

Methods for the prediction of low-molecular-weight occupational respiratory sensitizers

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Purpose of review

There is recognition that respiratory sensitization is an occupational hazard of high concern. Despite international regulatory requirements there is no established protocol for the efficient prospective identification of chemical respiratory sensitizers. We review the predictive behaviour of available methods and suggest a possible high-throughput protocol.

Recent findings

Animal or in-vitro tests specific to respiratory exposure and resulting in direct asthma-related outcomes have not been developed, although the use of a local lymph node assay originally designed for skin sensitization has been advocated in a respiratory context. Various methods have been used to develop quantitative structure–activity relationship models for prediction of low-molecular-weight organic chemical respiratory sensitizers. The estimated negative predictive value for all of the published models is 1, but their differences in positive predictive value can be exploited.

Summary

The most pragmatic as well as valid approach for screening large numbers of industrial chemicals for respiratory sensitization hazard is likely to consist of an algorithm starting with quantitative structure–activity relationship models. Further corroboration from animal or human data, however, may be required for chemicals with a positive result by quantitative structure–activity relationship.

Keywords

chemical respiratory sensitizer, occupational asthma, predictive values, quantitative structure–activity relationship

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Introduction

In parallel with the introduction of novel chemicals in industry, there is a steady emergence of novel chemical respiratory sensitizers capable of causing rhinitis or asthma. These become apparent when, after industrial use, human cases are reported either individually [1], in small clusters [2] or as large outbreaks [3]. The globally harmonized system (GHS) for classification and labelling of chemicals ranks respiratory sensitization with other toxic effects of highest concern: carcinogenicity, germ cell mutagenicity and reproductive toxicity [4]. There is currently no established test method, however, that would be widely applicable to the identification of the respiratory sensitization hazard of chemicals before their industrial usage. We present a review of the available prediction methods and propose a novel, high-throughput protocol.

Definition of respiratory sensitizers

For regulatory purposes a working definition of a respiratory sensitizer is ‘a substance that will induce a state of hypersensitivity of the airways following inhalation of the

substance’ [4]. Development of hypersensitivity requires a sensitization phase during a variable latent period of exposure; on subsequent reexposure to the substance, a sensitized individual exhibits the clinical features of rhinitis or asthma.

Agents responsible for occupational asthma arising *de novo* in the workplace are known as respiratory sensitizers, but this term is not used for workplace substances that aggravate preexisting asthma or cause acute irritant-induced asthma, also known as the reactive airways dysfunction syndrome (RADS) [5^{*}]. Similarly, occupational rhinitis may be due to either sensitization or irritation [6]. Whilst immunological mechanisms are often involved in respiratory sensitisation [7], there is now consensus that it can occur when an immune mechanism cannot be demonstrated [8^{*}].

Classification of respiratory sensitizers

This review focuses on low-molecular-weight (LMW) chemical respiratory sensitizers, defined as having molecular weight below 1 kDa [9]. An indication of the

growing importance of LMW chemicals in the causation of occupational asthma can be gained from occupational disease reporting schemes such as The Health and Occupation Reporting Network (THOR) in the UK [10]. This scheme receives case reports from a network of respiratory [11] and occupational physicians [12] with recent data indicating that LMW chemicals now account for more cases of occupational asthma due to sensitization than high-molecular-weight (HMW) agents [13].

HMW respiratory sensitizers are usually biological proteins which consistently cause rhinitis or asthma through type I hypersensitivity mechanisms associated with immunoglobulin E (IgE) antibodies [14]. IgE antibodies, however, have only been consistently demonstrated to hapten-protein conjugates of a small proportion of LMW respiratory sensitizers [15], for example platinum salts [16] and acid anhydrides [17]. For other LMW chemicals, such as isocyanates, as yet uncharacterized non-IgE-mediated mechanisms are thought to be important [18,19].

This mechanistic uncertainty is a significant challenge to the development of a single accepted test for the prediction of LMW respiratory sensitizers which can be further classified as organic or inorganic chemicals. The smaller inorganic group includes several important salts of transition metals whose asthmagenic mechanism is thought to involve coordination bonding with human proteins [20,21] and they will not be considered here.

Legislation and criteria for a good prediction method

The GHS for chemical classification and labelling aims to enhance the protection of human health and the environment through international harmonized criteria for classifying substances and mixtures according to their health, environmental and physical effects. There is a goal for as many countries as possible to implement the GHS through their own regulatory process by 2008. In the USA, this will be achieved by appropriate modification of its existing Hazard Communication Standard [22]. Similarly, there are plans for the existing European system to adopt the GHS under the new REACH legislation [23].

Current GHS criteria for labelling a chemical as a respiratory sensitizer are

- (1) 'if there is evidence in humans that the substance can induce specific respiratory hypersensitivity, and/or
- (2) if there are positive results from an appropriate animal test'.

The evaluation of a potential toxicity screening method is similar to that for a clinical diagnostic test and requires estimates of predictive values. For example, in radiologi-

cal screening for breast cancer it is important that the number of false negatives is minimal and that the negative predictive value (NPV) is high [24]. Since positive radiology is followed by a more diagnostic biopsy it is less critical, although still desirable, that false positives are minimal and estimates of positive predictive value (PPV) are less than 0.5 [25]. These values are determined for a given test from its sensitivity and specificity, together with the estimated disease prevalence [26]:

$$PPV = \frac{\text{Sensitivity} \times \text{Prevalence}}{\text{Sensitivity} \times \text{Prevalence} + (1 - \text{Specificity}) \times (1 - \text{Prevalence})}$$

$$NPV = \frac{\text{Specificity} \times (1 - \text{Prevalence})}{(1 - \text{Sensitivity}) \times \text{Prevalence} + \text{Specificity} \times (1 - \text{Prevalence})}$$

When applying these equations to screening techniques for the prediction of chemical respiratory sensitization hazard, the 'prevalence' equates to the proportion of chemicals used in industry that truly are potential respiratory sensitizers. In Europe, REACH requires the registration of around 30 000 existing chemicals [27]. Approximately 100 LMW organic chemicals had been reported to cause at least one case of occupational asthma in the peer-reviewed literature by the end of 2004 [28]. A most conservative estimate, therefore, is that approximately one in 300 chemicals is asthmagenic, but the true proportion is likely to be considerably higher.

Due to the large number of chemicals to be screened for respiratory sensitization hazard there is a need for a prediction system that is highly efficient. Even if a reliable validated animal model existed for this purpose it would be impractical to test every chemical entity in this way. Thus an initial screening test with high throughput and a high NPV is needed to identify chemicals that require further consideration.

Appropriate animal tests for identifying respiratory sensitizers

'At present recognised animal models for the testing of respiratory hypersensitivity are not available' [4]. The GHS criteria go on to state 'data which may be indicative of the potential of a substance to cause sensitisation by inhalation in humans may include: (a) measurements of immunoglobulin E (IgE) and other specific immunological parameters, for example in mice, (b) specific pulmonary responses in guinea pigs'.

Measurements of immunoglobulin E and other specific immunological parameters

In the mouse IgE test, dermal exposure of the mouse to a test chemical is followed by measurement of total serum

IgE concentration. An increase in total IgE is thought to imply that the chemical is a respiratory sensitizer [29]. As rationale for this method it has been argued that inconsistencies in detection of specific IgE to LMW respiratory sensitizers in human disease may be a result of technical difficulties in analytical methods [30]. Problems have been encountered, however, in attempts to replicate the results for specific chemicals in different laboratories and between mice of either the same or different species [31].

Regardless of whether the production of IgE antibodies is a necessary correlate of respiratory sensitization, the detection of Th2-type cytokines released from CD4 lymphocytes during sensitization of an animal to a chemical is considered to be indicative of respiratory sensitization [32]. Interleukin-4 (IL-4), IL-5, IL-10 and IL-13 cause class switching to IgE production by B lymphocytes. A Th2 lymphocyte-driven inflammatory response, however, could also be non-IgE mediated. Such 'cytokine profiling' has shown initial promise in that it positively identified two acid anhydrides and two diisocyanates as respiratory sensitizers whereas the IgE response in Brown Norway rats only identified trimellitic anhydride correctly [33]. Validation with larger numbers of chemicals which reflect the full spectrum of putative asthmagens is needed.

Cytokine profiling distinguishes Th2 responses, believed to be an integral mechanism in respiratory sensitization from Th1 responses which predominate in skin sensitization, known to involve type IV hypersensitivity. The murine local lymph node assay, a test that has been developed and validated for the identification of skin sensitizers [34] has also been shown to give positive results for a limited number of common respiratory sensitizers. Whilst this may have potential as a sensitive test for respiratory sensitizers no results have been obtained with any control chemicals known to be respiratory nonsensitizers [35^{••}]; there are, therefore, no specificity data from which to calculate its predictive values.

Specific pulmonary responses in guinea pigs

The guinea pig is the species of choice for measuring pulmonary responses of animals to inhaled chemicals [36]. Its development, however, has been hindered by numerous practical difficulties, such as performing plethysmography in the elicitation phase [37].

Attempts to validate any of these animal models for the purpose of predicting respiratory sensitization hazard have been limited to low numbers of chemicals not fully representative of the diverse range of industrial chemicals. There are insufficient sensitivity and specificity data for the determination of (negative or positive) predictive values.

In-vitro testing methods

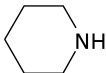
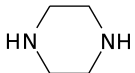
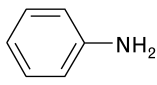
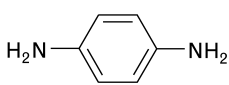
A single in-vitro test is unlikely to encompass the complexity of molecular mechanisms involved in the respiratory sensitization process. An important part of this mechanism is the covalent binding of a chemical to a protein molecule, known as haptentation. In-vitro tests of conjugation to whole protein molecules have been suggested as part of a tiered approach for evaluating respiratory allergy of LMW chemicals [38,39]. More recent approaches have been cell-based assays involving dendritic cells, respiratory epithelium and alveolar macrophages. Technical difficulties, for example, producing standardized human cell lines, however, have meant that as yet there is no single assay suitable for further validation [35^{••}].

Quantitative structure–activity relationship models for prediction of respiratory sensitization hazard

The current lack of suitable in-vitro or in-vivo methods as a means for the large-scale identification of respiratory sensitizers has been recognized by the European Centre for the Validation of Alternative Methods (ECVAM) [35^{••}]. (Quantitative) structure–activity relationships [(Q)SARs] link a chemical structure with a biological endpoint mathematically. Whereas SARs are qualitative associations between certain substructures and activity utilizing mechanistic knowledge, QSARs are developed by purely statistical analysis. Due to the uncertainties regarding molecular mechanisms for LMW respiratory sensitizers, QSARs are particularly appropriate as they make no a-priori mechanistic assumptions. As well as allowing prediction models to be developed, they can also lead to mechanistic insights and hypothesis generation.

Graham *et al.* [40] used the CASE and MultiCASE methodologies, both of which break down chemicals into fragments containing two to 10 (nonhydrogen) atoms. CASE identifies fragments associated with activity as biophores and those associated with inactive chemicals as biophobes. For these fragments, both probability of activity and potency estimates are determined. MultiCASE groups chemicals with a particular biophore and examines them for additional descriptors or modulators that increase or decrease the activity of chemicals containing the biophore. For their learning dataset 40 active chemicals that had been confirmed as a human respiratory sensitizer by bronchial challenge testing were used. Three sets of 40 control chemicals were taken from a database of dermal nonsensitizers on the assumption that dermal nonsensitizers are all respiratory nonsensitizers; however, there may be exceptions to this. For example, naphthalene diisocyanate is recognized as a human respiratory [41] but not skin sensitizer. Whilst the same group later used a set of controls better suited to respiratory sensitization based on human inhalation exposure

Figure 1 Structural comparison of asthmagenic with nonasthmagenic amines

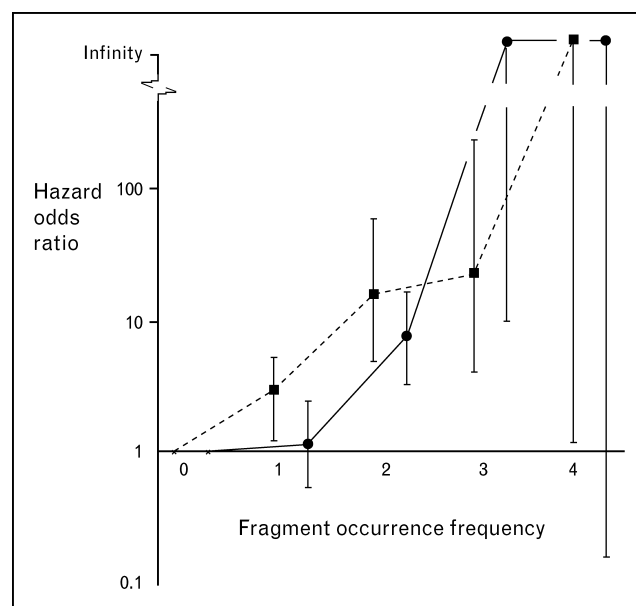
	Monoamines (nonasthmagenic)	Diamines (asthmagenic)
Aliphatic	<i>Ethylamine</i> $\text{H}_3\text{C}-\underset{\text{H}_2}{\text{C}}-\text{NH}_2$	<i>Ethylenediamine</i> $\text{H}_2\text{N}-\underset{\text{H}_2}{\text{C}}-\underset{\text{H}_2}{\text{C}}-\text{NH}_2$
Heterocyclic	<i>Piperidine</i> 	<i>Piperazine</i> 
Aromatic	<i>Aniline</i> 	<i>p-Phenylenediamine</i> 

tables [42], the resulting model was found to have a lower sensitivity and specificity on internal validation.

These same sets of 40 learning dataset chemicals have also been used to generate a predictive model using the more information-intensive cat-SAR program [43]. The greater number of active and inactive fragments generated by the cat-SAR program allows more complete correspondence between the fragments in the model and those in the test compound than with CASE/Multi-CASE. It is also flexible, such that the user can select the active and inactive learning dataset chemicals, types of fragment attributes to consider, and statistical considerations for development of the final model. 'Leave-one-out' validation methods were performed whereby test chemicals were removed in turn from the learning dataset so that they had not contributed to the development of the model they were validating.

A different methodology was used for development of an asthma hazard QSAR [28], following some initial observations on chemical structural differences between asthmagens and controls [44]. These included the presence of reactive groups such as isocyanate, carbonyl or amine, particularly when two or more groups were present in the same molecule (Fig. 1). This was demonstrated statistically in a study of the chemical substructures present in 78 LMW organic asthmagens compared with 301 control chemicals. The ratio of odds values (hazard odds ratio – HOR) that a particular chemical substructure was present in an asthmagenic compound compared with a control

were elevated for a range of fragments, suggesting their involvement in the biochemical processes leading to asthmagenesis [45]. Furthermore, for the carbonyl and amine groups, the HOR was found to increase with the number of fragments present in a molecule (Fig. 2).

Figure 2 Relationship between hazard odds ratio and fragment occurrence frequency for C=O ('carbonyl') and –NH₂ ('amine') groups

CI, confidence interval. Reproduced with permission from [45]. —○— Upper 95% C.I., Lower 95% C.I.; ●— Carbonyl; —■— Amine.

Table 1 Validation statistics for respiratory sensitization quantitative structure–activity relationship prediction models

Model	Sensitivity	Specificity	Predictive values		
			PPV ^a	PPV ^b	NPV ^c
Jarvis <i>et al.</i> [28]	0.86	0.99	0.22	0.75	1
Graham <i>et al.</i> [40]	0.95	0.95	0.06	0.40	1
Cunningham <i>et al.</i> [43]	0.89	0.95	0.06	0.38	1
Cunningham <i>et al.</i> [43]	0.94	0.87	0.02	0.20	1
Karol <i>et al.</i> [42]	0.85	0.74	0.01	0.10	1

PPV, positive predictive value; NPV, negative predictive value.

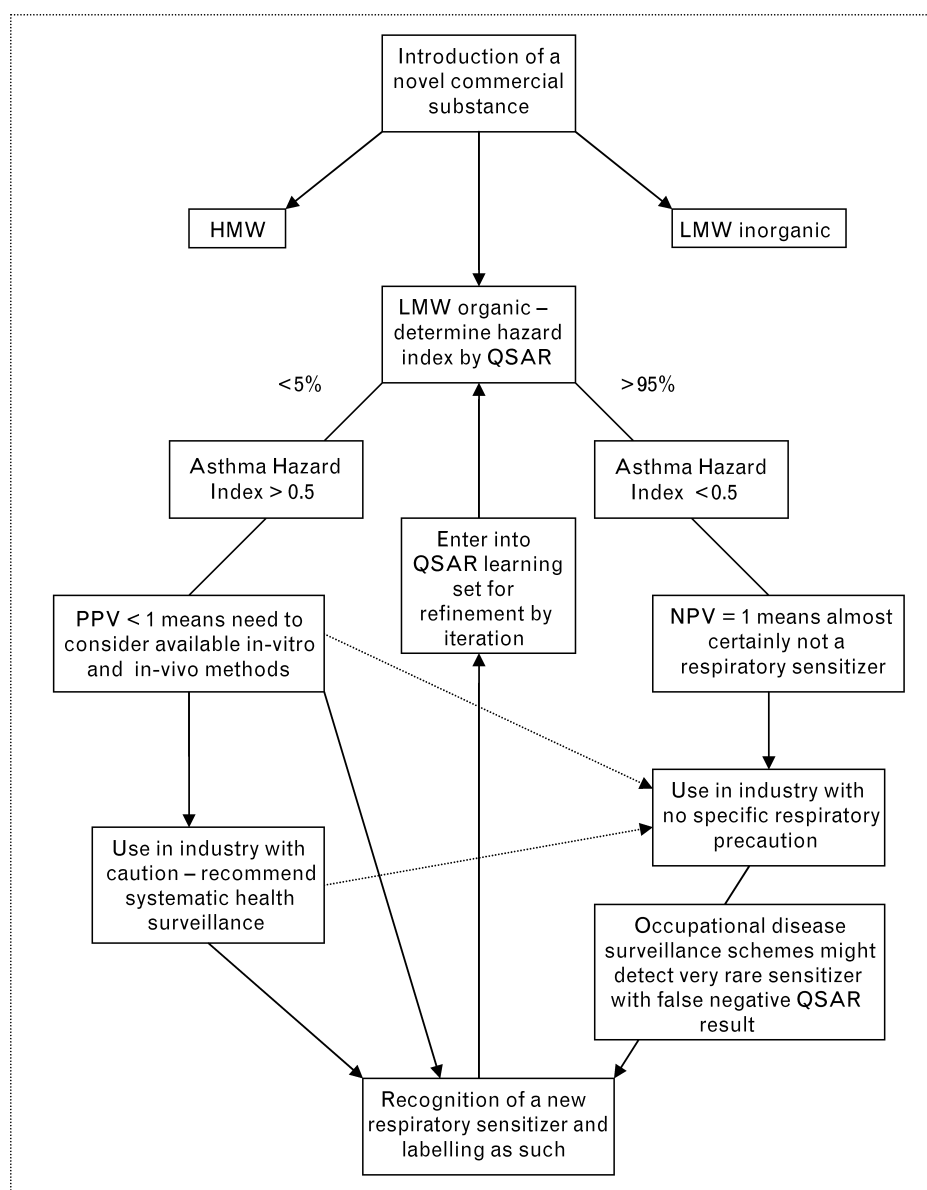
^a Using estimate that one in 300 chemicals are truly respiratory sensitizers.

^b Using estimate that one in 30 chemicals are truly respiratory sensitizers.

^c Using either of the above estimates.

A mechanistic inference of the findings shown in Figs 1 and 2 may be that when a chemical contains two or more reactive groups in the same molecule these groups can react simultaneously with different amino acids present on either the same native human protein or different protein molecules. This could lead to intra or intermolecular crosslinking, with the resulting conformational change exposing neoepitopes within the protein molecules. Such neoepitopes could be the immunological trigger leading to respiratory sensitization.

These HORs were the basis for identifying chemical substructural fragments for inclusion in a logistic

Figure 3 Possible protocol for screening novel commercial substances for respiratory sensitization hazard potential

HMW, high molecular weight; LMW, low molecular weight; NPV, negative predictive value; PPV, positive predictive value; QSAR, quantitative structure–activity relationship.

regression equation for determining the likelihood that an unknown chemical has asthmagenic potential [45]. The resulting model, which is freely available on the internet [46], has been externally validated for a cutpoint 'asthma hazard index' of 0.5 using 21 asthmagens not used in the model development. Its application to the corroboration of novel asthmagens following reports of human cases has already been described [47,48].

The validation statistics for each of these published QSARs are shown in Table 1. It can be seen that they all have a NPV of 1 with lower PPVs that vary considerably, with a 10-fold difference in the estimate used for the proportion of LMW chemicals that are truly respiratory sensitizers.

Possible screening protocol for identifying low-molecular-weight respiratory sensitizers

The current GHS for classification and labelling of respiratory sensitizers is inadequate; no definitive guidance is available on appropriate in-vivo or in-vitro tests; as a result, there is reliance on human epidemiological data. The high NPV of QSARs means that they are a possible first-stage screening method in an efficient protocol for prospective identification of respiratory sensitizers (Fig. 3). Clearly a valid QSAR presents an opportunity for cheaply and efficiently eliminating over 95% of these from the need for further testing. Further development and validation of in-vitro and in-vivo testing may provide means of confirming the respiratory sensitization potential for the small percentage of chemicals that have a positive result by QSAR.

This approach would help in the primary prevention of occupational asthma and rhinitis, but as no screening protocol is perfect there would still be occasional reported cases caused by a novel chemical. The detection of such sensitizers at an early stage would remain an important role of surveillance schemes such as THOR in the UK [10]. It should be emphasized that the QSAR models described have so far been developed for screening of LMW organic chemicals. Other methods would need to be considered, if necessary, for inorganic compounds or HMW substances. Epitope mapping is one potential means for prediction of allergenic proteins [49].

Conclusion

Many of the novel respiratory sensitizers that cause occupational rhinitis or asthma are LMW chemicals. No animal model has yet been validated for their prediction whilst all of the published QSAR models have high NPVs. This, together with their ease of use, makes them an ideal starting point for an efficient screening protocol for respiratory sensitization potential of industrial chemicals.

References and recommended reading

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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 190).

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