of the skin sensitisation process. As we define it, bioavailability is the proportion of the sensitiser applied to the outer skin which reaches the epidermis in the timescale of the skin sensitisation assay, thereby becoming available to contribute to sensitisation, in competition with other processes. As we will now discuss, the evidence suggests that, except in certain specific circumstances, bioavailability is a constant proportion of the dose applied to the skin.

Quantitative Mechanistic Models for Skin Sensitisation

Many quantitative models correlating sensitisation potential with chemical parameters have been reported, which do not require SC penetration to be invoked (Roberts and Williams, 1982; Roberts *et al*., 1983; Roberts, 1987, 1995; Roberts and Basketter, 1990 and 1997; Basketter *et al*., 1992; Franot *et al*., 1994; Mekenyan *et al*., 1997; Aptula *et al*., 2006). Most of these are based on a combination of a reactivity parameter with a hydrophobicity parameter. It is conceivable that the hydrophobicity parameter in these correlations could be serving a dual purpose, contributing firstly to modelling the competition between carrier protein binding and loss of sensitiser from the epidermis by epidermal partitioning, and secondly to modelling the bioavailability of the sensitiser, i.e. the extent to which it penetrates through the SC. However, ability to penetrate is a function of more than hydrophobicity alone. It can be expressed in terms of the permeability coefficient K_p, which can be related to log P and molecular weight (MW) (Potts and Guy, 1992):

$$\log K_p = 0.71 \log P - 0.0061MW - 2.72 \quad (4)$$

It follows that if the log P term in a correlation of sensitisation potency is modelling both epidermal partitioning and bioavailability, then the correlation should be improved by adding molecular weight as an extra parameter. Furthermore, the regression coefficient for the molecular weight parameter should be negative. To test this concept, we re-analysed the QMM reported by Aptula *et al*. (2006) for the 'Schiff base domain', i.e. compounds which are in principle capable of sensitising via reaction of aliphatic aldehyde or keto groups with protein nucleophiles. The reported correlation between LLNA pEC3 values and a combination of a reactivity parameter ($\Sigma\sigma^*$, the sum of Taft substituent constants of the groups bonded to the reactive carbonyl group) with log P is:

$$pEC3 = 1.12(\pm 0.07)\Sigma\sigma^* + 0.42(\pm 0.04)\log P - 0.62(\pm 0.13) \quad (5)$$

$n = 16$, $R^2 = 0.952$, $R^2_{adj} = 0.945$, $s = 0.12$, $F = 129.6$

Incorporation of MW gives the regression equation:

$$pEC3 = 1.09(\pm 0.07)\Sigma\sigma^* + 0.37(\pm 0.04)\log P + 0.002(\pm 0.001)MW - 0.80(\pm 0.14) \quad (6)$$

$n = 16$, $R^2 = 0.964$, $R^2_{adj} = 0.956$, $s = 0.11$, $F = 108.4$

The MW parameter has only a marginal effect on the regression, and its coefficient is positive. If MW was helping to model SC penetration, the coefficient should be negative. Thus we conclude that for this data set, with log P values ranging from −1.66 to 4.05, SC penetration is not a determining factor for sensitisation potency.

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Vehicle Effects

For different vehicles large differences between vehicle/SC partition coefficients would be expected and should lead to large differences in the rate of SC penetration as the vehicle is varied. However, changing the vehicle has relatively small effects on sensitisation potential — there appear to be no cases of a compound being classed as a strong sensitiser when tested in one vehicle and as a weak sensitiser when tested in another. The small vehicle effects which are observed, corresponding to variation in observed EC3 (this being the dose (expressed as percent concentration by weight) giving stimulation index, SI = 3) values by little more than an order of magnitude, and in many cases much less (Basketter *et al*., 2001; Wright *et al*., 2001), are more consistent with limited variation in bulk properties of the solutions — e.g. density, viscosity.

Timescale of Events following Topical Application

When 2,4-dinitrochlorobenzene (DNCB) is applied topically under LLNA conditions, significant activity of cytokines in the epidermis results within 15 min (Enk and Katz, 1992). Similarly Cumberbatch *et al*. (2005) reported significant cytokine activity within 30 min after topical application of DNCB or trimellitic anhydride (TMA). In the case of DNCB, significant migration of Langerhans cells from the epidermis to the draining lymph node occurs within 4 h (Cumberbatch *et al*., 2005). For these two compounds to reach the epidermis and stimulate cytokine release and Langerhans cell migration in such a short timescale does not support the concept of SC penetration being a major hurdle. It may be noted that while DNCB has a calculated log P value of 2.14, close to the value of 2 which is reported to be optimum for penetration (Smith and Hotchkiss, 2001), TMA is a strong acid (pK_a, 2.5), predominantly ionised at skin pH. The TMA anion has a calculated log P value of –2.5, and would be considered too hydrophilic to penetrate readily.

Strong sensitisers are known with log P values (P being the octanol/water partition coefficient) which are usually considered too low or too high for effective SC penetration. Thus poison ivy and poison oak urushiols (Fig. 1), for which the most hydrophilic components have log P values >7, should be too hydrophobic to penetrate, and *N*-methyl-*N*-nitroso urea (Fig. 2), with log P = –1.29 should be too hydrophilic to penetrate easily. All of these are strong sensitisers (Dupuis and Benezra, 1982). These cases are not unique: C16 alkene sultone and C16 bromosultone are extremely strong sensitisers with high log P values (4.60 and 5.12, respectively) (Roberts and Williams, 1982; Ritz *et al*., 1975; Basketter and Roberts, 1990). Towards the other end of the hydrophobicity/hydrophilicity spectrum, the compound streptozotocin (Fig. 1), which fails to sensitise in the murine local lymph node assay (LLNA) when the assay is conducted according to the normal protocol with topical application, but which does sensitise when applied by intradermal

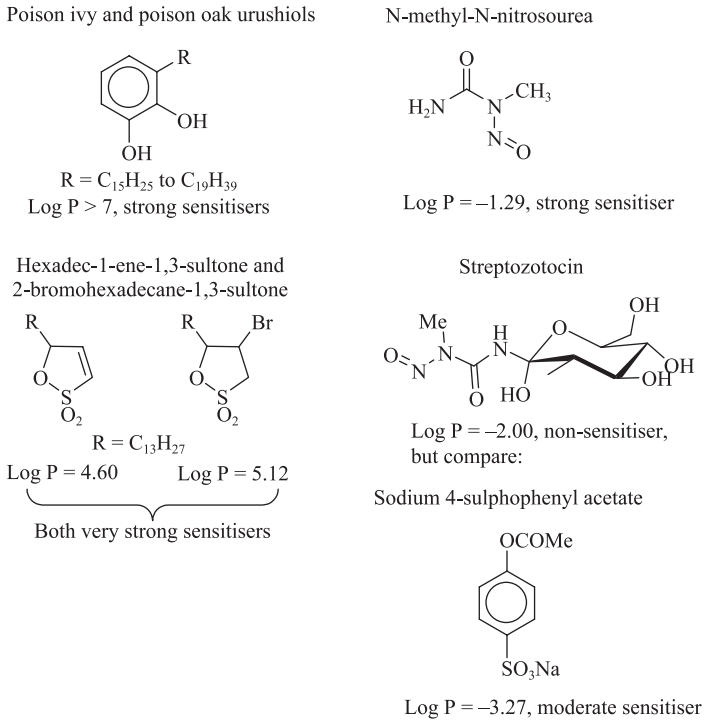

Figure 1. Sensitisers with sub-optimal and supra-optimal log P for penetration

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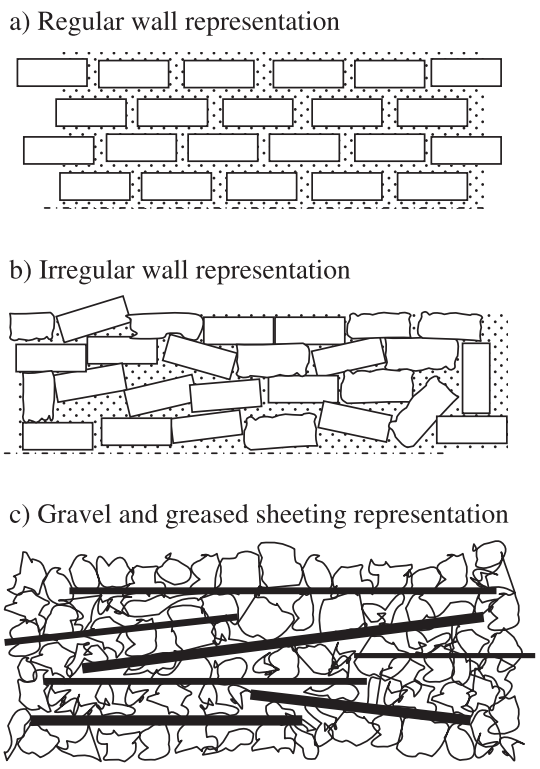

Figure 2. Building materials representations of the stratum corneum
In (a) and (b) the shaded zones (mortar) represents lamellar phase lipid and bricks represent dead cells. In (c) the straight lines (greased sheeting) represent lamellar phase lipid and irregular shapes (gravel) represent dead cells

injection, has been quoted as an example of a compound whose inability to penetrate due to its hydrophilicity (Clog P = −2.00) prevents it from sensitising (Ashby *et al*., 1995). However, there are compounds which are even more hydrophilic than streptozotocin which nevertheless are able to sensitise in the LLNA. An example is sodium 4-sulphophenyl acetate (Fig. 1), whose log P value, calculated by the Leo and Hansch method as used in ClogP software, is −3.27, which is clearly positive in the LLNA, giving SI values of 3.8, 3.9 and 10.1 at 5%, 10% and 25%, respectively (Ashby *et al*., 1995), corresponding to at least a moderate sensitiser classification. This positive result with sodium 4-sulphophenyl acetate makes it difficult to sustain the argument that streptozotocin's failure to sensitise in the LLNA is due to its being too hydrophilic to penetrate. Our interpretation is that streptozotocin, being not only hydrophilic but also a very hard electrophile, reacts with hard nucleophilic groups of hydrophilic proteins such as esterases, which have a reactive hard nucleophilic hydroxyl group as part of a serine unit (McMurry and Begley, 2005), during its passage through the SC. Esterases are known to be present in the stratum corneum (Menon *et al*., 1986). Thus streptozotocin is a special case: it fails to penetrate, and hence fails to sensitise via topical application in the LLNA, as a result of its high reactivity to SC nucleophiles. A similar special case has been observed with a series of aryl benzoates, which are activated esters and act as acyl transfer electrophiles. These compounds are also reactive to hard nucleophiles, although less so than streptozotocin, and their sensitisation potential, in a guinea-pig assay, has been found to be correlated with hydrophobicity but not with reactivity (Barratt *et al*., 1997). The rationale is that increasing reactivity towards carrier protein is cancelled out by increasing loss of sensitiser due to reaction in the SC.

At this point it is worth pointing out that sensitisation potential in the LLNA, but not in other tests, does seem to be to some extent dependent on ability to penetrate, for compounds with high log P values. Thus for alkyl alkanesulphonates with carbon numbers above C12 sensitisation potential is inversely correlated with log P (Roberts and Basketter, 2000). However, in guinea-pig assays the dependence on log P is positive (Roberts and Basketter, 1997). We consider this as an indication of underprediction by the LLNA for compounds of this type. We are not aware of any LLNA data on very hydrophobic compounds known to be strong sensitisers in man, such as C12–C16 alkene sultones and urushiols (Dupuis and Benezra, 1982).

Thus we conclude that SC penetration is not a significant hurdle in the skin sensitisation process, but is a potential limiting factor which only becomes important in specific circumstances — (a) compounds with high log P values; (b) compounds reactive to hard nucleophiles in the SC (streptozotocin, activated esters).

Why is SC penetration usually unimportant in determining sensitisation potential? The simplest interpretations are either:

– usually all of the compound penetrates the SC in the timescale of the test, or . . .
– a more or less fixed proportion, which could be small, reaches the epidermis rapidly, possibly by-passing the SC via 'shunt pathways' (Smith and Hotchkiss, 2001) such as hair follicles, sweat glands etc., while the remainder fails to penetrate significantly during the test timescale.

The first of these interpretations may appear contrary to what would be expected based on the Potts and Guy equation for percutaneous penetration (Potts and Guy, 1992). However, the Potts and Guy equation is derived from experimental data on penetration through all the layers of the skin, involving passage through lipid membrane barriers. If the SC is considered in terms of the frequently used 'bricks and mortar' representation (Smith and Hotchkiss, 2001), the bricks representing dead cells (hydrophilic) and the mortar representing the lamellar phase lipid separating them (Fig. 2a), then it seems

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reasonable to assume that the Potts and Guy equation, should be applicable to SC penetration, since the lamellar phase lipid in the SC will have similar barrier properties to lipid membranes. However, the 'bricks and mortar' analogy, implying a continuous lamellar phase barrier, should not be taken too literally, and in particular should not be represented as a regular recently built wall. In our view a more realistic analogy would be a brick wall which has been neglected, so that it has become irregular and in parts the mortar is missing (Fig. 2b). An alternative analogy would be a bed of coarse gravel (representing dead cells) in which discontinuous layers of greased sheeting (representing the lamellar phase lipid) have been interspersed (Fig. 2c). In Fig. 2 (b and c) the lamellar phase lipid serves to bind the dead cells together in a matrix, but it does not constitute a continuous barrier to the passage of liquids through the SC. However, it does provide a phase into which hydrophobic compounds can partition, thereby retarding their passage through the SC. This has been proposed as the mechanistic basis for the negative correlation of sensitisation potential with log P for hydrophobic alkyl alkanesulphonates in the LLNA (Roberts and Basketter, 2000).

Figure 2 could also be relevant to the second possible interpretation for the low relevance of penetration to skin sensitisation, i.e. that a fixed, possibly small, proportion of the applied sensitiser reaches the epidermis very rapidly. If most of the SC corresponds to Fig. 2a, but some proportion corresponds more to Figs 2b and/or 2c these latter could provide shunt pathways. It is conceivable that the proportion of 2b/2c-like SC would be greater in damaged, irritated or inflamed skin. A similar argument has been made for psoriatic and eczematous skin: these conditions are associated with a rapidly produced SC which may be structurally flawed with impaired barrier function (Kligman, 1983).

Migration of Langerhans Cells

For sensitisation to occur, Langerhans cells (LC) which have acquired and processed sensitiser-modified carrier protein have to migrate from the epidermis to the draining lymph node. Much progress has been made in recent years in understanding the rather complex details of this process, and has been well described by Corsini and Galli (2000), Kimber *et al*. (2000) and Cumberbatch *et al*. (2005). The LC have to detach themselves from the matrix of surrounding keratinocytes and travel along the basement membrane between the epidermis and the dermis, eventually penetrating through this membrane to reach the lymph node. In the course of this journey they develop into mature dendritic cells (DC), losing the ability to process sensitiser-modified carrier protein but acquire the ability to present the corresponding antigen to T-cells. In LLNA studies, significant LC migration to the draining lymph node is observed within 4 h of topical application of DNCB, and continues for up to 72 h (Cumberbatch *et al*., 2005).

These events are stimulated and controlled by several cytokines (these are glycoproteins released in the epidermis, which act on the LC via binding to their receptors). Notable among these cytokines are tumour necrosis factor α (TNF-α), interleukin (IL) 1β (IL-1β) and IL-18 (Cumberbatch *et al*., 1997, 2001). If the necessary cytokines are unavailable at the necessary concentrations, then the response of LCs to sensitisers is impaired (Kimber *et al*., 1998, 2000). An important stimulus for the production, or increased production, of such cytokines is dermal trauma (Kimber and Cumberbatch, 1992; Kimber *et al*., 2001) such as irritation. Many sensitisers, for example 2,4-dinitrochlorobenzene (DNCB), are to some extent irritant and may thereby be able to stimulate production of the cytokines necessary for sensitisation.

The ability of irritant trauma to facilitate sensitisation is well demonstrated by LLNA studies with DNCB (Cumberbatch *et al*., 1993). At reduced concentrations, DNCB alone gives only a minimal sensitisation response, but when the irritant sodium lauryl sulphate (SLS) is administered at the same time, the sensitisation response to DNCB is increased. At higher DNCB concentrations, at which the irritancy of DNCB is presumably sufficient to stimulate cytokine production, the response is not modified by SLS. This finding has been interpreted as suggesting that a certain level of skin irritation is required for the optimal acquisition of skin sensitisation, and that insufficiently irritant chemicals, in the absence of any other source of irritation, may not show their full sensitisation potency (Kimber and Dearman, 2003; Jowsey *et al*., 2006).

However, many very strong sensitisers, such as long chain alkene sultones and alkyl alkanesulphonates, are very mild, i.e. non-irritant, to the extent that they can be tested even at 100%. Furthermore, there appear to be no known cases where a compound which should otherwise sensitise fails to do so because it fails to stimulate the migration and maturation of LC. Possibly the stimulus can be produced by other processes which do not lead to clinically observable irritation. It may be that the acquisition by the LC membrane of a sufficient concentration of sensitiser-modified carrier protein is enough to stimulate the required cytokine production. In any event, it seems clear that LC migration can be taken for granted, and does not constitute a hurdle in the sensitisation process.

It is, however, interesting to note that the sensitiser trimellitic anhydride (TMA), which is also a respiratory allergen, produces a different pattern of LC and cytokine activity in LLNA studies when compared with DNCB. Whereas topical application of DNCB leads to rapid production of, *inter alia*, the cytokine IL-1β, TMA leads not

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to IL-1β but to rapid production of IL-10, which appears to be able to down-regulate production of IL-1β. Migration of LC to the lymph node occurs more slowly than with DNCB, and the T-cells which are stimulated to proliferate are of different types (Cumberbatch *et al.*, 2005). DNCB and TMA differ in their reaction chemistry and their physical chemistry (Roberts *et al.*, 2007a, 2007b): DNCB is an S_NAr electrophile, whereas TMA is an acyl transfer electrophile; DNCB is relatively hydrophobic, enabling it to partition well into membranes, whereas TMA, in its ionised form which will predominate at epidermal pH, is hydrophilic and less able to partition into membranes. It remains to be determined whether the different responses seen to DNCB and TMA represent a more general pattern of different LC/cytokine response depending on the reaction mechanistic domain of the sensitiser.

Antigen Recognition

The nature of the antigen which is presented by the matured Langerhans cells to naïve T-cells in the lymph node clearly depends on the nature of the sensitising compound: this is the basis of the specificity of the sensitisation. It seems reasonable therefore to consider that the ability of the antigen to stimulate proliferation of T-cell clones will depend on the nature of the antigen. It follows from this argument that the sensitisation potential of a compound should depend, *inter alia*, on its intrinsic antigenicity, i.e. how well the antigen derived from it is able to be recognised by T-cell receptors. This, it seems reasonable to suppose, should depend on the extent to which the sensitiser, after reaction with protein, produces groups which can bind strongly to T-cell receptors through complementary regions of polarity and molecular shape, as illustrated in Fig. 3.

Although this argument appears at first sight highly plausible, there is no convincing evidence to support it, and substantial evidence against it.

An early indication that intrinsic antigenicity might be less dependent on the structure of the sensitiser than previously assumed came from work with simple alkyl

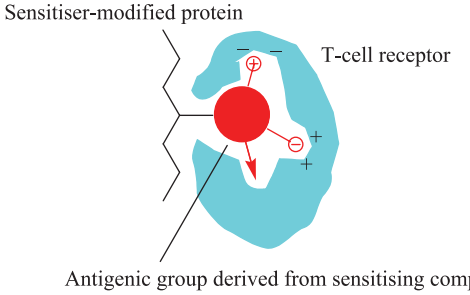

Figure 3. Antigen recognition. This figure is available in colour online at www.interscience.wiley.com/journal/jat

alkanesulphonates, $R^1SO_3R^2$, published in 1988 (Roberts *et al.*, 1988). These compounds are S_N2 electrophiles, and transfer the R^2 group to nucleophiles. If a compound of this type, with R^2 = methyl, reacts with a protein, that protein will become modified simply by covalent binding of methyl groups. Methyl groups are small, regularly shaped, and non-polar, so they would not be expected to bind strongly to T-cell receptors. Nevertheless, the compounds $R^1SO_3R^2$ (total carbon number 13 or higher) were found to be very strong sensitisers in guinea-pig tests. Subsequently, strong sensitisation has also been observed in the LLNA — methyl dodecansulphonate has an EC3 value of 0.39 (Gerberick *et al.*, 2005). Another finding from the guinea-pig work was that there was strong cross-reactivity between compounds $R^1SO_3R^2$ with R^2 = methyl and the compounds with R^2 = higher alkyl. It appears therefore that antigen recognition occurs readily with alkyl transfer agents, and does not vary as the alkyl group is changed.

Further evidence comes from LLNA data for a wider range of S_N2 electrophiles, covering not just alkyl group transfer agents but also compounds which transfer polar groups to the nucleophile. Figure 4 shows a graph in which the stimulation index values are plotted against RAI values, the latter being calculated from the test concentrations (dose term), rate constants for reaction with butylamine, and log P values (Roberts *et al.*, 2007a). The good fit to the curve indicates that there is no need to invoke differences in degree of antigen recognition to explain the data.

Even more compelling evidence comes from a recently published quantitative mechanistic model (QMM) for the 'Schiff base domain', i.e. compounds which are in

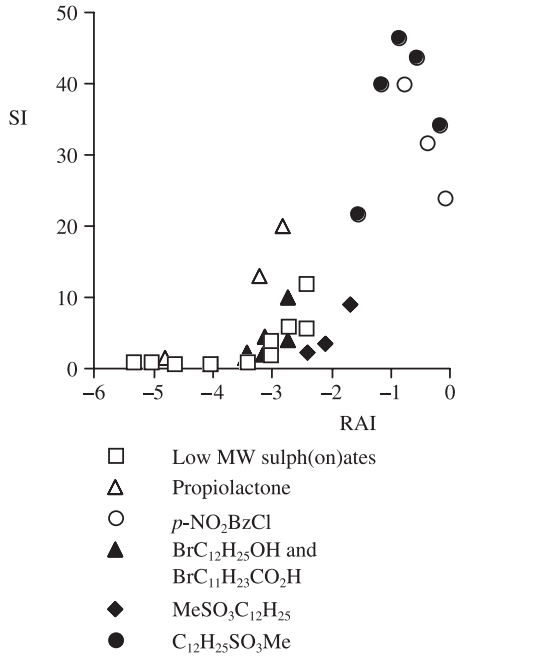

Figure 4. RAI plot for H-polar S_N2 electrophiles

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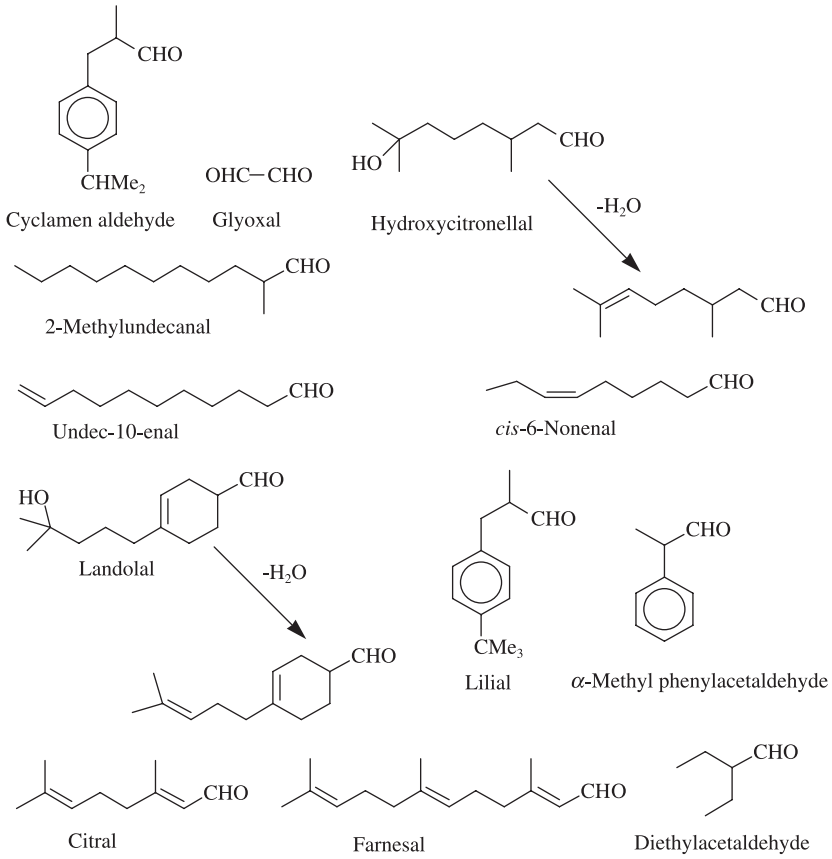

Figure 5. Training set carbonyl compounds

principle capable of sensitising via reaction of aliphatic aldehyde or keto groups with protein nucleophiles (Aptula *et al*., 2006). Figures 5 and 6 show the compounds studied, and Fig. 7 shows the correlation between LLNA pEC3 values and a combination of a reactivity parameter and a hydrophobicity parameter. The compounds in this dataset vary widely in structure, their only common feature being the presence of an aliphatic aldehyde or keto group in the molecule. If intrinsic antigenicity really varies according to the sensitiser structure, then the variation should be revealed in this dataset by failure to obtain a good correlation based on reactivity and hydrophobicity alone. However, there is no such failure. Sensitisation is well modelled without needing to take intrinsic antigenicity into account.

The evidence points to a clear conclusion: differences in degree of antigen recognition play no part in determining sensitisation potential.

However, there is an exception to this generalisation. Often it is found that cross-linking agents are significantly more potent than would be expected based on their reactivity relative to other compounds reacting by the same mechanism. For example, LLNA skin sensitisation potency of aldehydes and ketones, which can react via Schiff base formation, is well correlated with reactivity and hydrophobicity parameters, but the cross-linking agents formaldehyde and glutaraldehyde are, respectively, 10 times and 1000 times as potent as predicted from the correlation established with non-cross-linking homologues (Aptula *et al*., 2005, 2006). The very strong sensitisation potential in guinea-pig assays of alk-1-ene-1,3-sultones has also been attributed to cross-linking (Roberts *et al*., 2007c). Our interpretation is that cross-linking produces more extensive changes in the carrier protein tertiary structure, resulting in a larger number of different

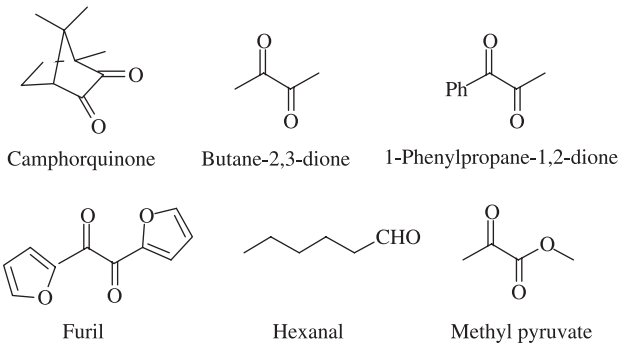

Figure 6. Test set carbonyl compounds. Note that the non-cyclic 1,2-diketones exist predominantly as their transoid conformers, as shown

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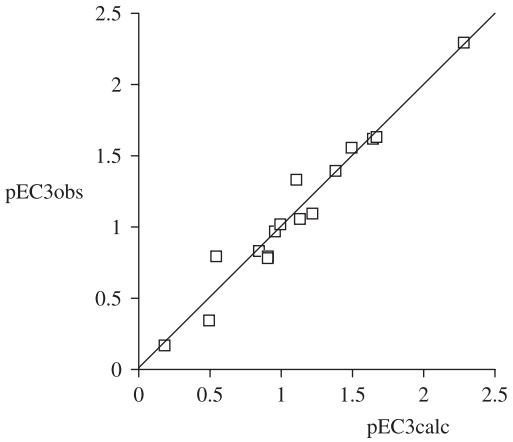

Figure 7. pEC3 observed vs pEC3 calculated for Schiff Base electrophiles. pEC3 calc is obtained from the QMM equation 5 for Schiff base electrophiles XCOY: pEC3 = 1.12 $\Sigma\sigma^*$ + 0.42 log P − 0.62, where $\Sigma\sigma^*$ is the sum of the Taft substituent constants for the groups X and Y

antigenic determinants and consequently proliferation of a larger number of T-cell clones. We are not aware of any definitive information as to whether skin sensitisation should be considered a monoclonal or a polyclonal phenomenon, but we are encouraged to assume the latter on the basis of what has been written about T-cell response to pathogens: '. . . a pathogen, even the smallest virus, offers not one but dozens of shapes (antigens) to be recognised by different lymphocytes . . . As a result of all this . . . about 1 in 10 000 to 1 in 1 000 000 lymphocytes will recognise *some part* of any one pathogen. With a population of 10 000 000 000 lymphocytes, we are in the range where there might be as many as a million lymphocytes per pathogen, *even before they start to proliferate*' (Playfair, 2004).

Practical Implications for Hazard and Risk Assessment

One of the conclusions from our analysis is that skin penetration ability does not normally constitute a significant hurdle in the skin sensitisation process. At this point it is useful to quote from a QSAR paper by Fedorowicz *et al.* (2005). They are discussing the rule within the DEREK expert system that for compounds with log $K_p < -5$ (K_p = permeability coefficient) the probability of skin sensitisation of the chemical is significantly lowered as the penetration of such chemicals across human skin is negligible, and thus, they are unable to exert their toxic effects:

'At first, the presence of this rule in DEREK for Windows seems to be justified, because both skin sensitisation and elicitation of the allergic response occur in and require the skin sensitisers to reach the viable epidermis. However, further considerations suggest caution about applying this rule at least in the area of workplace chemicals. Because this rule relies on the Potts-Guy equation, it applies to only intact skin. However, cuts, dermatitis, and other skin injuries are quite common in the workplace environment. For the same reason, this rule is unlikely to apply to workers wearing occlusive gloves. Additionally . . . in recent studies of DEREK for Windows's performance using the BgVV . . . database . . . it was shown that there are not any meaningful limiting values of skin permeation parameters that will have an impact on the induction of skin sensitisation.'

Approaching the issue from a different perspective, Fedorowicz *et al.* arrive at a similar practical interpretation to our own, which is that there is an important corollary to our conclusion that SC penetration is not a significant hurdle in the sensitisation process. When trying to predict sensitisation hazard, to assume that SC penetration is a sensitisation-determining factor is to risk underestimation of the sensitisation potency.

There is a similar corollary to our argument regarding migration and maturation of Langerhans cells. This is reinforced when we consider the implications of the facts that people are not usually exposed to one chemical at a time, and that cytokine production can be stimulated by a different, not necessarily allergenic, chemical. Consider a hypothetical compound which meets all the criteria to be a sensitiser except that it cannot produce a stimulus for cytokine production. Then all that would be needed to release its latent sensitisation potential would be simultaneous exposure to a compound which can produce a danger signal (e.g. a surfactant). Thus, to assume that, because a particular compound is unable to produce a danger signal, it cannot sensitise, could lead to underestimation of the risk of sensitisation.

Conclusions

Penetration of the stratum corneum, stimulation of migration and maturation of Langerhans cells and antigen recognition are important events in the induction of sensitisation, but they can be taken for granted. They are not important factors in determining whether a compound will be a sensitiser or not, nor are they important factors in determining how potent one sensitiser will be relative to another.

This leaves covalent binding to carrier protein as the key factor which determines sensitisation potential. For non-animal prediction of skin sensitisation potential, this is the process which needs to be modelled and the area where research effort should be focused. This is not a trivial problem but much progress has been made,

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building on the pioneering work reported by Landsteiner and Jacobs (1936). Compounds can be classified into a limited number of reaction mechanistic domains (Aptula and Roberts, 2006), and within these domains QMMs can be derived, based on the RAI model, relating sensitisation potential to a combination of electrophilic reactivity and hydrophobicity. Applying these mechanistic principles, the following strategy can be used (Roberts *et al*., 2007b).

Presented with a new compound:

1. The first step is to classify it into its reaction mechanistic domain. One domain is the 'unreactive' domain, populated by predicted non-sensitisers. For several mechanistic domains there are corresponding pro-electrophilic sub-domains. For example many sensitisers, such as hydroquinone and 3-alkyl/alkenyl catechols (active components of poison ivy) are thought to act as pro-Michael acceptors. Domain classification may often be possible by inspection of structure, but inevitably in some cases a confident prediction may not be possible. In such situations, experimental work will be needed to determine the reaction chemistry, in particular to determine if the compound is electrophilic or pro-electrophilic and the nature of the reactions.
2. Having assigned the compound to its reaction mechanistic applicability domain, the next step is to quantify its reactivity/hydrophobicity relative to known sensitisers in the same mechanistic applicability domain. These properties may sometimes be confidently predictable from structure, using physical organic chemistry approaches such as linear free energy relationships based on substituent constants or on molecular orbital parameters. In other cases it will be necessary to perform physical organic chemistry measurements, such as determination of reaction kinetics and measurement of partition coefficients.

Having assigned the compound to its reaction mechanistic applicability domain and quantified its reactivity/hydrophobicity relative to known sensitisers in the same domain, QMM or mechanistic read-across can be used to predict the sensitisation potential.

Although this strategy has only recently been presented as above, we have in practice been applying it for many years in the context of interpreting sensitisation data on raw materials, for example in considering whether a positive sensitisation test result should be taken as indicating that the chemical is a sensitiser or whether an impurity is responsible, and in the latter case identifying the sensitising impurity and setting specifications.

Of course the above strategy can only be applied with confidence for substances whose chemistry is sufficiently understood. There are still some general areas where further work is needed to better understand the chemical basis of sensitisation by certain structural classes of compounds. These include: aromatic compounds containing more than one hydroxyl and/or amino group, hydroperoxides and compounds which can readily give rise to them by autoxidation, epoxides and their autoxidation precursors.

Our ultimate vision for skin sensitisation prediction is that the animal testing laboratory should be replaced by the physical organic chemistry laboratory. Particularly bearing in mind that many compounds are easily predictable without experimentation, the experimental studies to generate the chemical data required should be no more costly or time consuming than the animal tests that have hitherto been used.

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