

What determines skin sensitization potency—myths, maybes and realities. Part 1. The 500 molecular weight cut-off

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Summary

Background. It is widely accepted that there is a molecular weight (MW) cut-off of 500, such that single chemicals with MWs higher than 500 cannot be skin sensitizers. If true, this could serve as a useful principle for designing non-sensitizing chemicals.

Objectives. To assess whether the 500 MW cut-off is a myth or a reality.

Methods. A database of 699 chemicals tested for skin sensitization in guinea pigs or mice was analysed to establish the number of tested chemicals with MW > 500, and to establish whether any of these were sensitizers.

Results. Only 13 (2%) of the 699 chemicals in the database have MW > 500. Of the 13 tested compounds with MW > 500 in the database, five are sensitizers and eight are non-sensitizers.

Conclusions. The 500 MW cut-off for skin sensitization is a myth, probably derived from the widespread misconception that ability to efficiently penetrate the stratum corneum is a key determinant of sensitization potency. The scarcity of sensitizers with MW > 500 simply reflects the general scarcity of chemicals with MW > 500.

Key words: bioavailability; molecular weight; penetration; sensitization; structure–activity.

There are strong pressures to reduce or even eliminate the use of animals for prediction of skin sensitization potency. Future risk assessments on new chemicals will have to be made on the basis of non-animal methods, such as *in vitro* assays (e.g. KeratinoSens (1)), and chemical information. Much is now understood concerning the mechanism of skin sensitization, and this understanding provides the basis for research initiatives aimed at replacing animal testing. It is important in this situation to ensure that research is appropriately directed towards the key steps in the sensitization process that depend on the nature of the

chemical, and to ensure that misconceptions that could lead to false predictions are identified.

In this article, we consider a common conception, that there is a molecular weight (MW) cut-off of 500 above which chemicals are non-sensitizing, and consider whether this is a myth or a reality.

The 500 MW cut-off is widely accepted among the immunology community. Two examples from publications are presented to demonstrate the statements in the scientific literature: 'It is generally believed that chemical allergens have low molecular weights (< 500) and a $\log K_{o/w}$ of > 1¹ that is thought to favour the

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¹ Although hydrophobicity ($\log K_{o/w}$ or $\log P$) is not the subject of the present paper, it is appropriate to point out here that the " $\log K_{o/w} > 1$ rule" does not appear to be well founded. For example, methylchloroisothiazolinone (extreme sensitizer, EC3 = 0.009%, calculated $\log K_{o/w}$ in the range −0.34 to 0.6, depending on calculation method) and methylisothiazolinone (moderate sensitizer, EC3 = 1.9%, calculated $\log K_{o/w}$ −0.11) are well recognized sensitizers that break this "rule". We suspected that the " $\log K_{o/w} > 1$

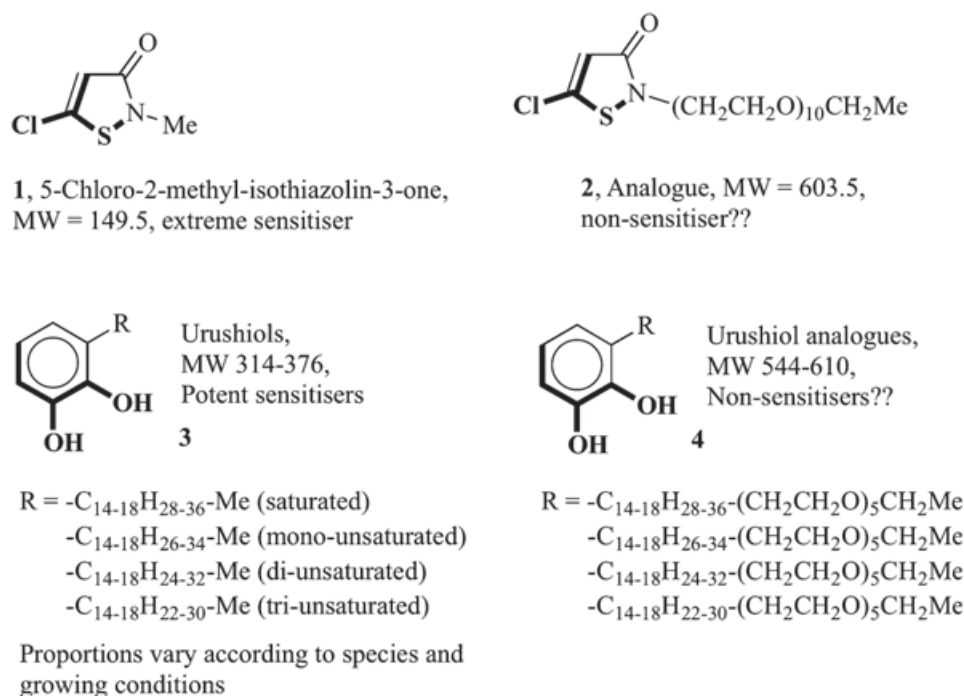


Fig. 1. Hypothetical applicability of the 500 MW cut-off for the design of non-sensitizers.

penetration of the chemical across the lipid-rich stratum corneum' (2); 'Virtually all common contact allergens are under 500 Da; larger molecules are unknown as contact sensitizers. They cannot penetrate and thus cannot act as allergens in man' (3).

Potential applicability of the 500 MW cut-off

If the 500 MW cut-off is real, it could provide a very useful design principle for new chemicals to be used in consumer products. Here, we give two hypothetical examples of how this principle could be applied.

First, consider the preservative methylchloroisothiazolinone (5-chloro-2-methyl-4-isothiazolin-3-one; CAS 26172-55-4), **1**, shown in Fig. 1. This compound is classed as an extreme sensitizer (5), having a local lymph node assay (LLNA) EC3 value of 0.009%, and can therefore only be used at very low concentrations in consumer

products. Its preservative properties and its sensitization potency are associated with the chemical reactivity of the section of the molecule highlighted in Fig. 1 (5, 6). A non-sensitizing analogue with the same reaction chemistry properties and the same hydrophobicity ($\log P$) that could be used at higher concentrations could be designed as follows. The MW of **1** is 149.5. According to the 500 MW cut-off principle, we need to increase the MW by at least 350 to make a non-sensitizing analogue. This can be done by inserting a sequence of ethylene oxide (EO) units ($-\text{CH}_2\text{CH}_2\text{O}-$)_n between the nitrogen atom and the methyl group. Each EO unit increases the MW by 44, so, with eight EO units, the MW would be increased to 501.5. To provide a greater margin, 10 EO units would give an MW of 589.5. As each EO unit reduces $\log P$ by 0.1, the addition of two methylene groups (each contributing 0.54 to $\log P$) would give a $\log P$ value almost identical to that of **1**. In this way, we have designed compound **2**, a compound that would be predicted to be non-sensitizing on the basis of the 500 MW cut-off, an analogue of methylchloroisothiazolinone, with an almost identical $\log P$ value and with the reaction centre unchanged.

As a second hypothetical example, we consider the case of urushiols, the allergenic natural products of poison ivy and related plant species (7). Urushiols are mixtures of C15–C19 3-alkyl and 3-alkenyl catechols, **3**, as shown in Fig. 1. These chemicals are highly potent skin sensitizers

rule" has become accepted because relatively few compounds with $\log K_{\text{O/W}} < 1$ have been tested, so we examined a published database (4) of 210 chemicals tested in the LLNA. There are 39 chemicals listed with $\log K_{\text{O/W}} < 1$ (19% of the total) and of these only 9 are non-sensitizers (23% of the compounds with $\log K_{\text{O/W}} < 1$). In the full dataset, 43 of the 210 are non-sensitizers and a further 6 are false positives, so the overall incidence of non-sensitizers is 21–22%. Thus the proportions of non-sensitizers to sensitizers are almost identical in the $\log K_{\text{O/W}} < 1$ subset and in the total dataset. Therefore, on the basis of the LLNA evidence, the " $\log K_{\text{O/W}} > 1$ rule" is a myth.

and a major cause of allergic contact dermatitis in the United States, where poison ivy and related plant species are native. Urushiol have been used for many centuries, and continue to be used, as lacquers in China and Japan. The MW of urushiol components ranges from 314 to 376, so incorporation into the alkyl or alkenyl chain of five EO units, plus a methylene group, would give a mixture of components all with MW > 500. In this way, we arrive at **4**, predicted (on the basis of the 500 MW cut-off) to be a non-sensitizing analogue of urushiol with the same reaction chemistry and hydrophobicity.

These examples illustrate how useful, and easy to apply, a design principle based on the 500 MW cut-off principle would be – but only if the 500 MW cut-off is valid. To make an assessment of validity, we first look for counter-examples, that is, known sensitizers with MW > 500.

Search for counter-examples

The training set of the Tissue Metabolism Simulator–Skin Sensitization (TIMES-SS) expert system (8) contains sensitization information on > 1000 chemicals in humans or animal models. Of these, 423 chemicals have been tested in the LLNA, and 276 have been tested in guinea pigs. The human data were, in many cases, based on a single clinical report, and as we could not be confident about these data, we restricted the analysis to the animal data. The animal data in the TIMES-SS training set come from several sources: predominantly LLNA datasets published by Gerberick et al. (4), Patlewicz et al. (8) Kern et al. (9), and Natsch et al. (10), with further in-house LLNA data provided by Givaudin and Unilever, and a guinea pig maximization test (GPMT) dataset published by Cronin and Basketter (11).

Compounds in the TIMES-SS training set are classified as: strong sensitizers (315 chemicals; EC3 up to 10% in the LLNA; > 30% of animals positive in guinea pig tests), weak sensitizers (155 chemicals; EC3 between 10% and 50% in the LLNA; 1–30% of animals positive in guinea pig tests), or non-sensitizers (229 chemicals; EC3 > 50% in the LLNA; no positives in guinea pig tests).

Of these total 699 chemicals with animal test data, 13 have MW > 500. Of these, eight are reported as non-sensitizers, four as weak sensitizers, and one as a strong sensitizer (Table 1). The compounds with MW > 500 that are reported to be positive all have structural alerts for sensitization, whereas all but one of the compounds with MW > 500 reported as non-sensitizers lack alerts that would lead to prediction of sensitization.

One of the non-sensitizers does have an alert for chemical reactivity that would be consistent with sensitization potential, but it is recognized that having

a reactivity alert does not mean that a chemical will necessarily be a sensitizer. A compound classified into one of the electrophilic reaction mechanistic domains may be a non-sensitizer because it is not reactive enough or not hydrophobic enough (12). An example of a non-sensitizer with a reactivity alert is trans-2-methylbut-2-enal (9), which is a Michael acceptor electrophile, but not reactive enough to be a sensitizer.

The lack of sensitization potential of these compounds with MW > 500 can be rationalized in terms of their lack of chemical reactivity (or, in one case, insufficient reactivity), without any need to invoke an MW cut-off.

The first five compounds in Table 1 all show reactivity alerts and are all sensitizers, despite having MW > 500. To rationalize these compounds being reported as sensitizers, there are only two options:

1. Despite the structural alerts, and despite the fact that at least one of the compounds, bisphenol A-glycidyl methacrylate (BisGMA) is a well-recognized clinical sensitizer in humans, these five compounds are all false positives.
2. Having MW > 500 does not prevent a compound from being a sensitizer. In other words, there is no 500 MW cut-off.

Bearing in mind that the only 'experimental' evidence in support of the 500 MW cut-off is the assertion that there are no skin sensitizers with MW > 500, option 2 seems the obvious choice.

Thus, the data do not support the existence of an MW threshold of > 500. It could be argued that they do not absolutely disprove it, because the number of compounds with MW > 500 is too small for proper statistical interpretation. However, for the MW > 500 rule to be supported, it would be necessary to invoke, with no supporting evidence, arguments that all five positives with MW > 500, for special and non-obvious reasons, are in fact false positives. False positives are sometimes encountered in skin sensitization testing, for example caused by a potent sensitizing impurity, but such cases are very much in the minority. If we assume, for the sake of argument, that no compounds with MW > 500 are sensitizers, then we have a set of 13 non-sensitizers, of which five are false positives. In the set, there are six compounds with reactivity alerts. The statistical odds of all five false positives having a reactivity alert can be calculated as:

$$(6/13)(5/12)(4/11)(3/10)(2/9) = 0.005, \text{ i.e., } 0.5\%$$

In other words, we can be 99.5% confident, solely on the basis of these five positives, that the 500 MW cut-off is not valid.

Table 1. Chemicals with molecular weight (MW) > 500

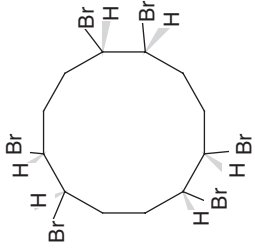
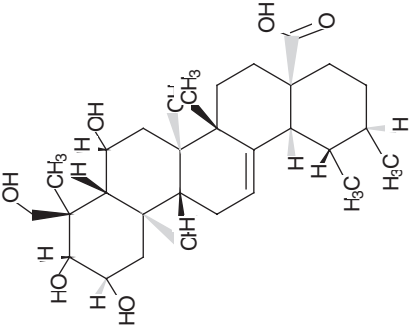
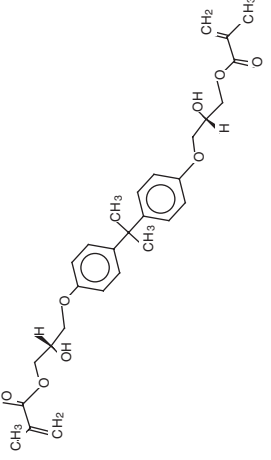
No.	CAS no.	Name	Two-dimensional structure	MW	Reactivity alerts	Potency class	Test method used
1	3194-55-6	1,2,5,6,9,10-Hexabromocyclododecane		641.70	Pro-S _N 2-allylic Br after partial dehydrobromination	Strong sensitizer	GPMT
2	18449-41-7	Madecassic acid		504.67	Pre-hapten-tert-allylic hydroperoxide by autooxidation	Weak sensitizer	GPMT
3	1565-94-2	Bisphenol A-glycidyl methacrylate		512.57	Michael acceptor	Weak sensitizer	LLNA

Table 1. *Continued*

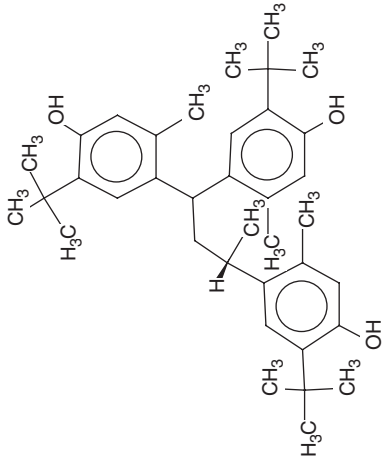
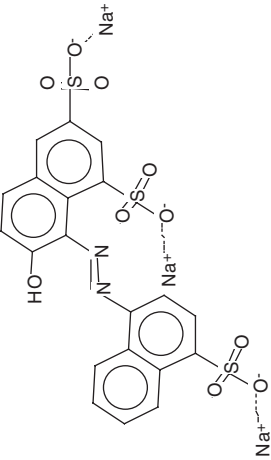
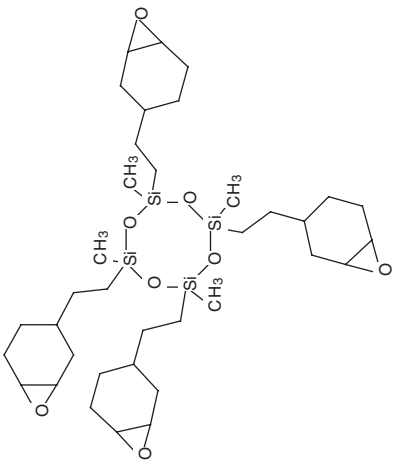
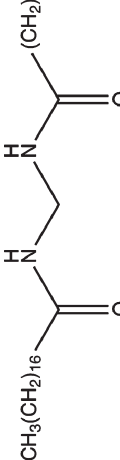
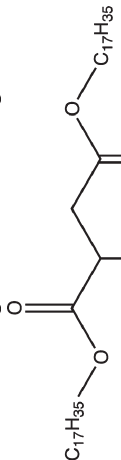
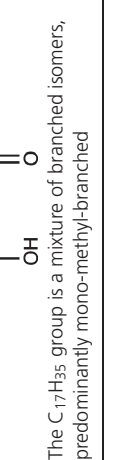
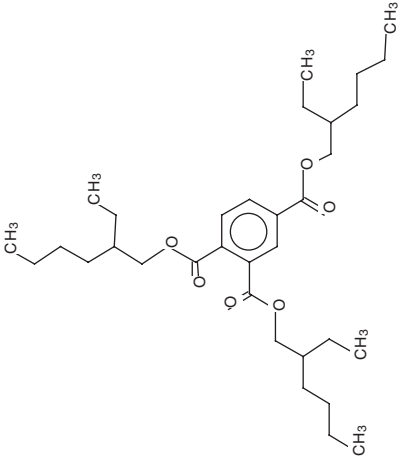
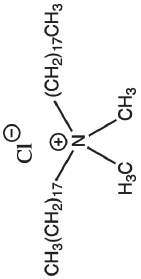
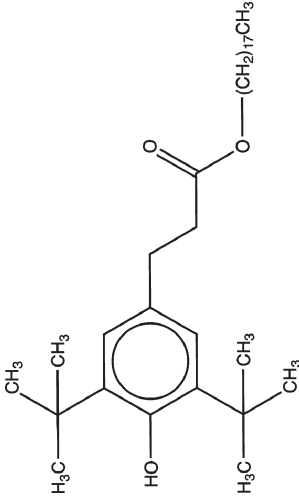
No.	CAS no.	Name	Two-dimensional structure	MW	Reactivity alerts	Potency class	Test method used
4	1843-03-4	1,1,3-Tris-(2-methyl-4-hydroxy-5-tert-butylphenyl)butane		544.78	Quinone-methide tautomer alert (aromatic -OH para to CH)	Weak sensitizer	GPMT
5	2611-82-7	Acid Red 18		604.45	N=N cleavage to NH ₂ to give ortho-aminohydroxy compound—pro-Michael acceptor	Weak sensitizer	GPMT

Table 1. Continued

No.	CAS no.	Name	Two-dimensional structure	MW	Reactivity alerts	Potency class	Test method used
6	121225-98-7	3,4-Epoxycyclohexylethylcyclopolymethylsiloxane		737.20	Epoxide units, but these are secondary, so only weakly chemically reactive	Non-sensitizer	LLNA
7	123-28-4	Dilauryl thiodipropionate		514.82	No alerts for chemical reactivity	Non-sensitizer	GPMT
8	25322-68-3	Poly(ethylene glycol)		546.62	No alerts for chemical reactivity	Non-sensitizer	GPMT
9	109-23-9	Methylene distearamide		578.97	No alerts for chemical reactivity	Non-sensitizer	GPMT
10	67763-18-2	Diisostearyl malate		639.01	No alerts for chemical reactivity	Non-sensitizer	GPMT

The C₁₇H₃₅ group is a mixture of branched isomers, predominantly mono-methyl-branched

Table 1. Continued

No.	CAS no.	Name	Two-dimensional structure	MW	Reactivity alerts	Potency class	Test method used
11	3319-31-1	Tris(2-ethylhexyl)benzene-1,2,4-tricarboxylate		546.75	No alerts for chemical reactivity	Non-sensitizer	Guinea pig (Buehler test)
12	107-64-2	Dimethyldioctadecylammonium chloride		586.47	No alerts for chemical reactivity	Non-sensitizer	GPMT
13	2082-79-3	Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate		530.84	No alerts for chemical reactivity (quinone-methide disfavoured by electronegative CH2COOR group)	Non-sensitizer	Guinea pig (Maurer optimization test)

GPMT, guinea pig maximization test; LLNA, local lymph node assay.

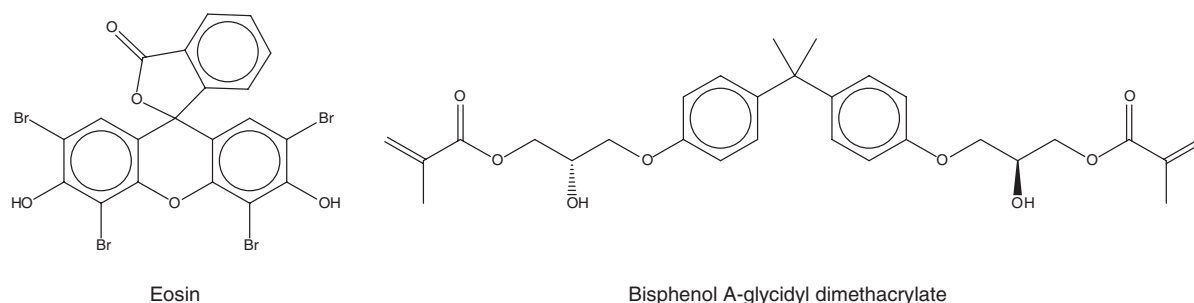


Fig. 2. The structural formulae of eosin and bisphenol A-glycidyl dimethacrylate.

In the literature, there are several reported cases where people exposed to chemicals with MW > 500 have a positive reaction when patch tested. Two examples are shown in Fig. 2. Eosin (C.I. 45380:2) (647.9 MW) is a red dye belonging to the xanthene group, and was used in lipsticks in the 1920–1930s (13). Currently, its use is regulated and it is less problematic. BisGMA (512.6 MW; this is also positive in the LLNA, and is included in the analysis above) has been shown to be a human sensitizer, and to be cross-reactive with epoxy resin (14). Other examples of reported human sensitizers with MW > 500 are chlorhexidine diacetate (625.6 MW), chlorhexidine digluconate (897.9 MW) (15), and methylene bis-benzotriazolyl tetramethylbutylphenol (658.9 MW) (16).

Why are there so few known sensitizers with MW > 500?

Of the 699 animal-tested chemicals in the TIMES-SS dataset, only five are sensitizers with MW > 500, but only eight with MW > 500 are non-sensitizers. Thus, the rarity of MW > 500 sensitizers does not imply a 500 MW cut-off. The reason why very few MW > 500 sensitizers are known is simply that there are very few reports of sensitization tests on any chemicals with MW > 500. To investigate why this should be so, we analysed the contents of the European Inventory of Existing Commercial Chemical Substances (EINECS) and the Toxic Substances Control Act (TSCA) inventory.

When mixtures and chemicals with no defined two-dimensional structure are eliminated from the analysis, then the total numbers of chemicals are:

- 66 368 structures in EINECS:
 - 8318 chemicals (12.53%) with MW > 500
 - 58 050 chemicals (87.47%) with MW < 500
- 31 120 structures in TSCA:
 - 4700 chemicals (15.10%) with MW > 500
 - 26 420 chemicals (84.90%) with MW < 500

It therefore seems clear that the universe of known and characterized single chemicals with known MWs is

populated predominately by chemicals with MW < 500, and this is the reason why so few sensitizers, and also non-sensitizers, with MW > 500 are known.

Why is the MW > 500 principle widely accepted?

The origin of the MW > 500 principle appears to be a paper by Bos and Meinardi (17) on the topic of skin penetration of chemicals and drugs. They argue that the MW of a compound must be < 500 to allow skin absorption, and that larger molecules cannot pass the corneal layer. Their arguments for this 500 MW rule are as follows: (i) almost all common contact allergens have MW < 500, and larger molecules are not known as contact sensitizers – they cannot penetrate, and thus cannot act as allergens in humans; (ii) the most commonly used pharmacological agents applied in topical dermatotherapy all have MW < 500; and (iii) all known topical drugs used in transdermal drug-delivery systems have MW < 500. In addition, clinical experience with topical agents such as cyclosporin, tacrolimus and ascomycins provides further arguments for the reality of the 500 MW rule. For pharmaceutical development purposes, it seems logical to restrict the development of new innovative compounds to a MW of < 500 when topical dermatological therapy, percutaneous systemic therapy or vaccination is the objective.

As discussed above, their argument (i) reflects the scarcity of MW > 500 chemicals to which consumers and test animals are exposed, rather than any inability of MW > 500 compounds to sensitize. It may be correct that large molecules tend not to efficiently penetrate the stratum corneum, and the overall conclusion of Bos and Meinardi regarding pharmaceutical development could be well founded.

The reason why the 500 MW rule has been generally accepted in the contact dermatitis world is probably lack of awareness that the scarcity of MW > 500 sensitizers

simply reflects the scarcity of MW > 500 chemicals in general, coupled with the widespread misconception that the ability to efficiently penetrate the stratum corneum is a key determinant of sensitization potency (2, 3, 17–19). This misconception continues to survive and to be accepted by many, although not all, in the contact dermatitis community. It is often stated but rarely challenged, despite the complete lack of evidence to support it and the evidence against it, discussed by Roberts and Aptula (20). As Roberts and Aptula argue, the small amount of chemical that reaches the viable epidermis rapidly via 'shunt pathways' may be responsible for the sensitization, rather than the material that penetrates more slowly by the 'classical' routes. These pathways are discussed in more detail by Schaefer, Redelmeier and Lademann (21). The intention is to discuss in more detail the issue of bioavailability in the context of skin sensitization in a separate paper. Of course, skin sensitization is not the only toxic effect that can be produced by skin contact, and therefore, in the wider toxicological context, it remains highly desirable to investigate and develop models for skin penetration and kinetics of intradermal distribution. For other endpoints, MW cut-offs are likely to apply, although, as we intend to discuss more fully elsewhere, MW is probably an oversimplistic parameter.

Conclusion

From the analysis of skin sensitization data above, it is clear that the 500 MW cut-off does not apply to skin sensitization. Chemicals with MW > 500 comprise only a small proportion of the universe of known and characterized chemicals, but five of the 13 MW > 500 compounds that have been tested are sensitizers. The widely accepted theoretical basis for the misconception that a 500 MW cut-off applies for skin sensitization is based on the mistaken concept that the ability to efficiently penetrate the stratum corneum is a relevant determinant of skin sensitization potency. It is quite clear from our analysis that the 500 MW cut-off rule cannot provide a useful tool for designing non-sensitizing analogues of reactive chemicals and therefore allergenic chemicals. Thus, we would expect that compounds **2** and **4** of Fig. 1, designed as non-sensitizers with the 500 MW cut-off, would, in fact, be potent sensitizers (because their reactivity has not been

altered). However, sensitization potency is not completely unaffected by MW. Skin sensitization is a molecular phenomenon, and the higher the MW, the fewer molecules per microgram are available on the applied area of skin. Although potency can be quantified on a molar basis, which is more scientifically meaningful, it is more usually reported on a weight basis, as in the LLNA EC₃, which is usually quoted as a weight percentage concentration [weight of compound (g) per 100 ml of vehicle + test compound, or weight (µg) per unit area ((cm²))] (22) to give a stimulation index of 3.0. Thus, modifying a chemical by increasing its MW without changing its chemical reactivity or partitioning characteristics will reduce its potency, if potency is expressed on a weight basis, simply because a greater weight of substance will be required to provide the same number of molecules. By default, any chemical that would need a concentration of > 100%, corresponding to ~250 µg/cm², to give a stimulation index of 3.0 would be a non-sensitizer in the LLNA, as such doses would be impossible to test.

We cannot exclude the possibility of an MW cut-off at some value above 600. However, in the absence of evidence for any such cut-off, we consider it unsafe to assume that any compound that, in other respects, has chemical properties associated with sensitization potency will be a non-sensitizer as a result of having a high MW. It is well known that many high-MW proteins can act as directly antigenic contact allergens. Although these may differ from lower-MW allergens in terms of the cells and cascades involved, in order to sensitize, these macromolecular compounds must be able to reach the viable epidermis. To reconcile this with the existence of a MW cut-off somewhere above 600, it would be necessary to postulate an MW range within which compounds cannot be sensitizers, but both below and above which sensitizers can be found. It seems more probable that the real situation is that there is a lower size or MW limit, whose value is not known precisely, below which macromolecular compounds such as proteins and oligosaccharides are unable to sensitize directly as antigens, in their own right. Any compound below this limit is unable to sensitize as a directly acting antigen, but may still be capable of being a sensitizer, provided that it is able to act directly or indirectly as a hapten, that is, react with *in cutaneo* protein so as to modify it to an antigenic derivative.

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