



Review

How to assess respiratory sensitization of low molecular weight chemicals?

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ABSTRACT

There are no validated and regulatory accepted (animal) models to test for respiratory sensitization of low molecular weight (LMW) chemicals. Since several decades such chemicals are classified as respiratory sensitizers almost exclusively based on observations in workers. However, both respiratory allergens (in which process the immune system is involved) as well as asthmagens (no involvement of the immune system) may induce the same type of respiratory symptoms. Correct classification is very important from a health's perspective point of view. On the other hand, over-classification is not preferable in view of high costs to overdue workplace engineering controls or the chemical ultimately being banned due to Authorities' decisions. It would therefore be very beneficial if respiratory sensitizers can be correctly identified and distinguished from skin sensitizers and non-sensitizers/respiratory irritants. The purpose of this paper is to consider whether LMW chemicals can be correctly identified based on the currently available screening methods in workers, and/or via *in silico*, *in vitro* and/or *in vivo* testing. Collectively, based on the available information further effort is still needed to be able to correctly identify respiratory sensitizers and to distinguish these from skin sensitizers and irritants, not at least because of the far-reaching consequences once a chemical is classified as a respiratory sensitizer.

1. Introduction

Under the terms of the Globally Harmonized System (GHS) of Classification, an LMW chemical currently classified as a respiratory sensitizer (H334) has as Hazard Statement: '*may cause allergy or asthma symptoms or breathing difficulties if inhaled*' (GHS, 2011). The guidance provided by the European Chemicals Agency (ECHA) for the implementation of REACH (ECHA, 2016) states that a respiratory sensitizer is defined '*as an agent that will lead to hypersensitivity of the airways following inhalation exposure of that agent*.' The guidance also indicates that: '*Respiratory sensitization (or hypersensitivity) is a term that is used to describe asthma and other related respiratory conditions (rhinitis, extrinsic allergic alveolitis), irrespective of the mechanism (immunological or non-immunological) by which they are caused*.'

Thus, this definition includes both true respiratory allergens which, by definition, induce effects via immunological mechanisms, and non-allergenic asthmagens in which case adverse effects are caused by non-immunological mechanisms (Kimber et al., 2001; North et al., 2016).

True chemical respiratory allergy is characterised by immunological priming that results in allergic sensitization of the respiratory tract. If the sensitized subject is exposed subsequently by inhalation to the same chemical then an accelerated and aggressive secondary immune response will be provoked that elicits a respiratory reaction recognised

clinically as respiratory allergy, and in a workplace setting described as occupational asthma. Reactions may become more severe with repeated exposure as the level of sensitization increases (Kimber et al., 2011).

It may be argued that because both types of chemicals, viz. respiratory allergens and asthmagens, may cause the same type of respiratory reactions there would be no harm to have these classified in the same category to protect workers or the general population. Or in other words, it would be better to over-classify rather than under-classify. However, classification for respiratory sensitization does not really take potency into account (see further) and asthmagens generally cause problems only at high and irritating concentrations. Thus, a chemical being classified as respiratory sensitizer but being an asthmagen will not only immediately require the implementation of highly protective measures at high costs; it will also be considered a Substance of Very High Concern (SVHC) under EU REACH with far going consequences such as restriction or authorization with the final aim of having the chemical banned.

In this paper, the scope and toxicology of chemical respiratory allergy will be briefly reviewed as well as the approaches currently available for the identification of LMW chemicals that have the ability to cause sensitization of the respiratory tract.

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2. Chemical respiratory allergy

Chemical respiratory allergy induced by Low Molecular Weight (LMW) chemicals is associated with a variety of occupational health and toxicological challenges, and these have been reviewed elsewhere (Holsapple et al., 2006; Kimber et al., 2007, 2014a; 2014b; Boverhof et al., 2008; Isola et al., 2008; Basketter and Kimber, 2011; Dotson et al., 2015; Cochrane et al., 2015; North et al., 2016; Chary et al., 2018).

LMW chemicals (those less than 500 Da) are not of sufficient size to engage effectively with the immune system in order to provoke an immune response; chemicals must be inherently electrophilic or must be transformed *in vivo* to an electrophilic species. Next, they should form a stable association with a protein to trigger an immune response and cause allergic sensitization (Kimber et al., 2011).

Chemical respiratory allergy resulting in rhinitis and asthma is an important occupational health issue. It is associated with high levels of morbidity and has significant financial costs. In contrast to skin sensitization and allergic contact dermatitis, where many hundreds of chemicals have been confirmed as causative agents, there are less than 100, and probably no more than 80, chemicals that have been implicated as having the potential to cause allergic sensitization of the respiratory tract. Among these are the diisocyanates, chlorplatinic salts, acid anhydrides, and some reactive dyes (Kimber et al., 2014b).

It has commonly been assumed that inhalation exposure is necessary for respiratory sensitization and elicitation. However, there is evidence from both experimental studies and clinical observations that effective sensitization of the respiratory tract can result from skin contact with chemical respiratory allergens. In susceptible individuals, dermal exposure to a chemical respiratory allergen can result in the class of immune response required for respiratory sensitization (Ratnayake et al., 1994; Arts et al., 1998; Kimber and Dearman, 2002; Kimber et al., 2014b). The relevance of skin exposure for respiratory sensitization to chemicals is an important consideration when interpreting data from sensitization studies in experimental animals.

A significant challenge to assess respiratory sensitization has been the absence of methods for the identification and characterisation of chemical respiratory allergens. Despite research for more than three decades, there are still no validated methods for the assessment of the respiratory sensitizing potential of LMW chemicals. Indeed, there are not even approaches that are widely recognised within the scientific community, or by regulatory agencies. Partly this is due to continuing uncertainty regarding the immunological mechanisms through which sensitization is acquired, and in particular about the role of IgE antibody (Kimber et al., 1998, 2014a; 2014b; Kimber and Dearman, 2002). However, there is a growing consensus about the cellular requirements: contact allergy is closely associated with the elicitation of selective T helper (Th)-1 type immune responses whereas chemical respiratory allergy is closely associated with T helper (Th)-2 type immune responses. This is based on experimental studies in which chemical respiratory allergens have been found to induce preferential Th2-type T cell responses (Dearman et al., 1995, 1996; 2005; Van Och et al., 2002; Kimber et al., 2011) and there are data indicating that a similar Th2 selectivity is found in humans also (Ouyang et al., 2013; Newell et al., 2013; Kimber et al., 2014c). See for more information on the mechanism of type 2 immunopathology e.g. the paper of Samuchiwal and Boyce (2018).

In the absence of (a) validated and universally recognised test(s) for the identification of chemical respiratory allergens, what can be done instead? The following main sources of information might inform decisions about the presence or absence of respiratory sensitizing potential: clinical observations and investigations in workers, and experimental (animal) studies including *in silico*, *in chemico* and *in vitro* testing. First the clinical observations and investigations in workers will be addressed.

3. Clinical observations and investigations

Information on LMW chemicals inducing respiratory allergy can be obtained from workplace studies in which exposed workers are investigated, from incidence data in national registers, and from case reports. Sensitization studies in volunteers are usually not performed (nor allowed in the EU) as these individuals may run the risk of becoming sensitized and develop respiratory allergy upon a subsequent exposure to that chemical.

The appearance of respiratory symptoms in workers during or after manufacturing may possibly point at occupational asthma but this alone is not sufficient for the diagnosis of allergic asthma. Occupational asthma can either be (a) sensitizer-induced asthma (allergic asthma), (b) irritant-induced asthma (non-allergic asthma, including Reactive Airways Dysfunction Syndrome (RADS)), or (c) work-exacerbated asthma (pre-existing asthma unrelated to work that is made worse by a workplace exposure; Arts and Kimber, 2017).

In order to confirm a diagnosis of allergic occupational asthma the following criteria must be met: (1) diagnosis of asthma by a physician, (2) a proven association between the symptoms of asthma and work, (3) workplace exposure to an agent or process previously associated with work-related asthma, and at least two of the following criteria: (4) significant work-related changes in spirometry, (5) significant work-related changes in non-specific airway hyperresponsiveness, and (6) a positive response to an inhalation provocation with the specific agent to which the individual is exposed at work (Klees et al., 1990).

In many worker studies associations between symptoms of asthma and work have been reported, but very often lung function tests and/or non-specific airway hyperresponsiveness and/or inhalation provocation tests with the suspected chemical (without impurities) have not been conducted. Additional immunological measurements are of considerable value in confirming a diagnosis of allergic asthma, as even with the six criteria mentioned it is difficult to discriminate between occupational asthma of types (a), (b) and (c). However, this is also not frequently done. In addition, information on atopy or predisposing factors is generally not or only partly available in case reports, and it is mostly absent in worker studies and for the cases included in the national registers. Also, bias may play a role: if one of the chemicals to which workers have been exposed has already been classified as a respiratory sensitizer, it will be difficult not to conclude it is that chemical that has caused the occupational asthma with as consequence that no further investigations will be done. And in how far can co-exposure to other substances be excluded during workplace exposure(s) (Arts and Kimber, 2017)?

Indeed, many work-related asthma cases may not be associated with exposure to allergens. Reinisch et al. (2001) reported that of the new asthma cases in California in 1993–1996, approximately one third (35%) of these new cases were identified as work-aggravated asthma. Individuals with predisposing risk factors for asthma, atopy and a family history of asthma are likely to first develop asthma due to non-work exposures, but then experience work-related exacerbation of pre-existing asthma. The other 65% consisted of new-onset work-related asthma cases. However, of these new-onset cases, only 13.4% was associated with exposure to a previously documented allergen, 77.2% was associated with exposure to agents that were unknown to be allergens, and 9.3% was based on an irritant mechanism. Also, for methyl methacrylate (MMA) cohort and cross-sectional worker studies reported irritation of eyes, nose, and upper respiratory tract associated with short-term peaks exposures, but little evidence for respiratory sensitization or asthma. Nineteen case reports described asthma, laryngitis, or hypersensitivity pneumonitis in MMA-exposed workers; however, exposures were either not well described or involved mixtures containing more reactive respiratory sensitizers and irritants. The authors indicated that the weight of evidence is not sufficient to conclude that MMA would be a respiratory sensitizer (Borak et al., 2011).

Thus, considering all available information presented in such

worker studies collectively, it is very difficult to conclude with certainty that a specific chemical has the potential to induce immunologically mediated respiratory allergy in humans.

4. Experimental studies

4.1. Animal testing

Several approaches have been taken to asses respiratory sensitization in test animals. Overviews have been presented by [Arts and Kuper \(2003, 2007\)](#), and more recently by [Chary et al. \(2018\)](#). In short, these approaches can be divided in sensitization tests such as the serum IgE test and cytokine fingerprinting, and sensitization plus elicitation tests using a single or repeated challenge exposures.

In analogy to the dermal Local Lymph Node Assay (LLNA), the respiratory LLNA was developed. Rather than applying the test material onto the skin of mice for three consecutive days, mice were exposed by inhalation head/nose-only to the test material during three consecutive days for 45, 90, 180 or 360 min/day. Ear application (skin LLNA) was used as a positive control. Negative controls were exposed to the vehicle. Three days after the last exposure, proliferation was determined in the draining mandibular lymph nodes, and the respiratory tract was examined microscopically. In the respiratory LLNA both typical contact and respiratory allergens tested positive ([Arts et al., 2008](#)) and both types of sensitizers could be identified by different cytokine profiles ([De Jong et al., 2009](#)). However, formaldehyde ([Arts et al., 2008](#)) and glutaraldehyde ([Van Triel et al., 2011](#)) tested negative as they did not induce a Stimulation Index (SI) ≥ 3 (a 3-fold or greater increase in proliferation (thymidine incorporation) compared with vehicle controls). It was first suggested that the tested formaldehyde (FA) concentration (3 ppm) may not have been sufficiently high per unit surface area, although slight irritation of the upper respiratory tract was seen. Glutaraldehyde (GA) was therefore tested at higher levels, viz. at 6 and 18 ppm, and both as vapour and aerosol, and distinct irritation was seen in the upper respiratory tract. It was hypothesized that the highly reactive and hydrophilic GA oligomerized in the lysine-rich protein mucous layer of the respiratory tract, thereby preventing GA from reaching the antigen presenting cells (APCs) in sufficient quantities to induce sensitization but still facilitating local irritation, and that the mucous lining of the airways may also protect against respiratory sensitization by FA as FA has solubility and reactivity characteristics similar to GA. The skin has no such aqueous mucous lining which may explain the positive results of FA and GA in the dermal LLNA ([Van Triel et al., 2011](#)).

The dermal LLNA has also been combined with gene expression analysis. Typical respiratory sensitizers such as trimellitic anhydride (TMA) and ortho-phthalaldehyde could be distinguished by differences in gene expression responses from the typical dermal sensitizers 2,4-dinitro-1-chlorobenzene (DNCB) and hexylcinnamic aldehyde (HCA), and from the non-sensitizing irritants methyl salicylate and nonanoic acid ([Boverhof et al., 2009](#); [Adenuga et al., 2012](#)).

It was also noted that reactions upon dermal sensitization and inhalation challenge with TMA and DNCB differed strongly in high IgE responding BN rats versus low IgE responding Wistar rats ([Arts et al., 1998](#)). In addition, it turned out to be possible to discriminate between respiratory allergic and respiratory irritation reactions ([Arts and Kuper, 2003](#)) and finally, the dermal sensitization route was to be preferred over inhalation sensitization, because of stronger reactions and because respiratory irritation may confound the allergic airway reactions (functional breathing responses as well as histopathologically).

Investigations of dose-response relationships and/or threshold levels in respiratory allergy were reviewed by [Arts et al. \(2006\)](#) and later by [Cochrane et al. \(2015\)](#).

However, although progress has been made ([Botham et al., 1988](#); [Griffiths-Johnson and Karol, 1991](#); [Sarlo and Clark, 1992](#); [Satoh et al., 1995](#); [Hilton et al., 1996](#); [Dearman and Kimber, 2001](#); [Arts et al., 2008](#);

[Pauluhn, 2008](#); [Lalko et al., 2011](#)), none of the respiratory sensitization tests has yet resulted in a standard approach that can be used with confidence for the identification of respiratory sensitizers and as such has been included in an official OECD guideline. Besides the earlier mentioned uncertainty regarding the immunological mechanism involved, other reasons may be lack of time, resources, the need to set up a validation round with other labs, and thus costs, and the need to operate an inhalation facility. Nevertheless, in view of the increasing reluctance to perform animal tests, the set up and validation of an OECD respiratory sensitization and challenge testing guideline in animals most probably is too late.

In the absence of validated methods for the identification of chemical respiratory allergens, one strategy has been to examine the behaviour of chemical respiratory allergens in test methods for the evaluation of skin sensitization potential such as the dermal LLNA in mice and rats ([Kimber and Weisenberger, 1989](#); [Kimber and Basketter, 1992](#); [Kimber et al., 1994, 2002](#); [Arts et al., 1996](#); [Basketter et al., 2002](#)), and tests in guinea pigs such as the GPMT ([Magnusson and Kligman, 1969](#)) and the occluded patch test ([Buehler, 1965](#)). According to [Dearman et al. \(2013\)](#), with only a single exception - piperazine - chemical respiratory allergens (such as acid anhydrides, amines, aldehydes, isocyanates, platinum salts, reactive dyes and uronium salts) also tested positive in these mouse and guinea pig test methods. However, piperazine showed signs of skin sensitization in 5 out of 20 animals (25%), just below the 30% limit used for classification ([Leung and Auletta, 1997](#)).

The LLNA is predicated on the fact that skin sensitizing chemicals will induce lymphocyte (primarily T cell) activation and proliferation in regional lymph nodes draining the site of skin exposure ([Kimber et al., 2011](#)). Chemicals that at one or more test concentrations elicit an SI ≥ 3 are classified as skin sensitizers ([Kimber et al., 2002](#)). Several hundreds of chemicals have been tested in the LLNA ([Kern et al., 2010](#)) and, in addition to skin sensitizers, all known chemical respiratory allergens that have been tested in the LLNA elicited positive responses ([Dearman et al., 2013](#)).

Although both contact allergens and respiratory allergens tested positive in the LLNA, it is known that they induce different qualities of T cell response. Chemical respiratory allergens elicit selective Th2-type responses, whereas contact allergens are associated with preferential Th1 responses ([Dearman et al., 1995, 1996; 2005](#); [Van Och et al., 2002](#); [Kimber et al., 2011](#)). However, such differential qualities of T cell response are not registered in the standard LLNA (OECD 429) which simply measures the proliferation of lymph node cells. Although the characteristics of immune responses elicited by contact allergens and respiratory allergens differ qualitatively, they are both associated with the activation and proliferation of T lymphocytes in lymph nodes draining the site of exposure. For this reason, chemical respiratory allergens and contact allergens elicit positive responses in the LLNA when administered topically. It is after that initial activation of responsive T lymphocytes that the immune responses induced by contact allergens and chemical respiratory allergens begin to diverge in a qualitative sense ([Cochrane et al., 2015](#)). And that's why typical contact and respiratory allergens also tested positive in the respiratory LLNA ([Arts et al., 2008](#)) as indicated above.

In addition, whereas the standard dermal LLNA is based on the evaluation of immune responses induced following skin contact, it is now established that the skin is a relevant route of exposure for sensitization of the respiratory tract to chemicals (summarized in [Kimber and Dearman, 2002](#); [Redlich and Herrick, 2008](#); [Redlich, 2010](#); [Kimber et al., 2014b](#)). Thus, there is good reason for chemical respiratory allergens to induce T cell activation and proliferation in regional lymph nodes following skin exposure, and to elicit positive responses in the LLNA. Moreover, it is assumed that respiratory allergens also elicit positive responses in guinea pig assays for the same reason; that they provoke T cell responses that drive skin reactions following challenge of sensitized animals.

Based on this mechanistic activity of chemical respiratory allergens in the LLNA (in mice and rats), and in guinea pig assays, it has become apparent that this activity provides a useful tool for examining the respiratory sensitizing potential of chemicals: chemicals that fail to elicit positive responses in the LLNA can therefore be regarded as lacking not only skin sensitizing activity, but also the potential to induce sensitization of the respiratory tract. That is, chemicals that test negative in the LLNA (and/or guinea pig tests) can be eliminated with confidence from further consideration as possible chemical respiratory allergens (Dearman et al., 2013). Bloemen et al. (2009) listed several other LMW chemicals as respiratory sensitizers based on human case studies showing these chemicals had caused occupational asthma. Based on differences in the outcome of LLNA results it could lead to the assumption that the LLNA would be of only limited predictive value for the absence of respiratory sensitization. Of course, the LLNA is no golden standard but it should be noted that LMW chemicals currently classified (or listed) as respiratory sensitizers only based on human clinical observations may be asthmagens and no respiratory allergens. Most likely this is the reason that the LLNA would not be of predictive value in case of assessment of asthmagens. Nevertheless, Dik et al. (2014) included 168 chemicals that tested negative in the LLNA in their database as respiratory non-sensitizers.

Although the LLNA (OECD 429) is a sensitization test - so no induction of clinical symptoms in animals as no subsequent elicitation/provocation test is performed - it is still an animal test which, according to the latest EU REACH requirements, has resulted in the need to test for skin sensitization *in vitro* first. However, in case some of these *in vitro* tests are positive, or in case not all 3 *in vitro* tests can be carried out (e.g. due to solubility issues), a LLNA may still need to be performed.

4.2. In silico/in chemico/in vitro testing

4.2.1. In silico sensitization testing

In silico testing can consist of using (Q)SAR (Quantitative Structure-Activity Relationship) models (expert models, knowledge based using structural fragments) and/or e.g. OECD QSAR toolbox (which includes read across, trend analysis and QSAR models; Patlewicz et al., 2013). However, neither a full positive nor a full negative will be obtained, and as such *in silico* analysis on its own will not be sufficient for correct classification. In addition, a (Q)SAR analysis is as good as its database is. For skin sensitizers there is a large database because many chemicals have been tested in dermal sensitization tests and/or for which human data are available. However, even here there may be doubt as f.i. positive results in the LLNA cannot always be used, such as in the case of surface-active substances or irritants which have resulted in false-positive outcomes (Kreiling et al., 2008; Basketter et al., 2009; Garcia et al., 2010; Ball et al., 2011). Taking this into account for skin sensitizers, and in view of the absence of validated tests for respiratory sensitizers, how accurate would the current database for respiratory sensitizers be? Can we be confident that all respiratory sensitizers listed are indeed respiratory sensitizers/allergens and not asthmagens/irritants?

As an example: ADCA (CAS no. 123-77-3) has officially been classified as a respiratory sensitizer but is negative in the dermal LLNA, GPMT, and Buehler test, and does not show hypersensitivity reactions in guinea pigs in a 28-day inhalation study in which also a challenge exposure had been included. Although the latter study is not a standard test, other studies with known respiratory sensitizers were positive. Also, symptoms reported in workers are far from conclusive (summarized in Arts and Kimber, 2017). Profiling using the OECD QSAR Toolbox (version 4.3) learns that ADCA could interact with proteins (lysine or cysteine) via a Michael addition mechanism (Hill and Vederas, 1999); chemicals with such a structural alert would be assessed in the Toolbox as respiratory sensitizer category 1A. However, according to Kimber et al. (2018) there are two features that are commonly identified as being associated with chemical respiratory

allergens: the first is 'hard' electrophilic activity and the second is an ability to cross-link proteins. Enoch et al. (2010) included ADCA in their SAR as it was listed as a respiratory sensitizer by the UK HSE and used as a rationale that ADCA is a very reactive Michael acceptor and therefore it would likely be sufficiently reactive towards lysine to cause respiratory sensitization. However, information on cross-linking potential is lacking. It was also noted in the Toolbox that the dataset from which the profiler was developed contained only a single chemical that featured this structural alert, viz ADCA itself, based on some observations in humans (Slovak, 1981; Normand et al., 1989; Kim et al., 2004).

Using Derek (version Derek Nexus 6.0.1; Nexus 2.2.1), which has rules for respiratory sensitization, ADCA pops up as an alert (alert 282) for plausible occupational asthma based on some same and other observations in humans (Ferris et al., 1977; Slovak, 1981; Malo et al., 1985; UK HSE, 1997), and most likely because of its official classification. Interestingly, however, ADCA has been included in Derek in the category 'occupational asthma'¹ and not in the category 'respiratory sensitization', but it is not clear whether this could be considered being equivalent to an 'asthmagen' versus 'respiratory allergen'. On the other hand, Derek predicts ADCA to be a non-sensitizer with regard to skin sensitization but remarks in addition that it contains 'unclassified features' (structural features that do not appear in the negative prediction dataset; or in other words, the prediction is negative as none of the features of the chemical structure appear in the positive prediction set).

Dik et al. (2014) evaluated existing *in silico* models for the identification of respiratory sensitization, viz. MultiCASE (Graham et al., 1997), cat-SAR (Cunningham et al., 2005) and a logistic regression model (Jarvis et al., 2005). In addition to these three SAR models, they added two sets of structural alerts: the combined respiratory sensitization and occupational asthma knowledge database of Derek and a set of alerts proposed by Enoch et al. (2012). The predictive performance of the SARs calculated from the training sets was compared to their performance on a dataset of newly identified respiratory sensitizers and non-sensitizers derived from literature. In total, a dataset of 138 respiratory sensitizers and 521 non-sensitizers was compiled. Remarkably, although the authors emphasized that not all asthmagens induce immunological mediated hypersensitivity, they assumed for the purpose of their investigation that all occupational asthmagens are also respiratory allergens.

As none of the single SAR models was considered sufficiently reliable a tiered approach was suggested by Dik et al. (2014) combining the two SARs with the highest positive and negative predictivity taking into account model specific chemical applicability domain issues. For only 26 of the 138 respiratory sensitizers (19%) all models gave a positive prediction, and for 76 of the 521 respiratory non-sensitizers (15%) all models agreed on a negative prediction. The tiered approach by Dik et al. (2014) provided reliable predictions for 35% of the respiratory sensitizers and non-sensitizers compiled (with a positive and negative prediction value of 96% and 89%, respectively) but it was not able to predict the other 65% of the chemicals.

With regard to ADCA, the example mentioned above, this chemical was included in all five datasets as a structural alert and as such came out positive in the tiered approach by Dik et al. (2014). However, because a reason why chemicals have been included consists of 'a case of occupational asthma was reported in a peer-reviewed report', it can be questioned whether it was wise to consider all occupational asthmagens as respiratory allergens. Also, according to Kimber et al. (2014b) there are probably no more than 80 chemicals that have been implicated as having the potential to cause allergic sensitization of the respiratory tract. This number is substantially lower than the 138 used by Dik et al. (2014) who finally concluded that based on the 65% that could not be predicted there is an urgent need for other test methods (*in chemico/in*

¹ Note in Derek: Examples of active compounds which fire the alert: azodicarbonamide – no other examples are known.

vitro) to reach a reliable conclusion.

According to Arts and Kimber (2017), ADCA does not have the ability, under physiological conditions, to form sufficiently stable associations with proteins and is not a true respiratory allergen. There will certainly be more examples in view of the discussion above and in order not to pollute any QSAR database, for each LMW chemical currently classified as a respiratory sensitizer, a careful (re-)examination should be made on its potential respiratory allergenicity before inclusion in a database for true respiratory sensitizers.

4.2.2. In chemico/in vitro sensitization testing

Because respiratory sensitizers tested in assays for skin sensitization have tested positive in such tests it may be worth to first regard the key biological events underlying skin sensitization which have been summarized in the form of an Adverse Outcome Pathway (AOP), starting with the molecular initiating event through intermediate events to the adverse effect, namely allergic contact dermatitis:

The molecular initiating event (i.e. the first key event) is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. This can be measured by the Direct Peptide Reactivity Assay (DPRA) or the Amino acid Derivative Reactivity Assay (ADRA) which are included in OECD testing guideline 422C. The second key event takes place in the keratinocytes and includes inflammatory responses as well as changes in gene expression associated with specific cell signalling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways. There are two keratinocyte activation assays viz. the KeratinoSens and the LuSens method (OECD 442D). The third key event is the activation of dendritic cells (DC), typically assessed by expression of specific cell surface markers, chemokines and cytokines. One of the test methods is the Human Cell Line Activation Test (h-CLAT) included in OECD testing guideline 442E. The h-CLAT method quantifies changes in the expression of cell surface markers (CD86 and CD54) associated with the process of activation of monocytes and dendritic cells (DC) in the human monocytic leukaemia cell line THP-1 following exposure to sensitizers. The measured expression levels of CD86 and CD54 are then used for supporting the discrimination between skin sensitizers and non-sensitizers. The fourth key event is T-cell activation and proliferation, which is indirectly assessed in the murine Local Lymph Node Assay (LLNA; see above).

An AOP has also been described for respiratory sensitization (Sullivan et al., 2017) consisting of: (1) covalent binding of chemicals to proteins, (2) activation of cellular danger signals (inflammatory cytokines and chemokines and cytoprotective gene pathways), (3) dendritic cell activation and migration, (4) activation, proliferation, and polarization of T cells, and (5) sensitization of the respiratory tract. These events mirror the skin sensitization AOP but with specific differences: respiratory sensitizers bind preferentially to lysine moieties, whereas skin sensitizers bind to both cysteine and lysine, and exposure to respiratory sensitizers seems to result in skewing toward cytokine secretions predominantly associated with T helper 2 (Th2) response; see for extended reviews Sullivan et al. (2017) and Samuchiwal and Boyce (2018). Except for the first step (protein binding) for which the DPRA can be used and step 4 for which the LLNA could be used, no specific OECD TGs are as yet available for step 2 and 3.

In the DPRA, chemical respiratory sensitizers – being hard electrophiles - have shown a preferential reactivity with lysine peptides which may result in different protein conjugates and thus influence the immune response (Lalko et al., 2012). However, the word ‘preferential’ indicates it is not conclusive. Dik et al. (2016) modified the DPRA protocol to identify respiratory sensitization potential by including two peptide depletion measurement time points and added high-performance liquid chromatography mass spectrometry analysis of reaction products, which improved predictive capacity for sensitization; however, this adapted assay could not be used to distinguish respiratory from skin sensitizers. In the review by Chary et al. (2018) it was indicated that typical respiratory allergens such as maleic anhydride

(MA), phthalic anhydride (PA), trimellitic anhydride (TMA), and toluene diisocyanate (TDI) all tested positive in the dermal LLNA. However, TMA was negative in the DPRA, PA was negative in the h-CLAT assay (using both CD86 and CD54 cell surface markers), whereas MA was negative in the h-CLAT assay using CD86 surface markers. TDI was positive in the DPRA but not tested in the h-CLAT assay, so there is currently too little information to also consider the existing OECD 442CDE tests, including the DPRA and h-CLAT assays, as a method to identify respiratory sensitizers.

During the last years, a few *in vitro* methods have been developed to study respiratory sensitization of LMW chemicals, generally focusing on one of the key events in the process of respiratory sensitization (Huang et al., 2013; Dik et al., 2015; Hermanns et al., 2015; Mizoguchi et al., 2017). An overview has been prepared by North et al. (2016); in the next section, a few *in vitro* methods are described in more detail:

Advances have been made *in vitro* to provide a realistic way for exposure of epithelial cells at the air-liquid interface (ALI). Because the respiratory tract is a complex organ with many cell types, it should be questioned which cells to include to study respiratory sensitization *in vitro*. Cells from the alveolar region could of course be used as they may be exposed (Roggen et al., 2006); however, in case of aerosol exposure (dust and droplets) the area of deposition may be in the upper respiratory tract only, depending on the particle size distribution. In the respiratory LLNA (Arts et al., 2008) using various skin and respiratory sensitizers, histopathological changes were only observed in the nasal area and larynx following a 3-day inhalation exposure regimen to vapours and respirable aerosols but not in the lungs which suggests the alveolar region had not been reached, or at least not by sufficient amounts.

A co-culture system for the detection of respiratory sensitizers has been developed by Chary et al. (2018, 2019) consisting of different cell types, viz. alveolar type II cells secreting lung surfactant, endothelial cells, dendritic cells and macrophages. The respiratory sensitizers TMA and PA were tested and the respiratory irritants acrolein and methyl salicylate served as negative control. They were tested at maximum 25% of cytotoxicity. Exposure to TMA and PA resulted in dendritic cell activation (TSLPr and CD54 cell surface markers expression) and a specific cytokine release pattern (upregulation of IL-10, GM-CSF and CCL20) whereas the irritants did not. The authors claimed that with this procedure – measuring cytokines at the alveolar barrier and activation of dendritic cells – 2 out of the 4 key events of the AOP for respiratory sensitization can be investigated (Chary et al., 2019). So far skin sensitizers have not been tested in this set up, and only a very limited number of respiratory sensitizers belonging to the acid anhydrides chemical group only. As such it is not known whether this system would be suitable to distinguish respiratory sensitizers from skin sensitizers.

The SENS-IS assay, currently under ECVAM validation and included in the OECD guideline preparation schedule, is an *in vitro* assay that aims to predict skin sensitization potency of chemical ingredients with a similar accuracy as the LLNA (although the LLNA cannot be considered a golden standard either as indicated previously). The assay uses human 3D epidermis as test system; the read-out uses the genomic signature measured by multi-target quantitative RT-PCR. According to the developers, the assay would have an accuracy of more than 90% compared to the LLNA and human data, not only for hazard assessment but also for classification of potency, viz. Cat 1A or Cat 1B. Because it uses human 3D epidermis, it would be possible with this assay to evaluate the hazard of a wide variety of chemicals regardless their solubility (Cottrez et al., 2016; Petry et al., 2018). However, so far it is not known whether in this assay typical respiratory sensitizers could be differentiated from typical dermal sensitizers.

In the *in vitro* Genomic Allergen Rapid Detection (GARDskin) test, instead of looking at common events such as total T-cell proliferation or upregulation of CD86 cell surface markers, this test aims to look directly at the mechanistic differences induced by skin-sensitizers and non-sensitizers on the transcriptomic level, using 200 genes. The test

was set up using a comparison between skin-sensitizers and non-sensitizers (Johansson et al., 2011, 2013), and also potency assessment has been claimed (Zeller et al., 2017). However, as indicated before, it is important to know whether the response from known respiratory allergens would be different from that of known dermal allergens in this assay. The developers of this test are currently collecting evidence that this assay does not falsely classify 'pure' respiratory sensitizers as skin sensitizers. F.i. phthalic anhydride was correctly classified as a non-sensitizer in GARDskin as well as a respiratory sensitizer in GARDair (The personal communication was with J. Schmidt, working at SenzaGen and took place in 2019). In the GARDair assay, the 28 genes selected are associated with the regulation of the TSLP pathway which is known to participate in T-cell polarization towards Th2, representing one of the key mechanistic events of allergic asthma. The test was set up with 10 known respiratory allergens and 20 non-respiratory sensitizers, the latter group including both irritants as well as skin sensitizers (Forreryd et al., 2015). Out of the 10 respiratory sensitizers 9 were correctly classified as such (except for TDI), whereas 7 out of 8 skin sensitizers (except for glyoxal) were correctly classified as non-respiratory sensitizers (The personal communication was with J. Schmidt, working at SenzaGen and took place in 2019). Although there seems to be high sensitivity, it is quite worrying that a potent respiratory sensitizer as TDI came out negative. Based on expected differences in cellular responses between the 3 groups (skin sensitizers, respiratory sensitizers, and irritants), it is very important to know whether these 3 groups can indeed be discriminated in both assays. So, when using the assays in combination, there are indications that the different responses can be distinguished but the two assays clearly need further investigations also in view of chemicals that are not so potent or poorly soluble (but it is fair to say this also applies to the *in vivo* tests).

5. Discussion and conclusion

The appearance of respiratory symptoms in workers in manufacturing or processing factories may possibly point at occupational asthma. However, this alone is not sufficient for the diagnosis of allergic asthma. As stated previously, the term occupational asthma embraces sensitizer-induced asthma (allergic asthma), irritant-induced asthma (non-allergic, including Reactive Airways Dysfunction Syndrome (RADS)), or work-exacerbated asthma (pre-existing asthma unrelated to work that is made worse by a workplace exposure). In order to confirm a diagnosis of allergic occupational asthma several criteria must be met (Klees et al., 1990).

In contrast to classification of skin sensitizers (H317; may cause an allergic skin reaction), classification of respiratory sensitizers (H334) according to GHS/CLP is not clear-cut. Any chemical that induces a specific respiratory hypersensitivity reaction at a low to moderate (cat. 1B) or high (cat. 1A) frequency in humans should, according to the criteria, be considered a respiratory sensitizer. However, low to moderate and high frequencies are not defined. Also, according to the criteria it is necessary to take into account the size of the exposed population and the extent and conditions of exposure, but this is not further clarified or explained. And finally, the word 'specific' is not clear as it can denote a response to a particular (specific) test substance or a specific immunological reaction (an immunological reaction specific for the substance); however, immunological mechanisms do not have to be demonstrated.

The guidance further indicates that appropriate lung function tests or bronchial challenge tests should be conducted according to accepted guidelines in order to provide evidence for respiratory sensitization. It is relevant to consider, therefore, whether any lung function tests conducted are appropriate and performed according to accepted guidelines using a pure form of the chemical under investigation. In addition, occupational, medical and smoking histories should be known. Also, according to the guidance, symptoms of asthma by irritation do not apply for classification in case of existing bronchial

hyperreactivity. If it can be demonstrated that these substances induce symptoms of asthma by irritation only in people with bronchial hyperreactivity, they should not be considered respiratory sensitizers. However, if only non-specific bronchial hyperreactivity tests will be carried out, it cannot be concluded nor excluded whether a specific chemical induced symptoms of asthma by irritation (Arts and Kimber, 2017).

In addition, in order not to pollute any QSAR database assessing respiratory sensitization, for each LMW chemical currently classified or listed as a respiratory sensitizer, a careful (re-)examination of its potential respiratory allergenicity should be done before inclusion in that database.

There are no validated *in vivo* animal models to assess respiratory sensitization of LMW chemicals, and most probably no effort will be put in validating any of the existing approaches in view of 'animal testing being the last resort'. Nevertheless, LMW chemicals that have tested negative in *in vivo* assays for skin sensitization should be considered to also lack the potential to induce respiratory sensitization (Arts and Kimber, 2017). Chemicals that have tested positive in such assays could either be a skin sensitizer or a respiratory sensitizer as these assays do not really allow to discriminate skin from respiratory sensitizers (Chary et al., 2018), which in case of a positive test also leads to the question whether classification as skin sensitizer would always be correct.

So for any new LMW chemical to be tested the hope is to have at least one appropriate, predictive and cost-effective *in vitro* assay (or a combination thereof) to assess respiratory sensitization. Efforts have been made using potentially relevant cell types and exposure conditions such as ALI, or to investigate differences in cell surface marker expression and cytokine production. However, it could be questioned whether lung tissue, lung epithelia/endothelia and ALI exposure are the most appropriate to investigate an allergic disease characterized by rhinitis and bronchoconstriction. And can elicitation be captured *in vitro*? Perhaps gene profiling may offer a solution, but further effort is needed to identify respiratory sensitizers and to distinguish these from skin sensitizers and irritants, not at least because of the far-reaching consequences once classified as respiratory sensitizer.

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