

An evaluation of selected valid and mechanistically based SARs for skin sensitisation

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Abstract

Skin sensitisation which clinically is manifested as allergic contact dermatitis is an important health endpoint to be taken into account in the risk assessment of chemicals. In the new EU chemical policy REACH, information on skin sensitisation potential will have to be provided for chemicals manufactured or imported equal to or above 1 tonne/year and it is estimated that the highest number of substances will need to be tested for skin sensitisation. Within REACH, the use of (Q)SARs and other alternative methods is strongly encouraged in order to reduce the number of animal tests. The goal of this project was to test the applicability of SARs and structural alerts for skin sensitisation in the REACH framework. SARs and structural alerts which were found in the literature were analysed and further refined by using a number of examples (substances) that had not been previously used in the derivation of the published SARs. For substances which fit within the boundaries of the proposed alerts, it is assumed that they would be classified as sensitising in accordance with EU classification and labelling rules.

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1. Introduction

Skin sensitisation which clinically is manifested as allergic contact dermatitis (ACD) is an important health endpoint to be taken into account in risk assessment of chemicals, since people are being continuously sensitised at the workplace and by coming into contact with consumer products. This may in part also be due to the fact that for a large number of substances, the sensitising potential is not yet known. In the new EU chemical policy REACH, information on skin sensitisation will have to be provided for chemicals manufactured or imported at or above 1 tonne/year (EC, 2006). According to the estimation carried out by the ECB (Pedersen et al., 2003), under REACH, the largest number of substances will need to be tested for skin sensitisation. The goal of REACH, however,

is not only to generate the lacking toxicity data, but also at the same time reduce animal testing as much as possible (EC, 2006). Therefore, within REACH the use of (Q)SARs and other alternative methods in order to reduce the number of animal tests and the costs is strongly encouraged.

In view of this changing legislation, several initiatives have recently emerged in order to further develop and increase the acceptance of (Q)SARs in hazard and risk assessment of chemicals (ECETOC, 2002). The principles for validation of (Q)SARs are generally referred as the “OECD Principles for (Q)SAR validation” (ECETOC, 2002; OECD, 2004, 2007). They are intended to give some guidance and increase consistency in development and validation of (Q)SARs in order to be used for regulatory purposes. According to the OECD principles, a (Q)SAR should be associated with: (1) a defined endpoint, (2) an unambiguous algorithm, (3) a defined domain of applicability, (4) appropriate measures of goodness of fit, robustness and predictivity and (5) a mechanistic interpretation, if possible. The main goal of applying the OECD principles

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in development and application of (Q)SARs is to assure that all necessary information is included and to facilitate consideration and validation of these models for regulatory purposes.

Although several SARs (Derekfw 8.0, Gerner et al., 2004; Zinke et al., 2002), and QSARs (Roberts and Williams, 1982; Einslein et al., 1997; Patlewicz et al., 2001, 2002, 2004; Roberts and Patlewicz, 2002; Rodford et al., 2003) for skin sensitisation are available in the literature, they have so far not been extensively used for regulatory purposes. The main reasons for this limited usage could be: a lack of description of the mechanisms of action, no insight into the domain of applicability, lack of external validation and very limited available guidance on how to develop and use (Q)SARs. The available SARs often require additional expert judgement and are therefore not easy to use for regulatory applications. Currently, no structural alerts for skin sensitisation are available in the TGD or EU classification and labelling (C&L) guidance (ECB, 2003; EC, 2004). The goal of this paper is to investigate what could be the evaluation essentials for possible acceptance of structural alerts for skin sensitisation in the REACH framework. The OECD principles on (Q)SARs were considered to be the guiding rules (OECD, 2004).

For several of the sensitisation (Q)SARs available in the literature, OECD principles are indicated, but not explicitly mentioned. Roberts et al. (2007) have evaluated a number of global (Q)SARs according to the OECD principles. This is the first time that local models are critically reviewed from a regulatory perspective using OECD principles. In the end a proposal is brought forward how to actually use these models in the regulatory context.

2. Material and methods

2.1. Regulatory needs

The standard tests for skin sensitisation used for regulatory purposes are the Local lymph node assay [LLNA; method OECD 429 (OECD, 2002)], Guinea pig maximisation test [GPMT; method OECD 406 (OECD, 1992)] and the Buehler test [method OECD 406 (OECD, 1992)]. The major differences between these three tests are in the animal species used, procedure of application of substances, use of adjuvant and in the interpretation of the results. In terms of interpretation of results, the guinea pig tests are somehow subjective, as they rely on interpretation of the skin reaction during challenge, which is not an immune specific endpoint, while in the LLNA cell proliferation is determined by measurement of incorporated radioactivity. Furthermore, the LLNA identifies the potential skin sensitizers by focusing only on induction, while the guinea pig tests consider both induction and elicitation (challenge).

It appears that different tests can give different results, especially in case of weak sensitizers (Basketter and Scholes, 1992; Basketter et al., 1996) and sometimes allergens

proved to be active in tests in humans are not detected by the animal tests (Dir 2004/73/EC). When comparing the results from the GPMT test (Basketter and Scholes, 1992) and Buehler test (Basketter et al., 1996) with those of the LLNA test, in general close concordance was shown in case of strong and moderate sensitizers, while weak sensitizers in GPMT test were usually not detected by the LLNA.

In our investigation, most of the substances included in training and external sets had information on the sensitising potential based on the LLNA or the GPMT test. For substances that had available information based on both test methods, no discordance in terms of the outcome (sensitising/not sensitising) between the two methods was found.

The skin sensitisation endpoint can be considered as a function of bioavailability, chemical reactivity and immune response (Jowsey et al., 2006). The reactivity of chemicals towards skin proteins that results in hapten formation is a crucial element in skin sensitisation potential. A short introduction on the role of chemical reactivity in relation to skin sensitisation and the selection of chemical reactivity types used in this paper is given below.

2.2. Role of chemical reactivity in skin sensitisation

Chemical substances that cause allergies are haptens. In order to stimulate the immune response, haptens have to bind with proteins in the body, to form hapten/protein conjugates, which can be recognised by the immune system. In general, a compound which is able to cause contact allergy must have electrophilic properties, since the skin is a kind of “nucleophilic environment”. Electrophilic properties enable the compound to form a covalent binding, but the protein binding can also be established through a radical mechanism (Karlberg et al., 1994; Aptula et al., 2005). In general it is assumed that protein-hapten conjugates need to be formed by covalent bonding, in order to be recognised by the immune system (Karlberg et al., 1994; Aptula et al., 2005; Smith Pease, 2003). However, the mechanisms of protein haptentation and the ability to cause skin sensitisation are not yet fully understood (Divkovic et al., 2005; Smith Pease, 2003). Some authors argue that “non-covalent modes” could also be involved (Pichler, 2001; Wulferink et al., 2002; reviewed in Divkovic et al., 2005 and Smith Pease, 2003; Basketter et al., 1995).

Also non electrophilic substances can become such by changing the structure through an enzymatic process in the skin or chemical transformation outside the skin. Such substances are called pro-haptens. The conversion from a pro-hapten to a hapten might take place by oxidation involving enzymes in the skin (among others cytochrome P-450, alcohol and aldehyde dehydrogenases), but the oxidation might also take place due to oxygen in air, often in combination with light and heat (Karlberg et al., 1994; Smith Pease et al., 2003; Smith Pease, 2003).

In summary, two main types of sensitizers can be distinguished

- *haptens* – usually electrophilic; direct binding to skin proteins,
- *pro-haptens*:
 - not directly reactive; need to be metabolised to reactive intermediates/species which bind to skin proteins, and
 - not directly reactive; reactive molecules created by e.g. exposure to air (oxidation).

Skin metabolism does not only play a role in activation of allergens, but also in detoxification (Smith Pease et al., 2003; Smith Pease, 2003). The balance between phase 1 reactions (activation) and phase 2 reactions (detoxification) in the skin also determines the sensitising potential. A well known example which shows how important skin metabolism may be in skin sensitisation is the potential activation of cinnamic alcohol to cinnamic aldehyde, a covalent binder to skin proteins, while further metabolism detoxifies the substance by converting it to the acid (Smith Pease et al., 2003).

The most common electrophile-nucleophile reactions involved in skin sensitisation are (Aptula et al., 2005):

- Michael-type reactions,
- Schiff-base formation,
- S_N2 reactions,
- S_NAr reactions,
- acylation reactions.

The Michael-type reactions, Schiff base formation reactions, and “ S_N2 reactions” are applicable for the chemicals selected.

The investigation of application of SARs for predicting skin sensitisation potential was done by extracting SARs from public literature or from DererKfW8.0 (only their SARs were used, not their predictions). These SARs were reanalysed using the training sets of the authors. Thereafter the SARs were refined. External sets of chemicals were used to evaluate the refined SARs and an additional refinement was done if appropriate. The intention was to refine or derive mechanistically based SARs so that they can be used for regulatory purposes. At the same time the applicability of the OECD principles (OECD, 2004) for the derivation of SARs was investigated as described below:

- (1) *A defined endpoint*: the sensitisation endpoint was described from a mechanistic point of view by considering chemical reactivity towards skin proteins, and from a regulatory point of view, for which a yes(sensitising)/no(not sensitising) answer is required;
- (2) *An unambiguous algorithm*: the algorithm was described as a 2D structure, including the environ-

ment of the alert; these 2D structures were found in the literature and DererKfW.

- (3) *A defined domain of applicability*: the description of the domain of applicability was based on bioavailability parameters e.g. hydrophobicity.
- (4) *Appropriate measures of goodness of fit, robustness and predictivity*: the full training set was presented, including chemical name, CAS Nr., 2D structure and outcome of the specific test.
- (5) *A mechanistic interpretation*: the chemical and biological mechanisms were thoroughly discussed within principle one.

3. Materials

Two chemical classes were chosen, aldehydes and haloalkanes. For both groups of chemicals, information on the mechanisms of reaction with proteins was found in the literature, and there was also a relatively large number of examples (structures) for which adequate information on sensitisation potential was available. To further define the domains of the refined SARs, $\log K_{ow}$ were calculated and analysed using EPI Suite v3.12 (US EPA, 2000).

Substances were included in the respective training and external sets based on the expected mechanism of reaction with proteins. Four training and external sets were established:

- aromatic aldehydes,
- “Schiff base” aldehydes,
- “Michael addition” aldehydes,
- haloalkanes

The chemicals selected are given in Tables 1–9. Substances were included in the training or external set if information on sensitisation potential was available and was based on at least one of the three animal tests most commonly used for assessment of sensitising potential and for classification and labelling (C&L) of chemicals.

4. Results

4.1. Aldehydes

4.1.1. Principle 1 and 5: review of reaction mechanisms

Possible mechanisms of action of aldehydes in skin sensitisation were up to date discussed in several papers (Patlewicz et al., 2001; Roberts and Patlewicz, 2002; Patlewicz et al., 2002; Patlewicz et al., 2003; Patlewicz et al., 2004; Estrada et al., 2003; Estrada et al., 2004; and Aptula et al., 2005).

Aldehydes are expected to react with proteins by two mechanisms: *Schiff base formation* and by *Michael addition reactions* (Patlewicz et al., 2001; Roberts and Patlewicz, 2002; Patlewicz et al., 2002; Patlewicz et al., 2003; Patlewicz et al., 2004; and Aptula et al., 2005). Both types of

reactions are further explained below and the site of nucleophilic attack is illustrated in Fig. 1.

Based on an evaluation of 17 aldehydes containing a *benzene ring* and taking into account mechanistic and chemical properties, Patlewicz et al. (2001) divided these aldehydes into 4 chemical groups. When aldehydes were considered as one chemical group, little correlation between sensitisation and generic chemical properties related to the aldehyde group was found, Patlewicz et al. (2001). The differences in sensitising potential could only be explained when the substances were grouped based on mechanisms of action, Patlewicz et al. (2001). Taking into account also the classification of Patlewicz et al. (2001) and based on structural similarities, three types of aldehyde groups can be distinguished:

4.1.1.1. Aromatic aldehydes. In case of *simple Aromatic aldehydes* the $-CHO$ group is attached directly to an aromatic ring and no other electron withdrawing functional groups are present. The double bond is in α position. The carbonyl group in these substances is deactivated, because the activation energy required in going from a resonance-stabilized parent compound to an intermediate which is not resonance-stabilized is higher than for other aldehydes (Patlewicz et al., 2001). All such substances in Patlewicz et al. were considered to be non-sensitising. However, in case of electron withdrawing substituents in aromatic aldehydes, these could stabilize the *Schiff base* transition state more than they stabilize the parent aldehyde and in this case such aldehydes would be expected to be sensitizers (Patlewicz et al., 2001). Examples can be found in Table 1.

In case of *Aromatic aldehydes with “features” that can be actively metabolised into more reactive species which influence the resonance effect*, the presence of an additional functional group can enhance the sensitisation potency from that of the unsubstituted parent aromatic aldehyde. These substances can become sensitising after metabolic activation. In vanillin, de-methylation of the methoxy group in *meta* position to the aldehyde group gives rise to possible orthoquinone mechanism (orthoquinone is a known structural alert for sensitisation). In case of an ethoxy group in *meta* position, as is the case of ethyl vanillin the de-alkylation occurs less readily than de-methylation; this might explain why vanillin is a weak sensitizer while ethyl vanillin is not sensitising (Patlewicz et al., 2001). According to Smith and Hotchkiss (2001) also heliotropin could become sensitising after metabolic activation.

4.1.1.2. Aliphatic aldehydes. (simple aliphatic and aryl-substituted aliphatic aldehydes). The $-CHO$ group is attached to a saturated carbon atom at least 1 carbon away from a possible conjugated system. The aldehydic carbonyl group in these molecules is strongly electrophilic (Smith and Hotchkiss, 2001), and these substances are thought to react with proteins by *Schiff base* formation mechanism. Patlewicz et al. (2001) included only aliphatic aldehydes with a benzene ring present in the molecule at least two

C-atoms away from the aldehyde group. However, all saturated aldehydes aryl-substituted and simple aliphatic aldehydes are expected to react with proteins by the same mechanism, see for example in Table 3. The majority of Schiff base aldehydes were shown to be not very strong sensitizers (Patlewicz et al. (2002, 2003, and 2004)).

4.1.1.3. α,β -Unsaturated aldehydes. Substances belonging to this class are thought to act by a Michael addition mechanism at the conjugated $C=C$ double bond. It is believed that such chemicals would favour this route of reaction rather than acting directly with lysine residues through the aldehyde group (Patlewicz et al., 2001). The nucleophilic groups that can be involved in Michael addition reactions with aldehydes are the amino, thiol, and hydroxyl groups, with the order of nucleophilicity: $SH > NH_2 > OH$ (Estrada et al., 2003). The differences in potencies observed (e.g. cinnamic aldehyde being more potent than α -hexyl cinnamic aldehyde) by Patlewicz et al. (2001) were attributed to possible influences of the alkyl group on lipophilicity or reactivity. According to Aptula et al. (2005), the double bond is deactivated by the alkyl group. However, in some cases α,β -unsaturated aldehydes react by Schiff base formation (Ege, 1994; Patlewicz et al., 2002). These findings were further elaborated in Patlewicz et al. (2002, 2003, and 2004) including more substances and also the potency aspect.

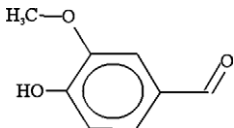
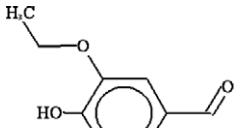
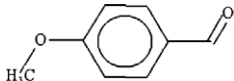
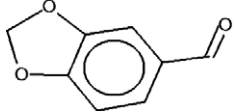
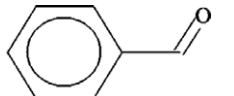
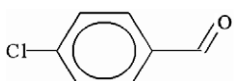
4.1.2. Principle 2: review of the structural alerts available in the literature

The structural alerts for aldehydes proposed by Gerner et al. (2004, 2005) and DerekfW8.0 (Langton et al., 2006) are reported in Table 10. Gerner et al. (2004, 2005) did not further define the domain of the proposed alerts.

DerekfW8.0 distinguishes two alerts, aldehydes with a *Schiff base* formation mechanism and α,β -unsaturated aldehydes with a *Michael addition* mechanism. The aromatic aldehydes are included in the reasoning of the alert for aldehydes with Schiff base mechanism. They are considered to be outside the applicability domain for the positives and are therefore not recognised by the program by themselves. DerekfW8.0 states that aromatic aldehydes are weak or non-sensitising based on the information of Patlewicz et al. However, in some cases conflicting results are reported or some aromatic aldehydes do show sensitising potential, for which justification is given. In DerekfW8.0, no examples of aldehydes with halogens on R1 positions were included (Table 10), therefore it is assumed that the inclusion of the halogens in the structural alert was based on expert judgement and not on actual examples. No examples of aldehydes reacting by Schiff base mechanism with halogens in the molecule were found in this investigation (except for one aromatic aldehyde).

In addition to α,β -unsaturated aldehydes with Michael addition mechanism also precursors that will be metabolised to α,β -unsaturated aldehydes were included in the alert in DerekfW8.0. According to the information reported it appears that this chemical class was added by

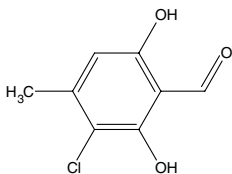
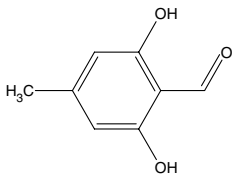
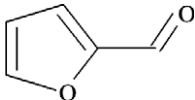
Table 1
Aromatic aldehydes – training set

Name	CAS RN	Structure	M_w	$\log K_{ow}$	Sensitisation		Reference (regarding sensitisation)
					LLNA (EC3)	GPMT/BT	
Vanillin	121-33-5		152.15	1.05	$\pm$ (>50%)	n.i.	Patlewicz et al. (2001), Basketter et al. (2002)
Ethyl vanillin	121-32-4		166.18	1.55	$\pm$ (>50%)	n.i.	Patlewicz et al. (2001),
Anisic aldehyde	123-11-5		136.15	1.79	–	n.i.	Patlewicz et al. (2001)
Heliotropin	120-57-0		150.13	1.77	–	n.i.	Patlewicz et al. (2001)
Benzaldehyde	100-52-7		106.13	1.71	– ^a	– ^a	Patlewicz et al. (2001)
4-Chlorobenzaldehyde	104-88-1		140.57	2.35	$\pm$ (>10%)	n.i.	Patlewicz et al. (2004)
4-Nitrobenzaldehyde	555-16-8		151.12	1.53	–	n.i.	Patlewicz et al. (2004)

– (negative; non-sensitising), $\pm$ (equivocal; weakly sensitising or non-sensitising), n.i. (no information).

^a The substance was tested in at least one of these tests; from the publication it is not clear in which one.

Table 2
Aromatic aldehydes – external set

Name	CAS RN	Structure	M_w	$\log K_{ow}$	Sensitisation		Reference (regarding sensitization)
					LLNA (EC3)	GPMT/BT	
Chloroatranol	57074-21-2		186.60	2.73	+(0.4%)	n.i.	SCCP (2004)
Atranol	526-37-4		153.16	2.2	+(0.6%)	n.i.	SCCP (2004)
Furfural	98-01-1		96.09	0.83	n.i.	–	EC (2004)

+ (positive; sensitising), – (negative; non-sensitising), n.i. (no information).

expert judgement, based on positive response observed in case of α,β -unsaturated esters in a modified single injection adjuvant test in guinea pigs. However, no examples (structures) were given in DerekfW8.0. The precursors were not included in the present study.

4.1.3. Principle 3 and 4: training and external sets

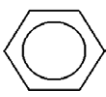
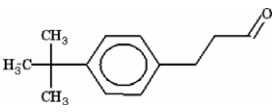
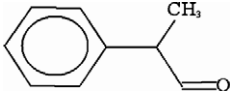
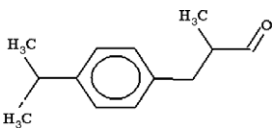
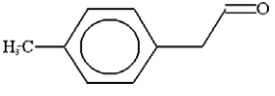
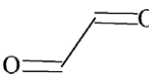
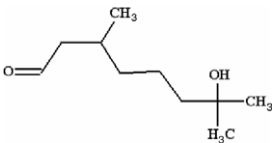
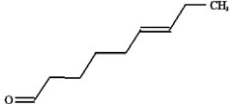
To assess whether the aldehyde alerts mentioned could be used for regulatory purposes and to further refine them, examples of substances already used by DerekfW8.0 and Gerner et al. (2004) for the derivation of these alerts, together with other aldehydes found in the literature were collected for the training and external set (Tables 1–6). In total 51 aldehydes, for which adequate information on their sensitising potential was available were included in this study. The following three training and external sets were established based on expected sensitising potential and the expected mechanism of reaction with proteins: *Aromatic aldehydes* (not or weak Schiff base), *Schiff base aldehydes* and *Michael addition aldehydes*.

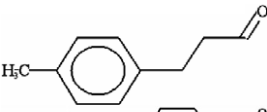
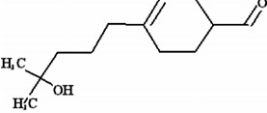
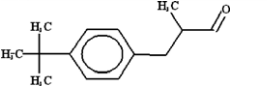
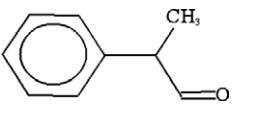
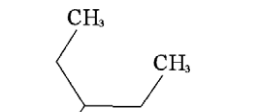
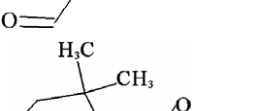
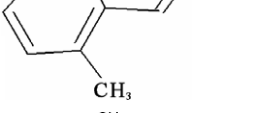
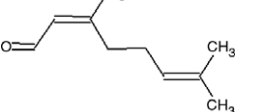
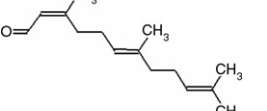
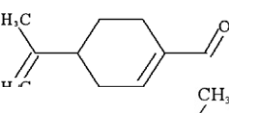
All aldehydes included in the respective training sets were taken from the publications of Patlewicz et al. (2001, 2002, 2004), and for some of them, additional information on sensitisation was available from other sources (see references in Tables 1, 3, 5). Most of the substances in the training sets had information on sensitisation based on LLNA test. In the external sets, substances not studied by Patlewicz et al. were included, and in this case, information on sensitisation was mostly based on the GPMT test. This could be a limitation of the external sets. However in all cases where information in the training set was available from both tests, no discrepancies between the results were found, therefore this is not expected to be a problem for the derivation of the SARs.

Several problems were encountered when selecting substances for the training and external set. For some substances it was not clear from the publications, in which test system they were tested and which criteria were used for classification as sensitizer/non-sensitizer. Most of the substances were tested to determine their sensitising potency and not for EU C&L purposes. In the papers there was no information on the scores in different tests for single substances and also often the criteria, based on which the substances were classified in strong, moderate, weak and non-sensitising, were often not mentioned. This was a problem especially in case of substances which were classified as weak and non-sensitising, and were tested in LLNA only up to 10% or 50%, with no detailed information on scores and dose response. Therefore, it was difficult to relate this information to the EU C&L rules. Some of these problems were solved by personal communication with the author of the papers, but in some cases the sensitising potential in terms of application of the EU C&L rules could not be firmly determined. These substances were then classified as equivocal (Table 1).

Another problem was in some cases to determine by which mechanism the aldehyde reacts with proteins, since it is assumed that α,β -unsaturated aldehydes can react by both Michael addition, as well as by Schiff base formation, and it is not always clear which reactivity is favoured (Ege, 1994; Patlewicz et al., 2002). Four α,β -unsaturated aldehydes were included in the QSAR for Schiff base aldehydes by Patlewicz et al. (2002), because they were considered to react by this mechanism due to steric hindrance effects (e.g. conformational constraint or presence of methyl groups at the site of nucleophilic attack), and they were therefore included in the training set of Schiff base aldehydes also for the purpose of this

Table 3
Schiff base aldehydes – training set

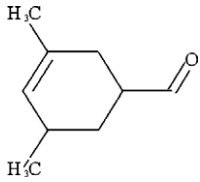
Name	CAS RN	Structure	M_w	$\log K_{ow}$	Sensitisation		Reference (regarding sensitisation)
					LLNA (EC3)	GPMT/ BT	
Phenylacetaldehyde	122-78-1		120.15	1.54	+ (3.1%)	n.i.	Patlewicz et al. (2001), Gerberick et al. (2004)
4- <i>tert</i> -Butyldihydrocinnamaldehyde	18127-01-0		190.29	3.94	(+) ^a	(+) ^a	Patlewicz et al. (2001)
Phenylpropionaldehyde	93-53-8		134.18	1.96	+ (6.3%)	n.i.	Patlewicz et al. (2001, 2002)
4-Propyl- α -methylhydrocinnamaldehyde	103-95-7		190.29	3.91	+ (20.5%)	n.i.	Patlewicz et al. (2001)
4-Methylphenylacetaldehyde	104-09-6		134.18	2.09	+ (6.3%)	n.i.	Patlewicz et al. (2001)
Glyoxal	107-22-2		58.04	-1.66	+ (1.4%)	+	Patlewicz et al. (2002), Kimber et al. (2002), ICCVAM (1999)
Hydroxycitronellal	107-75-5		172.27	2.11	+ (25.25%)	+	Patlewicz et al. (2002), ICCVAM (1999)
2-Methyundecanal	110-41-8		184.32	4.67	+ (10%)	n.i.	Patlewicz et al. (2002)
Undec-10-enal	112-45-8		168.28	4.12	+ (6.8%)	n.i.	Patlewicz et al. (2002)
3- <i>cis</i> -6-Nonenal	2277-19-2		140.23	3.06	+ (23.1%)	n.i.	Patlewicz et al. (2002)

4-Methylhydrocinnamaldehyde (<i>p</i> -methylhydrocinnamic aldehyde)	5406-12-2		148.21	2.58	+ (14%)	n.i.	Patlewicz et al. (2001), Kimber et al. (2002)
Landolal (lyral)	31906-04-4		210.32	3.32	+ (17.1%)	n.i.	Patlewicz et al. (2002)
Para- <i>tert</i> -butyl- α -methylhydrocinnamic aldehyde	80-54-6		204.31	4.36	+ (19.7%)	n.i.	Patlewicz et al. (2002)
α -Methylphenylacetaldehyde	93-53-8		134.18	1.96	+ (6.3%)	n.i.	Patlewicz et al. (2002)
Diethyl acetaldehyde	97-96-1		100.16	1.73	+ (68.15%)	n.i.	Patlewicz et al. (2002)
1,1,3-Trimethyl-2-formylcyclohexa- 2,4-diene# (safranal)	116-26-7		150.22	3.22	+ (7.5%)	n.i.	Patlewicz et al. (2002)
Citral#	5392-40-5		152.24	3.45	+ (13.2%)	+	Patlewicz et al. (2002)
Farnesal#	502-67-0		220.36	5.74	+ (11.7%)	n.i.	Patlewicz et al. (2002)
L-(–)-Perillaldehyde#	18031-40-8		150.22	3.34	+ (8.1%)	n.i.	Patlewicz et al. (2002)
Hexanal	66-25-1		100.16	1.80	+ (45%)	n.i.	Patlewicz et al. (2004)

+ (positive; sensitising), n.i. (no information), # (α , β -unsaturated aldehydes believed to react by Schiff base formation).

^a The substance was tested in at least one of these tests; from the publication it is not clear in which one.

Table 4
Schiff base aldehydes – external set

Name	CAS RN	Structure	M_w	$\log K_{ow}$	Sensitisation		Reference (regarding sensitisation)
					LLNA (EC3)	GPMT/BT	
Formaldehyde	50-00-0	<chem>O=CH2</chem>	30.03	0.35	+ (0.70%)	+	Gerberick et al. (2004)
Carbaldehyde phenylacetaldehyde	68039-48-5		138.21	2.85	n.i.	+	Cronin and Basketter (1994)
Propionaldehyde	123-38-6	<chem>CCC=O</chem>	58.08	0.33	n.i.	+	Cronin and Basketter (1994)
Glutaraldehyde	111-30-8	<chem>CCCCC=O</chem>	100.12	−0.18	+ (0.1%)	n.i.	Gerberick et al. (2004)
Butyraldehyde	123-72-8	<chem>CCCC=O</chem>	72.11	0.82	n.i.	+	ECB (2000g)
3-(Methylthio)propionaldehyde	3268-49-3	<chem>CCC(S)C=O</chem>	104.17	0.41	n.i.	+	ECB (2000d)
3-Methyl-2-butenal#	107-86-8	<chem>CC(C)=CC=O</chem>	84.17	0.92	n.i.	+	ECB (2000f)

+ (positive; sensitising), n.i. (no information), # (α , β -unsaturated aldehydes believed to react by Schiff base formation).

Table 5
Michael addition aldehydes – training set

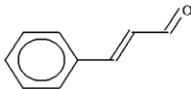
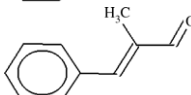
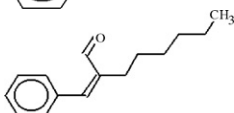
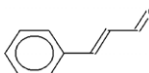
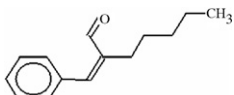
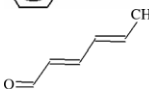
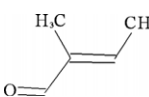
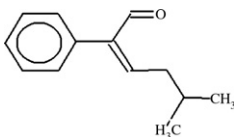
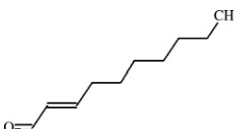
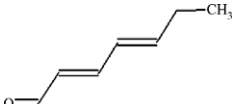
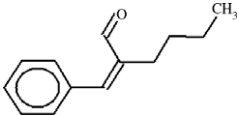
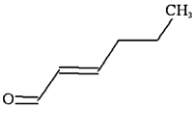
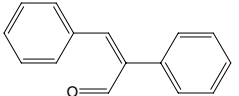
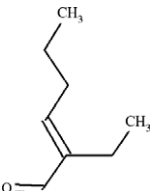
Name	CAS RN	Structure	M_w	$\log K_{ow}$	Sensitisation		Reference (regarding sensitisation)
					LLNA (EC3)	GPMT/ BT	
3-Phenyl propenal (<i>trans</i> -cinnamic aldehyde)	14371-10-9		132.16	1.82	+ (1.4%)	n.i.	Patlewicz et al. (2002)
Methyl cinnamic aldehyde	101-39-3		146.19	2.37	+ (4.5%)	n.i.	Patlewicz et al. (2002)
α -Hexyl cinnamaldehyde	101-86-0		216.33	4.82	+ (12%)	+	Patlewicz et al. (2002), ICCVAM (1999)
Cinnamaldehyde (cinnamal) (<i>cis</i> -cinnamic aldehyde)	104-55-2		132.60	1.82	+ (2.5%)	+	Patlewicz et al. (2001), Patlewicz et al. (2002), ICCVAM (1999)
α -Amyl cinnamaldehyde	122-40-7		202.30	4.33	+ (10.06%)	n.i.	Patlewicz et al. (2002)
2,4-Hexadienal	142-83-6		96.13	1.37	+ (3.5%)	n.i.	Patlewicz et al. (2004)
<i>trans</i> -2-Methyl-2-butenal	497-03-0		84.12	1.15	$\pm$ (> 50%)	n.i.	Patlewicz et al. (2004)
5-Methyl-2-phenyl-hex-2-enal	21834-92-4		188.27	3.77	+ (4.4%)	n.i.	Patlewicz et al. (2004)
<i>trans</i> -2-Decenal	3913-71-1		154.25	3.55	+ (2.5%)	n.i.	Patlewicz et al. (2002)
(<i>E,E</i>)-2,4-Heptadienal	4313-03-5		110.16	1.86	+ (4%)	n.i.	Patlewicz et al. (2002)

Table 5 (continued)

Name	CAS RN	Structure	M_w	$\log K_{ow}$	Sensitisation		Reference (regarding sensitisation)
					LLNA (EC3)	GPMT/BT	
2-Butyl-3-phenyl propenal	7492-44-6		188.27	3.84	+ (11.2%)	n.i.	Patlewicz et al. (2002)
<i>trans</i> -2-Hexenal	6728-26-3		98.15	1.58	+ (5.5%)	n.i.	Patlewicz et al. (2002)
β -Phenyl cinnamic aldehyde	13702-35-7		208.25	3.40	+ (2.5%)	n.i.	Patlewicz et al. (2004)

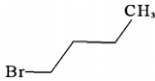
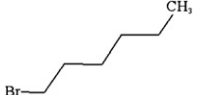
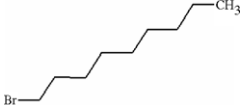
+ (positive; sensitising), n.i. (no information), $\pm$ (weakly or non-sensitising).

Table 6
Michael addition aldehydes – external set

Name	CAS RN	Structure	M_w	$\log K_{ow}$	Sensitisation		Reference (regarding sensitisation)
					LLNA (EC3)	GPMT/BT	
2-Ethylhex-2-enal	645-62-5		126.20	2.62	n.i.	+	ECB (2000c)

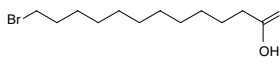
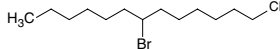
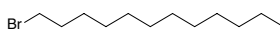
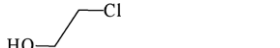
+ (positive; sensitising), n.i. (no information).

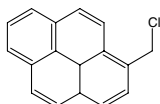
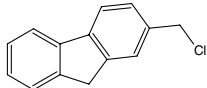
Table 7
Haloalkanes – training set

Name	CAS RN	Structure	M_w	$\log K_{ow}$	Sensitisation		Reference (regarding sensitisation)
					LLNA (SI at % or EC3 at %)	GPMT/BT	
1-Bromobutane	109-65-9		137.2	2.65	– (SI 1.0 at 25%)	n.i.	Basketter et al. (1992), Ashby et al. (1995)
1-Bromohexane	111-25-1		165.07	3.63	± (SI 1.4 at 25%), very slight dose-response Observed	+ ^a	Basketter et al. (1992), ICCVAM (1999), Gerberick et al. (2000)
1-Bromononane	693-58-3		207.16	5.10	+ (SI 2.8 at 25%) dose response observed	n.i.	Basketter et al. (1992), ICCVAM (1999), Gerberick et al. (2000)
1-Bromoundecane	693-67-4		235.21	6.09	+ (SI 3.9 at 25%)	n.i.	Basketter et al. (1992)
1-Bromododecane	143-15-7		249.24	6.58	+ (SI 4.5 at 25%)	+ ^a	Basketter et al. (1992), ICCVAM (1999)
1-Bromotridecane	765-09-3		263.26	7.07	+ (SI 10.4 at 25%)	n.i.	Basketter et al. (1992)
1-Bromotetradecane	112-71-0		277.29	7.56	+ (SI 11.4 at 25%)	n.i.	Basketter et al. (1992)
1-Bromopentadecane	629-72-1		291.32	8.05	+ (SI 19.6 at 25%)	n.i.	Basketter et al. (1992)
1-Bromohexadecane	112-82-3		305.35	8.54	+ (SI 15.6 at 25%,)	+	Basketter et al. (1992)

(continued on next page)

Table 7 (continued)

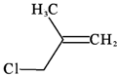
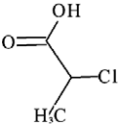
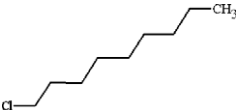
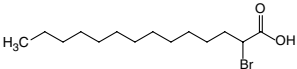
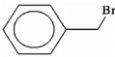
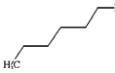
Name	CAS RN	Structure	M_w	$\log K_{ow}$	Sensitisation		Reference (regarding sensitisation)
					LLNA (SI at % or EC3 at %)	GPMT/BT	
1-Bromoheptadecane	3508-00-7		319.40	9.52	+ (SI 9.6 at 25%)	n.i.	Basketter et al. (1992)
1-Bromooctadecane	112-89-0		333.40	9.52	+ (SI 3.7 at 25%)	n.i.	Basketter et al. (1992)
12-Bromododecanoic acid	73637-80-3		279.22	5.34	+ (EC3 at 7.6%)	n.i.	Estrada et al. (2004), ICCVAM (1999)
1-Bromoeicosane	4276-49-7		361.45	10.51	+ (EC3 at 6.1%)	n.i.	Estrada et al. (2004)
1-Bromodocosane	6938-66-5		389.51	11.49	+ (EC3 at 8.3%)	n.i.	Estrada et al. (2004)
7-Tetrabromodecane	—		277.28	7.95	+ (EC3 at 21.3%)	n.i.	Estrada et al. (2004)
12-Bromo-1-dodecanol	3344-77-2		265.23	4.91	+ (EC3 at 6.9%)	n.i.	Estrada et al. (2004), ICCVAM, 1999
2-Chloroethanol	107-07-3		80.51	0.11	—	—	Ashby et al. (1995), ECB (2000a), ICCVAM (1999)
1-Chlorohexadecane	4860-03-1		260.89	8.45	+ (EC3 at 9.1%)	n.i.	Estrada et al. (2004)

1-Chlorooctadecane	3386-33-2		288.95	9.44	+ (EC3 at 16.3%)	n.i.	Estrada et al. (2004), ICCVAM (1999)
1-Chlorotetradecane	2425-54-9		232.84	7.47	+ (EC3 at 20.2%)	n.i.	Estrada et al. (2004), ICCVAM (1999)
1-Iodononane	4282-42-2		254.16	5.52	+ (EC3 at 24.2%)	n.i.	Estrada et al. (2004), ICCVAM (1999)
1-Iododecane	2050-77-3		268.18	6.01	+ (EC3 at 13.1%)	n.i.	Estrada et al. (2004)
1-Iodotetradecane	19218-94-1		324.29	7.98	+ (EC3 at 13.8%)	n.i.	Estrada et al. (2004), ICCVAM (1999)
1-Chloromethylpyrene (1-CMF)	1086-00-6		253.74	—	+ (SI 1.6 at 0.025%)	n.i.	Ashby et al. (1995)
2-Chloromethylfluorene (2-CMF)	91679-67-3		214.69 (CS)	4.64 (CLOG)	+ (SI 5.7 at 0.1%)	n.i.	Ashby et al. (1995)

+ (positive; sensitising), – (negative; non-sensitising), ± (equivocal; weakly sensitising or non-sensitising), n.i. (no information).

^a (Non-standard GPMT test).

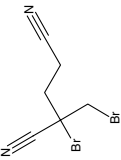
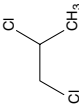
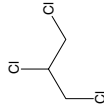
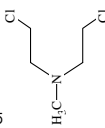
Table 8
Haloalkanes – external set

Name	CAS RN	Structure	M_w	$\log K_{ow}$	Sensitisation		Reference (regarding sensitisation)
					LLNA (SI at % or EC3 at %)	GPMT/BT	
3-Chloro-2-methylpropene	563-47-3		90.55	2.48	n.i.	+	ECB (2000e)
2-Chloropropanoic acid	598-78-7		108.53	0.76	n.i.	–	ECB (2000b)
1-Chlorononane	2473-01-0		162.70	5.02	+	n.i.	ICCVAM (1999), Gerberick et al. (2000)
2-Bromotetradecanoic acid	10520-81-7		307.27	6.25	+	n.i.	ICCVAM (1999)
Benzyl bromide	100-39-0		171.04	2.88	+	n.i.	ICCVAM (1999), Gerberick et al. (2000)
4-Nitrobenzyl bromide	100-11-8		216.04	2.70	+	+ ^a	Geberick et al. (2000), ICCVAM (1999)
4-Nitrobenzyl chloride	100-14-1		171.58	2.61	+	+ ^a	Geberick et al. (2000), ICCVAM (1999)
1-Iodohexane	638-45-9		212.08	4.05	±	n.i.	ICCVAM (1999), Gerberick (2000)
1-Iodohexadecane	544-77-4		352.35	8.96	+(EC3 at 19.1)	n.i.	ICCVAM (1999), Estrada et al. (2004)
1-Iodoctadecane	629-93-6		380.40	9.94	±	n.i.	Geberick et al. (2000), ICCVAM (1999)

+ (positive; sensitising), – (negative; non-sensitising), ± (equivocal; weakly sensitising or non-sensitising), n.i. (no information).

^a Non-standard GPMT test.

Table 9
Haloalkanes – more than one halogen in the molecule

Name	CAS RN	Structure	M_w	$\log K_{ow}$	Sensitisation		Reference (regarding sensitisation)
					LLNA (SI at % or EC3 at %)	GPMT/BT	
1,2-Dibromo-2,4-dicyanobutane	35691-65-7		265.94	1.65	+	+	ICCVAM (1999), Gerner et al. (2004)
1,2-Dichloropropane	78-87-5		112.99	2.25	–	n.i.	OECD-HPV (2003)
1,2,3-Trichloropropane	96-18-4		147.43	2.50	n.i.	–	OECD-HPV (2004)
N-Methyl-2,2'-dichloro-diethyl amine	51-75-2		156.10	1.53	a	a	Gerner et al. (2004)

+ (positive; sensitising), – (negative; non-sensitising), n.i.(no information).

^a Human allergen included in the BgVV list of human allergens.

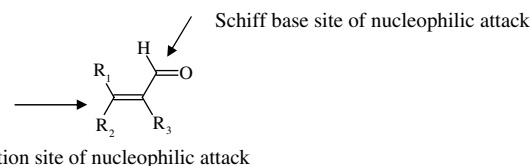


Fig. 1. Schiff base and Michael addition site of nucleophilic attack.

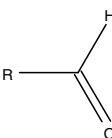
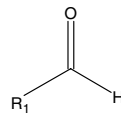
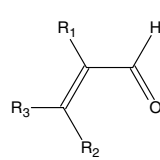
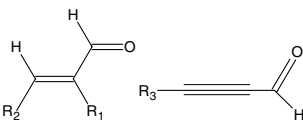
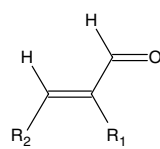
investigation. These substances were: safranal, citral, Farnesal, and L-(–) perillaldehyde (last four chemicals in Table 2).

The substance 3-methyl-2-butenal (CAS: 107-86-8) was first selected to be included in the external set of Michael addition aldehydes, but since it also contains two methyl groups at the site of the nucleophilic attack, it was then included in the external set of Schiff base aldehydes (Table 4).

4.1.3.1. Aromatic aldehydes. Seven aromatic aldehydes were included in the training set (Table 1) and three in the external set (Table 2). All substances included in the training set were considered to be weak or non-sensitising. The problem with this chemical group was that while it is quite obvious, that the aldehyde group is deactivated due to attachment to the benzene ring, it is not clear for all the substances with different substituents, whether this is deactivated to the point that it will not be sensitising at all or whether it will be a weak sensitizer, which would still fit the EU C&L criteria. This is especially uncertain in case of presence of electron withdrawing groups. However, 4-nitrobenzaldehyde (having an $-\text{NO}_2$, a potent electron withdrawing group attached), was negative in LLNA (tested up to 50%, with no dose response). 4-chlorobenzaldehyde was tested only up to 10% and did not reach the SI (stimulation index) of 3, therefore it is expected to be weak or non sensitizer, but no firm conclusion in terms of C&L can be drawn for this substance. The conclusion based on the substances in the training set could be, that aromatic aldehydes with or without other functional groups present are not expected to be strong sensitizers, but rather weak or non-sensitizers. In case of weak sensitizers, these are expected to react with proteins by Schiff base formation.

In the external set (Table 2), two aromatic aldehydes were included, atranol and chloratranol, which based on results in LLNA test are very strong sensitizers. These two substances are metabolites of atranorin (Fig. 2) and chloratranorin and are thought to be responsible for sensitizing effects of these two substances (SCCP, 2004). Gerner et al. (2004) used atranorin as an example for derivation of the structural alert for aldehydes. According to Bernard et al., 2003 atranol and chloroatranol (Table 2) are 1,3-benzenediol derivatives that can be considered as pro-haptens which require biotransformation in order to become sensitizers (aromatic hydroxyl functions could be oxidized enzymatically to form quinones, which are also known to be sensitizers). Beside the phenolic feature, the aldehydic

Table 10
Refined structural alerts for aldehydes compared to the literature and DerekfW8.0 alerts

Structural alert	Application of OECD principles	
<i>“Schiff base”: Aliphatic and aryl-substituted aldehydes</i>		
Former alerts	New alert	Defined endpoint
		• EU C&L for skin sensitisation for positives
DerektfW 8.0 (includes aldehydes (non-aromatic) with Schiff base formation), R = non-aromatic and may be halogens	R1: alkyl	Algorithm
Gerner et al. (2004) R = can be any type of carbon atom linked to any other atom, except the –N-CH=O combination, which is not allowed		• aldehydes with no other functional groups present in the molecule (except S; no examples with other functional groups were available); • aromatic ring can be attached at β position
Gerner et al. (2005) R = –C (any oxidation state, linked to any atom)		Validation using training set and external set (Tables 1 and 2)
<i>“Schiff base”: hindered α, β-Unsaturated aldehydes</i>		
Former alert was not defined in the literature	New alert	Defined endpoint
		• EU C&L for skin sensitisation for positives
	R ₁ and = H, alkyl, aryl; R ₂ and R ₃ = alkyl or aryl	Algorithm
		• aldehydes with no other functional groups present in the molecule (no examples with other functional groups were available)
<i>“Michael addition”: α, β-Unsaturated aldehydes</i>		
Former alert	New alert	Defined endpoint
		• EU C&L for skin sensitisation for positives
R ₁ = H, C, F, Cl, Br, I, R ₂ , R ₃ = any, except O	R1, R2 = H, alkyl, aryl	Algorithm
DerektfW 8.0 includes aldehydes that are thought to react via Michael addition mechanism		• aldehydes with no other functional groups present in the molecule (no examples with other functional groups were available)
R ₁ = H, C, F, Cl, Br, I R ₂ , R ₃ = any, except O		Mechanism
		• Michael addition mechanism of reaction with proteins is assumed

function of atranol and chloroatranol could also be of importance, but since it was shown in some examples that aldehydes directly conjugated to the aromatic ring are weak sensitizers or non-sensitizers, the importance of this functionality is not very clear. It appears that the precise structure of the hapten that causes sensitisation is not yet known. It is likely, that these substances should not be even used in the training and external set for aldehydes, but rather for example for precursors of quinones. This case shows the difficulty in determining, in case of more than

one functional group in the molecule, which of them is really causing the effect, how different functional groups interfere with each other, and how important the metabolism is for the sensitising potential of a substance.

A third substance, furfural (CAS: 98-01-1), was included in the external set. Furfural is according to the draft EU Risk Assessment Report (RAR) (EU, 2004) not sensitising to skin. This conclusion is based on a negative Buehler test, an acceptable GPMT test (conducted according to GLP) which was also negative. Based on the conclusions of the

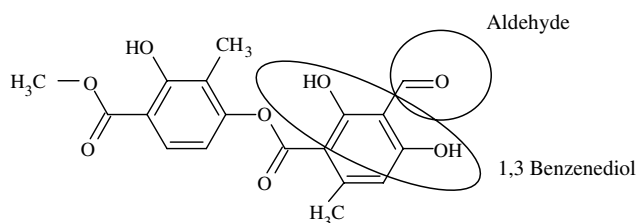


Fig. 2. The chemical structure of Atronorin (Cas No. 479-20-9).

draft EU RAR, this substance is considered to be non-sensitising. Furthermore, it was included in the external set of aromatic aldehydes because the aldehyde group is attached to furan (an aromatic compound) and it is believed that this could also be an explanation of its inactivity, as it is the case for some other aromatic aldehydes in which the aldehyde group is attached to a benzene ring.

In summary, based on information that is available, for some aromatic aldehydes no firm conclusion can be made on sensitising potential, in terms of C&L. As concluded by Patlewicz et al. (2001), aromatic aldehydes without any other functional groups present in the molecule are expected to be very weak or non-sensitising. Aldehydes with other functional groups attached (e.g. electron withdrawing groups) could be sensitising. Nevertheless when taking into account the information available for 4-nitrobenzaldehyde and 4-chlorobenzaldehyde are not expected to be strong but rather weak sensitizers. In cases where functional groups attached on the aromatic ring can be metabolised to more reactive species these aromatic aldehydes could become very strong sensitizers, but in this cases the role of the carbonyl group in the reactivity of the substance is not expected to be very important.

4.1.3.2. Schiff base aldehydes. To evaluate the aldehydes which are thought to react by Schiff base mechanism twenty aldehydes studied by Patlewicz et al. (2001, 2002, 2004) were included in the training set (Table 3) and 7 aldehydes which were not included in the work of Patlewicz et al., were included in the external set (Table 4). The sets included aliphatic and aryl substituted aliphatic aldehydes, and α , β -unsaturated aldehydes, which due to steric hindrance of the site of the nucleophilic attack are in some cases expected to react by Schiff base formation instead of Michael addition mechanism.

All substances investigated were relatively small, “simple molecules” with molecular weight range from around 60 up to 220, and $\log K_{ow}$ from -1.66 up to 5.75 . No other functional groups beside the aldehyde group and alkyl groups were present in the molecules, except for sulphur in 3-(methylthio)propionaldehyde in the external set (Table 4). The substances from the external set fitted within the domain of the training set.

All the substances in the training and external set were classified as sensitising. Differences in potency between the substances were observed, but it is generally difficult

to determine, what causes the reduction or increase in sensitisation potential. It could be due to differences in reactivity, distribution or metabolism of the compounds (Estrada et al., 2004).

4.1.3.3. Michael addition aldehydes. In case of aldehydes that are believed to react by Michael addition mechanism thirteen aldehydes studied by Patlewicz et al. (2001, 2002, 2004) were included in the training set (Table 5) and only one aldehyde was found to be included in the external set (Table 6). The sets included aliphatic and aryl substituted aliphatic α , β -unsaturated aldehydes, which are thought to react with proteins by Michael addition mechanism. The substance 3-methyl-2-butenal was initially selected to be included in the external set of “Michael addition aldehydes”, but since it contains two methyl groups at the site of the nucleophilic attack, it was then added to the external set of “Schiff base” aldehydes (Table 4).

All substances investigated were relatively small, “simple molecules” with molecular weight range from around 84 to 216, and $\log K_{ow}$ from 1.15 up to 4.82 . No other functional groups beside the aldehyde and alkyl groups were present in the molecules. There was only one substance available for the external set and fitted within the domain of the training set.

All the substances in the training and external set were classified as sensitising, except for *trans*-2-methyl-2-butenal (CAS: 497-03-0), which based on an LLNA test was considered as weak to non-sensitising (Patlewicz et al., 2004) with $EC3 > 50\%$. According to the author (personal communication), this substance was tested at concentrations up to 50% . It can not be completely excluded that at higher concentrations a $SI \geq 3$ could be achieved. In any case, this substance appears to be significantly less potent than other substances investigated (all of them have $EC3$ values $\leq 12\%$). The explanation that was given in Patlewicz et al. (2004) for the lower potency of *trans*-2-methyl-2-butenal, was very low molecular weight (84.12) and $\log P$ (1.15) relative to other aldehydes. However, when comparing it with 2,4-hexadienal ($M_w = 96$, $\log K_{ow} = 1.37$) and *trans*-2-hexenal ($M_w = 98.15$, $\log K_{ow} = 1.58$), which have relatively low $EC3$ values of $<6\%$, the difference in molecular weight and $\log K_{ow}$ does not appear to be so big. It might be that there is also some difference in reactivity, perhaps due to the presence of a methyl group on α carbon in *trans*-2-methyl-2-butenal. In fact, in an investigation of effects on sensitisation potency of α substitution in cinnamaldehyde using LLNA test, all α substituted cinnamic compounds showed lower sensitisation potency than non-substituted ones (Smith and Hotchkiss, 2001). Furthermore, the presence of a phenyl group in α position appears to lower the reactivity of the Michael addition aldehydes (Patlewicz et al., 2004).

4.1.4. Regulatory application: refining structural alerts

In the present study three groups of aldehydes were investigated of which one was possibly indicating the

absence (aromatic aldehydes) and two the presence of sensitisation potential which was based on “Schiff base”, “Michael addition” mechanisms of reaction with proteins.

No structural alert is proposed for “aromatic aldehydes”, as based on the available information, no firm conclusion can be drawn on their sensitising potential in terms of C&L. It should be noted however that based on available information, aromatic aldehydes which do not contain any other functionalities can be regarded as *non-sensitising* or *weak sensitizers*. It appears that this is the case also in cases where electron withdrawing groups are present in the molecule.

In cases in which other functionalities are present, these substances can sometimes become very strong sensitizers, if the attached functional groups can be actively metabolised to reactive species. However, in such cases, it should be considered that it is very likely that other functional groups are responsible for sensitising effects, rather than the aldehyde group. Metabolism plays an important role in the evaluation of sensitising potential of aromatic aldehydes.

In Table 10 structural alerts for aldehydes reacting by Schiff base formation and those reacting by Michael addition are proposed based on the findings on structural similarities, reactivity and reaction mechanisms.

It appears that there is no standardised way of discrimination between the elements that should be reported as part of the structural alert (OECD principle 2) and the elements that belong to the description of the domain (OECD principle 3). In this investigation indication of the possible substituents in the molecule was considered to be part of the alert and all other descriptors of the alert and its environment, as part of the domain.

To further define the domain of applicability of the SARs for aldehydes $\log K_{ow}$ values were used (EpiWin software US EPA, 2000). This descriptor appeared not to be useful to further define the domain of applicability of the proposed SARs for positive classification according to EU C&L rules, because all chemicals found except one were considered to be clearly sensitising.

Based on available information and due to limitations of the training and external sets, only SARs which cover specific groups of aldehydes, with narrow domain of applicability could be derived. It is assumed that substances which fit within the domain of the proposed alerts would all be classified as sensitising according to EU C&L rules (EC, 2001).

In case of structures, which do not fit into the narrow domain of the proposed alerts, expert judgement or other means (e.g. *in vivo* or if possible *in vitro* assays) should be applied in order to be able to take a decision on whether the substance is sensitising or not.

The proposed alerts for aldehydes could be used as a stand alone method for positive EU C&L purposes if applied within the structural domain described. A broader application using mechanistic boundaries is possible but needs to be further validated.

4.2. Haloalkanes

4.2.1. Principle 1 and 5: review of reaction mechanisms

In skin sensitisation, the most important reaction mechanism by which haloalkanes react with proteins is the “ S_N2 substitution” (“substitution, nucleophilic, bimolecular”, Fig. 3); examples are represented in Tables 8 and 9 (Aptula et al., 2005; Gerner et al., 2004; Roberts, 2005). Primary alkyl halides undergo “ S_N2 reactions” with strong and weak nucleophiles, secondary alkyl halides also react principally by this path (Fessenden and Fessenden, 1990). Tertiary alkyl halides can not undergo “ S_N2 reactions” due to steric hindrance (Fessenden and Fessenden, 1990; Ege, 1994). Allylic and benzylic halides undergo “ S_N2 reactions” faster than primary alkyl halides, (Fessenden and Fessenden, 1990). “ S_N1 reactions” are not common, because the ionic state of the C-cation is energetically difficult to achieve and is very unstable, however the reaction mechanism is described for other toxicological endpoints (LoPachin and DeCaprio, 2005).

4.2.2. Principle 2: review of structural alerts available in the literature

Two structural alerts for haloalkanes were found in the literature (Table 7), one derived by Gerner et al. (2004), and one derived in DerekfW8.0. The domains of these structural alerts were not further defined by the authors.

4.2.3. Principle 3 and 4: training and external set

To refine the available alerts and further define the domain, 25 haloalkanes (structures) were selected in the present study for the training and 11 for the external set (Tables 8, 9). Some of these substances were also reported as examples by DerekfW8.0 and Gerner et al. (2004): 1-bromododecane, 1-bromohexadecane, 1-chloromethylpyrene, and 2-chloromethylfluorene, and p-nitrobenzylbromide for the derivation of their structural alerts (Table 11). Gerner et al., included as examples for her structural alert also 2 haloalkanes with more than one halogen in the molecule [1,2-dibromo-2,4-dicyanobutane (CAS: 35691-65-7), and N-methyl-2,2'-dichloro-diethylamine (CAS: 51-75-2)]. When Zinke et al. (2002) compared the data on sensitisation of the new chemicals notified in EU with the structural alerts from DEREK (different program than DerekfW), he suggested that haloalkanes containing one halogen should be dealt with separately from those containing more than one halogen, because they have different properties. To check this, in our investigation four substances (Table 9) with more than one halogen in the

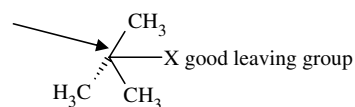
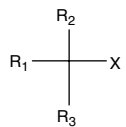
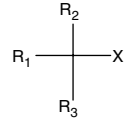


Fig. 3. “ S_N2 ” site of nucleophilic attack.

Table 11
Refined structural alerts for haloalkanes compared to the literature and DerekfW8.0 alerts

Former alerts	Refined Structural alert	Description of the structural alert using OECD principles
<i>DerefW8.0</i>  R₁–R₃ = any except F, Cl, Br, I, X = Cl, Br, I	Alert based on training set and external set  X = Br, Cl, I; R₁/R₂/R₃ = alkyl, aryl; at least one of them has to be H.	Endpoint: • EU C&L for sensitising substances Algorithm: • only one halogen present in the molecule • ≥7C atoms present in the molecule <i>and</i> • log <i>K</i>_{ow} > 3 to up to 12 (the highest log <i>K</i>_{ow} in the training set) • for R₁/R₂/R₃ = benzyl, allyl; log <i>K</i>_{ow} > 2 • other functional groups in the molecule allowed: COOH, OH (no examples of other functional groups in the training set) and NO₂ External set: • For substances outside this domain (<7C atoms), equivocal and negative substances were found in the training and external set. Below the log <i>K</i>_{ow} of 1, only two negative substances were found. Mechanism: S_N2 reaction
Gerner et al. (2004) R-CH₂-Hal R = alkyl, aryl, Hal = Cl, Br	and R₁/R₂/R₃ = benzyl, allyl; two of them have to be H;	

molecule (including the ones from [Gerner et al., 2004](#)) were checked, and two out of four substances appeared to be sensitising, while the others not. Only haloalkanes with one halogen in the molecule were taken into account for further investigation.

Substances and information on sensitisation included in the training and external set were found in several publications (Tables 8, 9 – references). A major problem for some haloalkanes was that different publications had conflicting and confusing information on sensitisation. This was the case for example for 1-bromohexane (111-25-1) from the training set and 1-iodohexane (638-45-9) and 1-iodooctadecane (629-93-6) from the external set.

For 1-bromohexane, [Basketter et al. \(1992\)](#) reported SI in LLNA of 0.9, 1.3, and 1.4 at 5%, 1.3%, and 25%, respectively. [Ashby et al., 1995](#) reported for this same substance SI of 1.2, 1.4, and 2.1 at 5%, 1.3%, and 25%, respectively. This substance was considered to be negative by these two authors, although it was tested only up to 25% and a very slight dose response was observed. In the ICCVAM report ([NIEHS, 1999](#)), which is referencing [Basketter et al. \(1992\)](#), and [Ashby et al. \(1995\)](#), this substance was reported to be positive in LLNA and in a non-standard GPMT test. The same was reported also in [Gerberick et al. \(2000\)](#). In view of these discrepancies in the literature and in view that in [Basketter et al. \(1992\)](#) this substance was tested only up to 25%, not reaching the SI of 3, but with a very slight dose-response observed, the sensitising potential of 1-bromohexane is considered to be equivocal in this investigation.

In case of 1-iodohexane and 1-iodooctadecane, these two substances were reported to be negative in LLNA in the ICCVAM ([NIEHS, 1999](#)) report, referencing [Basketter et al. \(1993\)](#) – this reference was not available), while in [Gerberick et al. \(2000\)](#); [Basketter as co-author](#)), the same two substances were reported to be positive in LLNA. For the purpose of this investigation the sensitising potential for both substances is considered to be equivocal.

It appears that there is no standardised way of discrimination between the elements that should be reported as part of the alert and the elements that belong to the description of the domain. In this investigation indication of the possible substituents in the molecule was considered to be part of the alert and all other descriptors of the alert and its environment, as part of the domain.

Taking into consideration only the 25 substances selected for the training set Table 8 and the structural alert proposed by [DerekfW8](#) and [Gerner et al. \(2004\)](#), the domain of the structural alert was refined (Table 11).

4.2.4. Regulatory application: refining the structural alert

No examples of haloalkanes with F (fluoride) were available. However, since F is not a good leaving group it is expected that these substances would be much less reactive compared to haloalkanes containing other halogens, and are expected to be weak or non-sensitising.

The majority of haloalkanes investigated are skin sensitizers. It appears that haloalkanes with short alkyl chains are non-sensitising or weaker sensitizers than haloalkanes with longer chain. This is evident in case of bromoalkanes studied by [Basketter et al. \(1992\)](#), for which the sensitising potential increases from 1-bromohexane (C₆), up to 1-bromohexadecane (C₁₆), and then decreases again for 1-bromoheptadecane (C₁₇) and 1-bromooctadecane (C₁₈). [Basketter et al. \(1992\)](#) and [Aptula et al. \(2005\)](#) attributed these differences in sensitising potential to increasing hydrophobicity and skin penetration. However, [Estrada et al. \(2003\)](#) reported an EC₃ values for 1-bromoeicosane (C₂₀) and 1-bromodocosane (C₂₂) of 6.1 and 8.3, respectively, which indicates that these substances could be more potent than 1-bromoheptadecane (C₁₇) and 1-bromooctadecane (C₁₈). According to [Barratt \(2000\)](#), all linear primary bromoalkanes with more than 2C atoms in the molecule are essentially equally reactive. In the training and external set, all clearly sensitising substances have the log *K*_{ow} above 2 and more than 7C atoms in the molecule.

Two of the non-sensitising substances, 2-chloroethanol and 2-chloropropanoic acid, have the $\log K_{ow}$ lower than 1, while the third non-sensitizer 1-bromobutane has a $\log K_{ow}$ of 2.65. All negative substances have less than 7C atoms in the molecule and none of the non sensitising haloalkanes has a $\log K_{ow} > 3$.

3-Chloro-2-methylpropene is according to available data a sensitising substance, which contains less than 7C atoms in the molecule and has a $\log K_{ow}$ of 2.5. This substance might be sensitising because it is an allylic halide, and therefore may undergo the “S_N2 reaction” with proteins at faster rate than primary and secondary alkyl halides (Fessenden and Fessenden, 1990). The same might be true for the three benzylic substances 4-nitrobenzyl bromide, 4-nitrobenzyl chloride and benzyl bromide (all of them have the $\log K_{ow} < 3$). Furthermore, 4-nitrobenzyl bromide and 4-nitrobenzyl chloride contain the nitro group (–NO₂), a very potent electron withdrawing group which is expected to increase their electrophilicity. Although the literature data were not found an “S_N1 mechanism” may be applicable as was shown for other toxicological endpoints by LoPachin and DeCaprio (2005).

When the validation of the alert and its domain was performed with examples not included in the training set (Table 11), some of the substances from the external set were outside the domain of the proposed SAR. This was due to more diverse representation of haloalkanes in the external set compared to the training set. Therefore, the structural alert was further defined and the domain of applicability was extended as presented above. However, the new (extended) alert and its domain of applicability need to be validated with new examples. The proposed alert could be used as a stand alone method for positive prediction of sensitisation according to EU C&L rules, if applied within the structural domain described.

5. Discussion

Structure–activity relationships (SARs) for skin sensitisation were evaluated and refined using the OECD principles on the validation of (Q)SARs in order to be used for regulatory purposes (OECD, 2004). The so far limited use of (Q)SARs for regulatory purposes has various reasons. The most important one appears to be the lack of structured communication on strengths and limitation of specific (Q)SARs. The application and documentation of the five OECD principles will contribute to structure the communication on these issues.

5.1. Application of the OECD principles for (Q)SAR validation

5.1.1. Defined endpoint

The defined endpoint is the prediction of the presence or absence of skin sensitisation according to the EU C&L rules for skin sensitizers, it does for example not give the number of sensitised animals or an EC3. The information

on the sensitising potential of substances evaluated was obtained using information derived from the following test protocols: LLNA, GMPT and BT test. There are several methodological differences between these tests, however in this evaluation this did not appear to be a problem, since most of the data were based only on LLNA. Moreover those that had been obtained from both the LLNA and guinea pig test did not show any discrepancies concerning classification.

5.1.2. Defined algorithm

Four structural alerts for aldehydes were discussed and refined SARs were proposed. One structural alert was present for haloalkanes and this was refined as well. For all alerts explicit descriptions of the substructures are identified.

5.1.3. Defined domain of applicability

According to Patlewicz et al. (2001, 2002, 2004), Aptula et al. (2005) and Roberts et al. (2006) the domains of applicability of SARs are defined by both the assumed mechanisms of reaction with proteins as well as structural limitations. For haloalkanes, apart from the assumed mechanisms of reaction with proteins and structural limitations, the domain of applicability was determined also by the $\log K_{ow}$. For aldehydes the $\log K_{ow}$ descriptor did not improve the definition of the domain. In the present paper the chemical reactivity domain was used for refining the SARs.

5.1.4. Internal performance and predictivity

Full details of the training and the external sets for both aldehydes and haloalkanes are given in the tables (Tables 1–9). The external sets were established independently from the training sets. Observations on the quality of the training and external sets are also given in the evaluation. The information on sensitisation potential was mostly obtained from open literature. Therefore, it was sometimes difficult to verify the criteria that were used to classify the substances as sensitizers or non-sensitizers and compatibility with EU C&L rules. However, in most cases this could be solved.

For aldehydes, the substances included in the external set were found to be within the domain based on the training set, while in case of haloalkanes, some substances were outside the domain of the proposed SAR. Therefore, taking into account substances from external set, the SAR was further defined and the domain of applicability extended. However, this “extended” SAR for haloalkanes needs to be validated with new examples.

5.1.5. Mechanistic interpretation

For aldehydes, as well as for haloalkanes, the reaction mechanisms with proteins are explained and the literature references that support these mechanistic bases were given. The reaction mechanisms were determined a priori, which means that the inclusion of substances in respective training and external sets was based on the expected mechanisms of reaction with proteins.

6. Conclusion

Within REACH information on skin sensitisation potential will have to be provided for chemicals manufactured or imported equal to or above 1 tonne/year. Alternative methods such as (Q)SARs could be used for identification of skin sensitizers to limit animal testing.

Although several (Q)SARs for skin sensitisation are available in the literature, they have not been extensively used for regulatory purposes, because of lack of description of the OECD principles on the validation of (Q)SARs. Furthermore, very limited guidance is available for development and use of (Q)SARs.

The goal of the present paper was to refine a number of mechanistically based SARs for skin sensitisation for regulatory purposes. OECD principles were applied to facilitate possible validation and regulatory use of these alerts.

SARs for aldehydes and haloalkanes, which were found in the literature and DerekfW, were rather general and their limitations and domains of applicability were not clearly explained. Taking into account structural similarities, chemical mechanisms by which these substances are expected to react with proteins to induce sensitisation, three structural alerts were refined for aldehydes and one for haloalkanes. From this study it can be concluded that the proposed alerts could be used as a stand alone method for EU C&L positive identification of sensitisation if applied within the boundaries described. This means that they can only be used for a limited number of substances. Apart from the use of SARs as stand alone methods (which at this moment is rather limited), SARs could have an important role in the future, if used as part of a Weight of Evidence and “Integrated Testing Strategies”.

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