Selection of Chemicals for the Development and Evaluation of *In Vitro* Methods for Skin Sensitisation Testing

Silvia Casati,[1] Pierre Aeby,[2] Ian Kimber,[3] Gavin Maxwell,[4] Jean Marc Ovigne,[5] Erwin Roggen,[6] Costanza Rovida,[1] Luca Tosti[1] and David Basketter[7]

[1]*In Vitro Methods Unit/ECVAM, Institute for Health and Consumer Protection, European Commission Joint Research Centre, Ispra, Italy;* [2]*Procter & Gamble Company (Wella-Cosmital), Marly, Switzerland;* [3]*Faculty of Life Sciences, The University of Manchester, Manchester, UK;* [4]*Unilever, Sharnbrook, Bedfordshire, UK;* [5]*L'Oréal Research, Aulnay-sous-Bois, France;* [6]*Novozymes A/S, Bagsvaerd, Denmark;* [7]*DABMEB Consultancy Ltd, Sharnbrook, Bedfordshire, UK*

Preface

Advances in the understanding of the biological mechanisms underlying skin sensitisation, and the need to comply with recent regulatory requirements, have favoured the development of alternative approaches, some of which may provide promising screening or partial replacement methods. However, efforts are still required to identify more-robust predictive endpoints, and to further optimise existing methods to be integrated into a testing strategy, with a view to achieving the full replacement of the current animal tests. The European Centre for the Validation of Alternative Methods (ECVAM) and the European Cosmetics Association (Colipa) have been collaborating with the aim of identifying a core set of reference chemicals for test method development and/or optimisation. The use of a common set of reference chemicals in the method development phase, would facilitate an early assessment of the performance of a method with respect to existing tests, and of its possible contribution to a testing strategy. By applying pre-defined criteria, existing databases were mined, and a list of 16 chemicals, including 12 positive controls, of which four require metabolic activation to act as sensitisers, and four negative controls, was collated. The chemicals and the criteria used for their selection are presented.

Introduction

Animals are used in the currently-accepted regulatory test methods for the identification and characterisation of skin sensitisation hazard (1–3). However, with the entry into force of the 7th Amendment to the EU Cosmetics Directive (4) and the new regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (5), there is an acute need for alternative methods which are capable of characterising the potential of substances to cause skin sensitisation in humans. In addition to the provisions introduced by the regulatory framework, there is worldwide growing awareness of the need for substantial changes in toxicity testing, as described in a recent report from the US National Research Council (6). Despite the complexity of the endpoint, advances in the development of alternative methods are being achieved, thanks to extensive research efforts at both the industry and university levels (7–9). Nevertheless, up to now, none of these methods could fully replace the current animal tests. It is more likely that a combination of tests, each covering a key biological mechanism of skin sensitisation, will be required (10). For this, further efforts are needed toward the identification of predictive biomarkers. New tests must incorporate the most recent advances in the understanding of the toxicological mechanisms involved, and must generate information which is complementary to that provided by existing test methods. New toxicological test methods evolve through a series of phases, from development, protocol refinement and optimisation, to the formal evaluation, through a validation exercise, of their reproducibility and relevance, before they can be used to generate data to support regulatory decisions (11–13). Each of these phases requires the availability of suitable test compounds. Specifically, the appropriate selection of substances in the development phase is crucial for the early identification

Address for correspondence: Silvia Casati, In Vitro Methods Unit/ECVAM, Institute for Health and Consumer Protection, European Commission Joint Research Centre, Via E. Fermi, 21027 Ispra (VA), Italy.
E-mail: *silvia.casati@jrc.ec.europa.eu*

of the test's strengths and limitations, and for an initial appreciation of its performance. This would also allow for the adoption of improvement measures in subsequent phases, if these are needed. Moreover, encouraging, as far as is possible, the use of a common set of test compounds by test method developers, would allow for a comparative assessment of the data generated and for the identification of methodologies which are complementary to existing ones or that have the potential to provide improved predictions of adverse health effects.

For these reasons, ECVAM, as part of the European Commission's Joint Research Centre (JRC) Institute for Health and Consumer Protection (IHCP), together with the Colipa Skin Tolerance Project Team and their associates, have worked on the identification of a list of chemicals that should provide a valuable reference for the development of new methods or for the improvement of existing ones, for targeting the assessment of the skin sensitisation potential of chemicals.

The Chemicals Selection Strategy

Inclusion criteria

Good reference chemicals must fulfil the following criteria:

1. *High quality* in vivo *data must be available for them.* This is a fundamental prerequisite for any chemical selection process. To properly evaluate the responses in the test system, the chemicals should possess unequivocal sensitising properties, or there should be consistent evidence that such a property is lacking. For the purpose of this exercise, chemicals were considered for inclusion, if they were backed by concordant results in the mouse Local Lymph Node Assay (LLNA) and in the Magnusson Kligman Guinea-pig Maximisation Test (GPMT), and/or in the Buehler occluded patch test. Furthermore, careful consideration was given to the availability of human evidence, from diagnostic patch test data and/or epidemiological studies, which matched the results from animal tests. On this last point, however, whilst it is self-evident that it is the hazard to humans that is paramount, human evidence itself requires a greater element of expert judgement. Often, it is complicated by uncertainties about the degree of exposure. For example, unless there is good evidence of extensive exposure, which was prolonged, involved a large number of individuals, and was at a reasonable concentration, an absence of clinical evidence cannot be taken to indicate an absence of skin sensitisation hazard. Similarly, clear clinical evidence of skin sensitisation must also be judged against the extent of exposure, so that substances which are of too low potency are not regarded as classifiable positives. As a guide, the criteria for the use of human data for classification purposes, which are set out in the REACH guidance documents (14) and in the Globally Harmonised System of Classification and Labelling of Chemicals (GHS; 15), have been used.

2. *They should be easily purchased from commercial sources.* In order to favour widespread use, chemicals must be readily available from commercial sources, be of defined purity (preferably $> 95\%$), and at a reasonable price.

3. *They should cover, as far as possible, the dynamic range of responses that can be assessed with the LLNA.* The LLNA has been formally validated for the purpose of hazard identification. However, if conducted according to current regulatory guidelines (16), it permits an estimation of relative potency — the EC3 value (the estimated concentration of a chemical required to induce a 3-fold stimulation of draining lymph node cell proliferation, as compared with concurrent controls). For both regulatory purposes and for risk assessment, this information is useful to categorise chemicals according to their sensitisation potencies. Nevertheless, it has to be acknowledged that such categorisation may be associated with a certain level of uncertainty. In the evaluation of any emerging alternative approach, attention should be paid as to whether such a method would be able to make correct predictions for a range of sensitising chemicals which differ in their intrinsic sensitising potency. However, bearing in mind that the selected chemicals are proposed for development and/or optimisation purposes, chemicals which are classified as very weak sensitisers in the LLNA, were deliberately excluded from the selection, since these might give equivocal responses in *in vitro* tests. Such materials must, of course, be assessed later on in the process of evaluation of new test methods.

4. *They should represent a relevant range of chemical classes.* It is recognised that, with a limited set of substances, is almost impossible to cover all the variety of molecular structures relevant to skin sensitisation. However, an attempt was made to represent such diversity as much as was possible, by the inclusion of alcohols, aldehydes, aromatic amines, halogenated compounds, thioorganics, and a nitrile.

5. *They should cover the range of chemical reaction mechanisms for the modification of proteins.* It has recently been proposed that skin sensitisers react with proteins through six major different chemical mechanisms. The

reactive domains include: Michael acceptors, SN2 electrophiles, SNAr electrophiles, Schiff's base formers, and acyl transfer electrophiles. One domain is proposed to categorise non-reactive or non-pro-reactive compounds (17). The categorisation of chemical sensitisers into reaction mechanistic domains is suggested for the development and/or application of mechanistic read-across and quantitative mechanistic models (QMMs; 18).

6. *They should embrace a wide range of physicochemical parameters.* In screening candidate chemicals, an attempt was made to cover, to the greatest possible extent, the range of physicochemical diversity relevant to skin sensitisers. The molecular weight (MW), physical state (solid, liquid), water solubility and octanol–water partition coefficient (log $P_{o/w}$), were considered for this purpose. However, it is recognised that fully meeting this challenge is beyond the scope of this limited set of substances.

7. *They should require activation before reacting with proteins.* It has been estimated that about one third of skin sensitisers require either metabolic activation by skin enzymes (pro-hapten) or biochemical activation (e.g. air oxidation), in order to be transformed into a reactive species able to bind to skin proteins (19). The inclusion of these categories of sensitisers into the list was deemed important for the preliminary evaluation of the ability of a test method to successfully detect these substances.

Exclusion criteria

Compounds falling in one or more of the following categories were not considered for inclusion:

1. *Gases and highly volatile chemicals.* Such chemicals present handling and testing difficulties. Volatilisation of the test chemical from the exposure medium poses experimental problems. This may result in a significant loss of the test chemical and/or cross contamination of test concentrations, both of which can lead to interpretational errors.

2. *Insoluble chemicals.* Chemicals with water solubility of < 5mg/L were deliberately excluded, to avoid the technical hurdles to obtaining the relevant concentrations in the plate wells.

3. *Metals.* Metals have been extensively characterised with regard to their skin sensitisation potentials. They induce sensitisation through mechanisms which are not similar to those of the vast majority of organic chemicals. For this reason, it was not considered of value to insist on their inclusion in a reference list against which new methods should be calibrated.

4. *Typical respiratory sensitisers.* Being able to distinguish between skin and respiratory sensitisers could be a very important and interesting feature of a new method. However, it would be too demanding at the development stage of a test method to expect this level of performance. Moreover, most of respiratory sensitisers are highly unstable (e.g. anhydrides and isocyanates), and degrade in contact with either water or air. It is important to recognise that the decision to exclude both metals and respiratory sensitisers was made only in the context of collating an initial small set of substances for recommended use in the primary development of an *in vitro* assay. It is probable that these two categories of substance will have to be appropriately assessed during the later stages of the evaluation of a new test method.

5. *High molecular weight chemicals (> 500Da).* It has been previously demonstrated (20) that it is highly improbable that proteins and high molecular weight chemicals would penetrate the intact epidermis, so they are therefore highly unlikely to be capable of causing sensitisation through the skin.

Reference Data Sources

Databases are available, which encompass a number of substances with documented contact allergenic properties in animals and/or in humans. The reference data source for the purpose of our selection was a compilation of LLNA data (21), which contains information on 211 chemicals. Such a database represents a unique source of *in vivo* data for skin sensitisers, having been generated by using an OECD standard method which yields an objective, quantitative endpoint. Consequently, it is proposed as a reference list for the development, evaluation and validation of alternative approaches for the assessment of skin sensitisation. We reviewed the LLNA database, and, by applying the pre-defined criteria, a short-list of 16 chemicals fulfilling the inclusion criteria was established. In this phase of identification of the candidate chemicals, the positive and negative controls recommended by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC; 22) were taken into account, as well as the chemicals selected as training compounds within the EU integrated project, Sens-it-iv (23).

Reference Chemicals

The suggested 16 reference substances are listed in Table 1. These consist of eight positive controls,

Table 1: Chemical structures, physicochemical parameters, *in vivo* data and mechanistic domains

Substance name	CAS No.	MW (g/mol)	Physical form	Water solubility mg/L	log $P_{o/w}$	LLNA EC3 (Refs*)	GPMT/ BT[1] (Refs*)	HMT/ HPTA[2] (Refs*)	Hypothetical chemical reaction involved in the sensitisation process
Positive controls									
Oxazolone	15646-46-5	217	solid	1850	1.51 (est)	0.003 (24)	+ (25)	+ (26)	Acyl transfer agent
4-Nitrobenzylbromide	100-11-8	216	solid	72.6 (est)	2.70 (est)	0.05 (21)	+ (27)	+ (28)	Electrophile-H-polar compound
2,4-Dinitrochlorobenzene (DNCB)	97-00-7	203	solid	8	2.17	0.06 (25)	+ (1, 29)	+ (30)	SN2Ar electrophile
Methyldibromoglutaronitrile (MDGN)	35691-65-7	266	solid	1280	1.63	0.9 (21)	– (31)	+ (32)	pro-Michael acceptor
Glyoxal	107-22-2	58	liquid	1.00×10^{6}	–1.66	1.4 (21)	+ (33)	+ (34–36)	Schiff's base former
2-Mercaptobenzothiazole (MBT)	149-30-4	167	solid	120	2.42	1.7 (21)	+ (37, 38)	+ (39, 40)	Acyl transfer agent
Cinnamal	104-55-2	132	liquid	1420	1.9	3 (21)	+ (38, 41)	+ (39, 42)	Michael acceptor and/or Schiff's base former
Tetramethyl thiuram disulphide (TMTD)	137-26-8	240	solid	30	1.73	5.2 (21)	+ (43)	+ (39, 40)	SN2 reaction at the S-atom can be proposed
Pre/pro haptens									
Paraphenylenediamine (PPD)	106-50-3	108	solid	3.70×10^{4}	–0.3	0.16 (21)	+ (38, 44)	+ (40, 45, 46)	Probably/possibly pro-Michael acceptor
Isoeugenol	97-54-1	206	liquid	356	3.04	1.2 (21)	+ (47, 48)	+ (39, 49)	Probably/possibly pro-Michael acceptor
Eugenol	97-53-0	164	liquid	2460	2.27	13 (21)	+ (38)	+ (39–42)	Probably/possibly pro-Michael acceptor
Cinnamic alcohol	104-54-1	134	solid	6190	1.95	21 (21)	+ (48)	+ (39, 50)	Probably/possibly Michael acceptor
Negative controls									
Glycerol	56-81-5	92	liquid	1.00×10^{6}	–1.76	NC (21)	– (1)	– (51)	Neither reactive nor pro-reactive domain
Salicylic acid (SA)	69-72-7	138	liquid	2240	2.26	NC (21)	– (52)	– (40, 51)	Neither reactive nor pro-reactive domain
Lactic acid	50-21-5	90	liquid	1.00×10^{6}	–0.72	NC (21)	– (52)	– (53)	Neither reactive nor pro-reactive domain/toxic
Sodium lauryl sulphate (SLS)	151-21-3	288	solid	1.00×10^{5}	1.6	14 (21)	– (44)	– (40, 55)	Neither reactive nor pro-reactive domain/irritant

*BT = Buehler test; CAS No. = Chemical Abstract Service Registry Number; (est) = estimated; GPMT = Guinea-pig maximisation test; HMT = human maximisation test; HPTA = human patch test allergen; LLNA = local lymph node assay; MW = molecular weight; NC = not calculated. [1]Results obtained from Guinea-pig maximisation test and/or Buehler test. [2]Results obtained from human maximisation test and/or human patch test allergen.*References given are not an exhaustive list.*

four chemicals requiring activation to act as sensitisers, and four negative controls. For each substance, the chemical name and the chemical abstract service (CAS) number is reported, as well as the main physicochemical properties: molecular weight, physical state, water solubility and the octanol–water partition coefficient. Moreover, the *in vivo* references (21, 24–55), and the probable chemical reactions involved in the sensitisation process, are listed. The observed range of physicochemical properties for the reference set is reported in Table 2.

Positive controls are chemicals that should be detected as positive by any proposed *in vitro* skin sensitisation test. Chemicals in this group are extreme, strong and moderate sensitisers, according to the ECETOC potency classification proposal (55), with EC3 values in the range of 0.003–5.2. Among these, two aldehydes, namely cinnamal and glyoxal, were deliberately selected, because they are hypothesised to react via different reaction mechanisms.

Isoeugenol, eugenol, and cinnamic alcohol need to be metabolised by cutaneous enzymes into protein-reactive species (pro-haptens), whereas *p*-phenylenediamine (PPD) requires a physicochemical reaction to be transformed into a reactive species. It is assumed that any alternative method would have the capacity to correctly identify them as skin sensitisers. The inclusion of such chemicals in the reference list will allow for an early appreciation of the extent to which the test system is able to metabolically activate inert substances.

As indicated in Table 1, the suggested 12 positive controls are all positive in standard *in vivo* predictive tests, with the exception of methyldibromoglutaronitrile (MDGN) in the guinea-pig tests. Furthermore, for most of them, there is convincing human evidence that they should be regarded as significant skin sensitisers, oxazolone being the exception. MDGN, PPD, cinnamal, cinnamic alcohol, isoeugenol, eugenol, tetramethylthiuramdisulphide (TMTD) and mercaptobenzothiazole (MBT) are well recognised clinical allergens. MDGN is an important preservative allergen (32); PPD is the most important hair-dye allergen (45); TMTD and MBT are well recognised rubber chemical allergens, being key agents which are used diagnostically in the thiuram mix and mercapto mix, respectively (39); the other four substances mentioned are common fragrance allergens, being part of the standard test series known as *Fragrance Mix 1* (39). Dinitrochlorobenzene (DNCB) has been used for many years as an experimental human sensitiser (30); skin sensitisation in humans to glyoxal has been reported, and, although less common generally, it appears to be significant in relation to the modest scale of exposure (34, 35); for 4-nitrobenzylbromide, there have only been isolated case reports (summarised in 28), but again, given the very modest scale of exposure, these serve to confirm the significant hazard. In addition, it is worth noting that, for most of these positive chemicals, there is also evidence of skin sensitising activity that has been derived from historical human predictive assay results, as is indicated in the Table.

The negative controls are chemicals with no reported skin sensitisation potential; indeed, glycerol was mentioned as a potential vehicle for use in the GPMT (1). Among these, lactic acid is slightly toxic and corrosive, and sodium lauryl sulphate (SLS) is a substantial skin irritant, which is characterised as a false positive in the LLNA (note that it is by no means clear that its irritant activity is the reason that it is a false positive). These chemicals have been selected, in order to assess the ability of a method to distinguish between sensitisers and irritants or toxic compounds. Consequently, it is interesting to ask whether there is substantive human evidence that, even with the occurrence of considerable exposure, these substances fail to cause skin sensitisation. For SLS, its overwhelming use for decades, as a classical skin irritant in clinical testing of many kinds, without any concomitant evidence of skin sensitisation, is probably reason enough (54). Similarly, for glycerol, skin exposure has been a common occurrence via its use as a humectant and potential anti-irritant in skin care products; no cases of skin sensitisation have been reported. For lactic acid, which is both a sensory and physically irritant substance at high concentrations, skin exposure is generally rather lower, but again, no evidence of human skin sensitisation could be found. However, of these selected negatives, salicylic acid is perhaps the most interesting. It has widespread use at high concentrations (up to 40%) in a variety of topical medicaments (reviewed in 51); skin sensitisation has been reported, but is so rare in relation to the substantial exposure that this substance must be regarded as a skin sensitiser which is too weak to classify. Confirmation of this view comes from the finding that the material was negative at high concentrations in the human maximisation test (40).

Table 2: The range of the physicochemical properties covered by the selected chemicals

Physicochemical properties	Observed range
Molecular weight	58 to 288
log $P_{o/w}$	–1.76 to 3.04
Water solubility[a]	10^0 to 10^6 mg/L

[a]*Measured at temperatures ranging from 19 to 25°C.*

Summary

There is a general, indeed pressing, need to develop, validate and bring into common use alternative methods to obviate the use of animals in toxicity testing. Specifically in the field of skin sensitisation, considerable investments are being made, at both the industry and university levels, in the development of alternative approaches to incorporate novel biomarkers or to enhance the functioning of existing models. In the development and/or optimisation phases of a method, the testing of reference chemicals with well characterised responses in predictive *in vivo* tests, would allow for an early appreciation of the utility of a method. More specifically, challenging a method with the same set of chemicals would facilitate the assessment of its possible contribution to an integrated testing approach. With such a small number of substances, the limited feasibility of fully representing the chemical and biological diversity of known chemical (skin) allergens is acknowledged. However, an attempt was made to cover, as much as possible, the range of known potencies for skin allergens, and to reflect the variety of physicochemical and reaction mechanisms involved in the sensitisation process. Furthermore, chemicals known to require biotransformation or other types of activation were included in the list, in order to evaluate the potential of the test system to detect them correctly. It is acknowledged that other chemical sets also might be appropriate for test development purposes. However, it was deemed important both to make public this recommendation and to encourage the use of a core set. It is therefore proposed that these selected substances are used as reference compounds for the primary development of new tests, or for the improvement of existing tests. In our view, a test that is able to correctly identify these chemicals should be considered very promising and worthy of further evaluation.

References

1. Magnusson, B. & Kligman, A.M. (1970). *Allergic Contact Dermatitis in the Guinea Pig*, 141pp. Springfield, IL, USA: Charles C. Thomas.
2. Buehler, E.V. (1965). Delayed contact hypersensitivity in the guinea pig. *Archives of Dermatology* **91**, 171–177.
3. Kimber, I. & Basketter, D.A. (1992). The murine local lymph node assay; collaborative studies and new directions: A commentary. *Food & Chemical Toxicology* **30**, 165–169.
4. EU (2003). *Directive 2003/15/EC* of the European Parliament and the Council of 27 February 2003 amending *Council Directive 76/768/EEC* on the approximations of laws of the Member States relating to cosmetic products. *Official Journal of the European Union* **L66**, 26–35.
5. EC (2006). *Regulation No 1907/2006* of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending *Directive 1999/45/EC* and repealing *Council Regulation (EEC) No 793/93* and *Commission Regulation (EC) No 1488/94* as well as *Council Directive 76/769/EEC* and *Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC* and *2000/21/EC*. *Official Journal of the European Union* **L396**, 1–849.
6. US National Research Council (2007). *Toxicity Testing in the Twenty-first Century: A Vision and a Strategy*. Committee on Toxicity and Assessment of Environmental Agents, 191pp. Washington, DC, USA: National Research Council.
7. Basketter, D. & Maxwell, G. (2007). *In vitro* approaches to the identification and characterization of skin sensitizers. *Cutaneous & Ocular Toxicology* **26**, 359–373.
8. Aeby, P., Ashikaga, T., Diembeck, W., Eschrich, D., Gerberick, F., Kimber, I., Marrec-Fairley, M., Maxwell, G., Ovigne, J-M., Sakaguchi, H., Tailhardat, M. & Teissier, S. (2008). The Colipa strategy for the development of *in vitro* alternatives: skin sensitization. *AATEX* **14**, 375–379.
9. *Sens-it-iv: Novel Testing Strategies for* In Vitro *Assessment of Allergens* website. Available at: http://www.sens-it-iv.eu/ (Accessed 11.03.09).
10. Jowsey, I.R., Basketter, D.A., Westmoreland, C. & Kimber, I. (2006). A future approach to measuring relative skin sensitising potency: a proposal. *Journal of Applied Toxicology* **26**, 341–350.
11. Curren, R.D., Southee, J.A., Spielmann, H., Liebsch, M., Fentem, J.H. & Balls, M. (1995). The role of prevalidation in the development, validation and acceptance of alternative methods. ECVAM Prevalidation Task Force Report 1. *ATLA* **23**, 211–217.
12. Balls, M., Blaauboer, B.J., Fentem, J.H., Bruner, L., Combes, R.D., Ekwall, B., Fielder, R.J., Guillouzo, A., Lewis, R.W., Lovell, D.P., Reinhardt, C.A., Repetto, G., Sladowski, D., Spielmann, H. & Zucco, F. (1995). Practical aspects of the validation of toxicity test procedures. ECVAM Workshop Report 5. *ATLA* **23**, 129–147.
13. OECD (2005*). OECD Series on Testing and Assessment. No. 34. Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment*, 96pp. Paris, France: Organisation for Economic Cooperation and Development.
14. ECHA (2008). *Guidance on Information Requirements and Chemical Safety Assessment. Chapter R.4: Evaluation of Available Information*, 23pp. Helsinki, Finland, European Chemicals Agency. Available at: http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_r4_en.pdf?vers=20_08_08 (Accessed 13.02.09).
15. Anon. (2003). *Globally Harmonised System of Class-*

ification and Labelling of Chemicals (GHS). Part 3: Health and Environmental Hazards, pp. 151–158. New York, NY, USA, and Geneva, Switzerland: United Nations Organisation.

16. OECD (2002). *OECD Guideline for the Testing of Chemicals. No. 429. Skin Sensitisation: Local Lymph Node Assay,* 7pp. Paris, France: Organisation for Economic Cooperation and Development.
17. Aptula, A.O., Patlewicz, G. & Roberts, D.W. (2005). Skin sensitisation: reaction mechanistic applicability domains for structure activity relationships. *Chemical Research in Toxicology* **18**, 1420–1426.
18. Roberts, D., Patlewicz, G., Kern, P., Gerberick, F., Kimber, I., Dearman, R., Ryan, C., Basketter, D. & Aptula, A. (2007). Mechanistic applicability domain classification of a Local Lymph Node Assay dataset for skin sensitization. *Chemical Research in Toxicology* **20**, 1019–1030.
19. Lepoittevin, J.P. (2006). Metabolism versus chemical transformation or pro- versus prehaptens? *Contact Dermatitis* **54**, 73–74.
20. Bos, J.D. & Meinardi, M.M.H.M. (2000). The 500 Dalton rule for the skin penetration of chemical compounds and drugs. *Experimental Dermatology* **9**, 165–169.
21. Gerberick, G.F., Ryan, C.A., Kern, P.S., Schlatter, H., Dearman, R.J., Kimber, I., Patlewicz, G.Y. & Basketter, D.A. (2005). Compilation of historical local lymph node data for evaluation of skin sensitisation alternative methods. *Dermatitis* **16**, 157–202.
22. ECETOC (1999). *Technical Report No. 77 — Skin and Respiratory Sensitisers: Reference Chemical Data Bank*. ISSN-0773-8072-77, 85pp. Brussels, Belgium: European Centre for Ecotoxicology and Toxicology of Chemicals.
23. Sens-it-iv (2007). *WP 1: set of reference compounds.* Newsletter Number 02, 26 January 2007. Available at: http://www.sens-it-iv.eu/files/newsletter/Sens-it-iv_Newsletter_02.pdf (Accessed 05.02.09).
24. Basketter, D.A., Wright, Z.M. & Warbrick, E.V. (2001). Human potency predictions for aldehydes using the local lymph node assay. *Contact Dermatitis* **45**, 89–94.
25. Basketter, D.A. & Kimber, I. (2006). Predictive test for irritants and allergens and their use in quantitative risk assessment. In *Contact Dermatitis, 4th Edition* (ed. P.J. Frosch, T. Menné & J-P. Lepoittevin), pp. 179–188. Heidelberg, Germany: Springer Verlag.
26. Baer, H., Stone, S.H. & Malik, F. (1976). Early and late contact sensitivity reactions in guinea pigs sensitized to oxazolone. *Journal of Immunology* **117**, 1159–1163.
27. Roberts, D.W., Goodwin, B.F.J., Williams, D.L., Jones, K., Johnson, A.W. & Alderson, J.C.E. (1983). Correlation between skin sensitisation potential and chemical reactivity for *p*-nitrobenzyl compounds. *Food Chemical Toxicology* **211**, 811–813.
28. Thompson, R., Finn, A.M. & Rycroft, R.J.G. (1998). Allergic contact dermatitis from *para*-nitrobenzyl bromide. *Contact Dermatitis* **38**, 232.
29. Basketter, D.A., Gerberick, G.F., Kimber, I. & Loveless, S.E. (1996). The local lymph node assay: A viable alternative to currently accepted skin sensitization tests. *Food & Chemical Toxicology* **34**, 985–997.
30. Friedmann, P.S. (2007). The relationships between exposure dose and response in induction and elicitation of contact hypersensitivity in humans. *The British Journal of Dermatology* **157**, 1093–1102.
31. Wahlkvist, H., Boman, A., Montelius, J. & Wahlberg, J.E. (1999). Sensitising potential in mice, guinea pig and man of the preservative Euxyl K400 and its ingredient methyldibromo glutaronitrile. *Contact Dermatitis* **41**, 330–338.
32. Wilkinson, J.D., Shaw, S., Andersen, K.E., Brandao, F.M., Bruynzeel, D.P., Bruze, M., Camarasa, J.M., Diepgen, T.L., Ducomns, G., Frosch, P.J., Goossens, A., Lachappelle, J.M., Lahti, A., Menné, T., Seidenari, S., Tosti, A. & Wahlberg, J.E. (2002). Monitoring levels of biocide sensitivity in Europe. A ten year overview (1991–2000). *Contact Dermatitis* **46**, 207–210.
33. Gerberick, G.F., Ryan, C.A., Kimber, I., Dearman, R.J., Lea, L.J. & Basketter, D.A. (2000). Local lymph node assay validation assessment for regulatory purposes. *American Journal of Contact Dermatitis* **11**, 3–18.
34. Elsner, P., Pevny, I. & Burg, G. (1990). Occupational contact dermatitis due to glyoxal in health care workers. *American Journal of Contact Dermatitis* **1**, 250–253.
35. Uter, W., Schwanitz, H.J., Lessman, H. & Schnuch, A. (2001). Glyoxal is an important allergen for (medical care) cleaning staff. *International Journal of Hygiene & Environmental Health* **204**, 251–253.
36. Aalto-Korte, K., Mäkelä, E.A., Huttunen, M., Suuronen, K. & Jolanki, R. (2005). Occupational contact allergy to glyoxal. *Contact Dermatitis* **52**, 276–281.
37. Basketter, D.A., Selbie, E., Scholes, E.W., Lees, D., Kimber, I. & Botham, P.A. (1993). Results with OECD recommended positive control sensitisers in the maximisation, Buehler and local lymph node assays. *Food & Chemical Toxicology* **31**, 63–67.
38. Basketter, D.A. & Gerberick, G.F. (1996). Interlaboratory evaluation of the Buehler test. *Contact Dermatitis* **35**, 146–151.
39. Andersen, K.E., White, I.R. & Goossens, A. (2006). Allergens from the standard series. In *Contact Dermatitis, 4th Edition* (ed. P.J. Frosch, T. Menné & J-P. Lepoittevin), pp. 453–492. Heidelberg, Germany: Springer Verlag.
40. Kligman, A.M. (1966). The identification of contact allergens by human assay. III: The maximisation test: a procedure for screening and rating contact sensitizers. *Journal of Investigative Dermatology* **47**, 393.
41. Basketter, D.A. & Scholes, E.W. (1992). Comparison of the local lymph node assay with the guinea pig maximisation test for the detection of a range of contact

allergens. *Food & Chemical Toxicology* **30**, 65–69.
42. Marzulli, F.N. & Maibach, H.I. (1980). Contact allergy: predictive testing of fragrance ingredients in humans by Draize and maximization methods. *Journal of Environmental Pathology & Toxicology* **3**, 235–245.
43. Ziegler, V., Suess, E., Standav, H. & Hasert, K. (1972). Der Meerschweinchen — Maximisations Test zum Nachweis der sensibilisierenden Wirkung wichtiger Industrieprodukte. *Allegie und Immunologie* **18**, 203.
44. Wahlberg, J.E. & Boman, A. (1985). Guinea pig maximisation test. In *Contact Allergy Predictive Tests in Guinea Pigs*. Current Problems in Dermatology Vol. 14 (ed. K.E. Andersen & H.I. Maibach), p. 59. Basel, Switzerland: Karger.
45. Thyssen, J.P. & White, J.M.L. (2008). Epidemiological data on consumer allergy *p*-phenylenediamine. *Contact Dermatitis* **59**, 327–343.
46. Marzulli, F.N. & Maibach, H.I. (1974). The use of graded concentrations in studying skin sensitizers: experimental contact sensitization in man. *Food & Cosmetics Toxicology* **12**, 219–227.
47. Maurer, T., Thomann, P., Weirich, E.G. & Hess, R. (1979). Predictive evaluation in animals of the contact allergenic potential of medically important substances. II: Comparisons of different methods of cutaneous sensitisation with 'weak' allergens. *Contact Dermatitis* **5**, 1.
48. Klecak, G., Geleik, H. & Frey, J.R. (1977). Screening of fragrance materials for allergenicity in the guinea pig. I: Comparison of four testing methods. *Journal of the Society of Cosmetic Chemists* **28**, 53–64.
49. Thompson, G.R., Booman, K.A., Dorsky, J., Kohrman, K.A., Rothenstein, A.S., Schwoeppe, E.A., Sedlak, R.I. & Steltenkamp, R.J. (1983). Isoeugenol: a survey of consumer patch-test sensitisation. *Food & Chemical Toxicology* **6**, 735–740.
50. Jordan, W.P. Jr & King, S.E. (1977). Delayed hypersensitivity in females. The development of allergic contact dermatitis in females during the comparison of two predictive patch tests. *Contact Dermatitis* **3**, 19–26.
51. Rietschel, R.L. & Fowler, J.F. (2008). *Fisher's Contact Dermatitis, 6th Edition*, pp. 129–130. Hamilton, ON, Canada: BC Decker, Inc.
52. Cronin, M.T.D. & Basketter, D.A. (1994). Multivariate QSAR analysis of a skin sensitization database. *SAR & QSAR in Environmental Research* **2**, 159–179.
53. Basketter, D.A., Gerberick, G.F. & Kimber, I. (1998). Strategies for identifying false positive responses in predictive skin sensitization tests. *Food & Chemical Toxicology* **36**, 327–333.
54. Tupker, R.A., Willis, C., Berardesca, E., Lee, C.H., Fartasch, M., Agner, T. & Serup, J. (1997). Guidelines on sodium lauryl sulfate (SLS) exposure tests. A report from the Standardization Group of the European Society of Contact Dermatitis. *Contact Dermatitis* **37**, 53–69.
55. ECETOC (2003). *Technical Report No. 87 — Contact Sensitisation: Classification According to Potency*. ISSN-0773-8072-87, 24pp. Brussels, Belgium: European Centre for Ecotoxicology and Toxicology of Chemicals.