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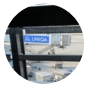

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A Mechanistic Approach to Modeling Respiratory Sensitization

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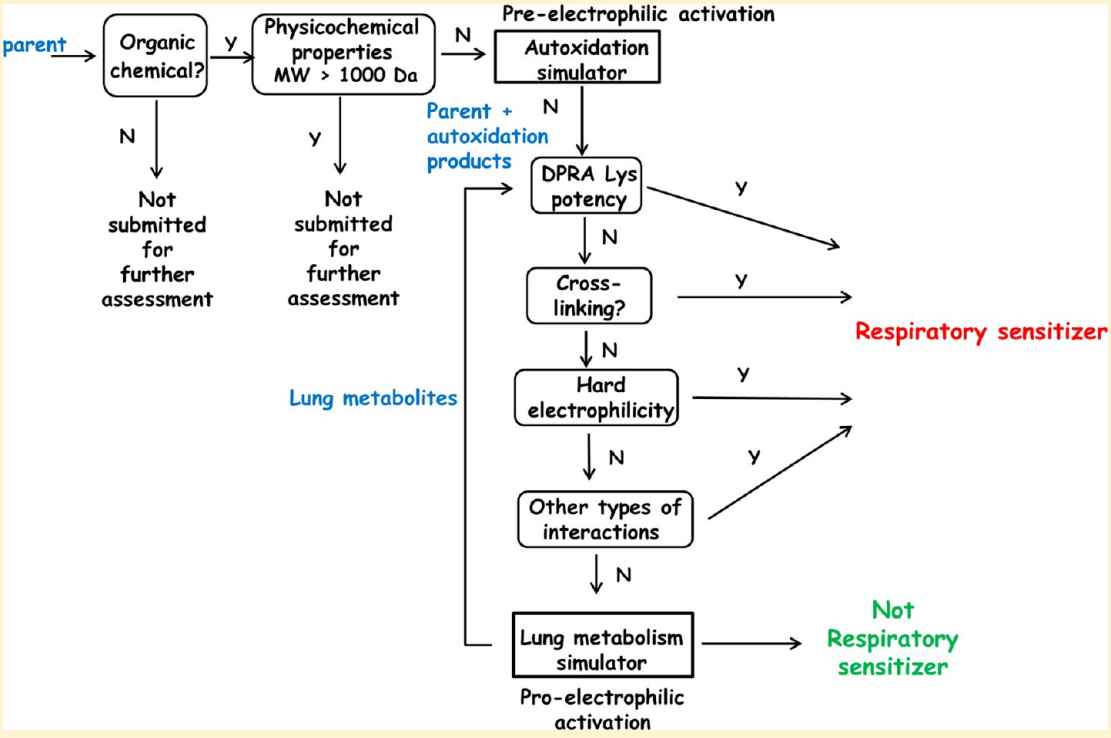

ABSTRACT: Chemical respiratory sensitization is an important occupational health problem which may lead to severely incapacitated human health, yet there are currently no validated or widely accepted models for identifying and characterizing the potential of a chemical to induce respiratory sensitization. This is in part due to the ongoing uncertainty about the immunological mechanisms through which respiratory sensitization may be acquired. Despite the lack of test method, regulations such as REACH still require an assessment of respiratory sensitization for risk assessment and/or for the purposes of classification and labeling. The REACH guidance describes an integrated evaluation strategy to characterize what information sources could be available to facilitate such an assessment. The components of this include a consideration of well-established structural alerts and existing data (whether it be derived from read-across, (quantitative) structure–activity relationships ((Q)SAR), *in vivo* studies etc.). There has been some progress in developing SARs as well as a handful of empirical QSARs. More recently, efforts have been focused on exploring whether the reaction chemistry mechanistic domains first characterized for skin sensitization are relevant for respiratory sensitization and to what extent modifications or refinements are needed to rationalize the differences between the two end points as far as their chemistry is concerned. This study has built upon the adverse outcome pathway (AOP) for skin sensitization that was developed and published by the OECD in 2012. We have structured a workflow to characterize the initiating events that are relevant in driving respiratory sensitization. OASIS pipeline technology was used to encode these events as components in a software platform to enable a prediction of respiratory sensitization potential to be made for new untested chemicals. This prediction platform could be useful in the assessment of respiratory sensitization potential or for grouping chemicals for subsequent read-across.

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INTRODUCTION

Chemical respiratory sensitization is an important occupational health problem, which may lead to severely incapacitated human health, yet there are currently no validated or widely accepted models for identifying and characterizing the potential of a chemical to induce respiratory sensitization. This is in part due to the ongoing uncertainty about the immunological mechanisms through which respiratory sensitization may be acquired.[1,2]

Despite the lack of test methods, regulations such as REACH still require an assessment of respiratory sensitization to be made for risk assessment and/or for the purposes of classification and labeling. The REACH technical guidance describes an integrated evaluation strategy (IES) to characterize what information sources could be available to facilitate such an assessment.[3] The components of this include a consideration of well-established structural alerts as well as existing data whether it be derived from read-across, (Q)SAR, or *in vivo* studies. Investigations of the underlying mechanisms that lead to respiratory sensitization have shown that there is strong evidence to support an IgE antibody dependent mechanism of action; however, not all sensitized individuals show a detectable level of IgE antibody. This is certainly true for diisocyanates where symptomatic individuals are reported to lack measurable levels of IgE antibodies.[2] This lack of association with IgE antibody might be due to technical difficulties in measurement, but it might also suggest that other mechanisms play a role. There is general consensus that respiratory sensitization to chemicals is associated with a selective T helper 2 (Th2-type) immune response. This has prompted study to investigate the extent to which it might be possible to distinguish skin sensitizers from respiratory sensitizers due to the discrete patterns of cytokines and antibody produced in mice that would be reflective of the development of selective Th cell responses.[4] Although the local lymph node assay (LLNA) does not represent a method for the specific identification of chemical respiratory allergens, there is evidence that chemical respiratory allergens will also elicit positive responses in this assay. This is of course a reflection of the fact that both classes of sensitizers are able to cause the activation of T lymphocytes and proliferative responses in draining lymph nodes.[4] The LLNA indicates that an immune response has been triggered but cannot distinguish the specific mechanisms inherent to skin or respiratory sensitization.[5] The REACH technical guidance proposes that if a chemical fails to induce a positive response in the LLNA (at an appropriate test concentration) it will probably lack the potential for respiratory allergy.[3] Such an assessment is of course built upon a weight of evidence (WoE) of various pieces of information. Thus, predictive approaches that seek to combine *in vitro*, *in chemico*, and *in silico* information might provide a means to effectively discriminate likely skin sensitizers from respiratory sensitizers.[6]

Efforts to model respiratory sensitization *in silico* have been variable and to some extent mirror those for skin sensitization itself. Structural alerts have been developed notably by Aguis et al.[7,8] Examples include diisocyanates, cyclic anhydrides, and diamines, which are also illustrated in the REACH technical guidance.[3] Other characteristic respiratory sensitizers include some platinum salts, certain reactive dyes, and chloramine T.[2] Typical alerting groups have been encoded into Derek Nexus, the knowledge based expert system developed by LHASA Ltd.[9] Other efforts have been focused on trying to establish statistical QSAR models; examples include those first derived by the developers of MCASE,[10] Jarvis et al.,[11] and more recently by Warne et al.[12] who investigated the use of pattern recognition methods to discriminate between skin and respiratory sensitizers.

An alternative approach has been to evaluate the extent to which predictive insights gained in the skin sensitization arena are relevant and applicable for respiratory sensitization, i.e., can approaches be refined or adapted to provide more useful indicators of likely respiratory sensitizers? This was the strategy favored by Enoch et al.[13−15] who exploited the concepts of reaction mechanistic domains for skin sensitization that Aptula and Roberts[16] had first described but investigated their applicability to respiratory sensitizers. The use of electrophilic reaction chemistry principles proved helpful for respiratory sensitizers, though not all the domains were well represented. For instance, hard electrophiles such as acylating agents and Schiff base formers were found to be more prevalent than softer mechanisms such as Michael addition. It was suggested that this was related to the predominance of lysine, the key biological nucleophile, as well as the assumption that cysteine was unavailable because of it being oxidized in the respiratory tract to a disulfide. The importance of lysine has since been confirmed experimentally in a number of studies, e.g., Holsapple et al.[17]

Lalko et al.[18] subsequently acknowledged the potential value of using chemical reactivity information to characterize respiratory sensitization, much in the same way as has been done for skin sensitization. Their publication investigated and evaluated the potential utility of the direct peptide reactivity assay (DPRA),[19,20] one of several peptide reactivity assays that have been developed and the one that has undergone an ECVAM validation study. Subsequent studies have explored reactivity in the peroxidase peptide reactivity assay (PPRA)[21] and have investigated the selectivity of chemicals to different amino acids by comparing the relative depletion of lysine and cysteine peptides.[22,23]

Thus, predictive *in silico* approaches have so far relied upon structural alerts either characterized by chemical classes or reaction chemical domains, QSAR models that are empirical in nature and *in chemico* reactivity data. The expected correlation between skin sensitization and respiratory sensitization and the work to date particularly by Enoch et al.[13] that has shown merit in exploring the underlying chemistry, prompted the present study. We sought to exploit the AOP for skin sensitization as published by OECD[24] to consider the extent to which early events for skin sensitization could be relevant for respiratory sensitization. Without a defined test method for predicting respiratory sensitization potential, an AOP could serve as a pragmatic framework for evaluating the utility and applicability of skin sensitization knowledge. Given the extensive work that this group has already undertaken in the area of skin sensitization leading to the development of the expert system, tissue metabolism simulator for skin sensitization (TIMES-SS),[25] a consideration of the relevance of the knowledge contained within TIMES-SS was also made. The AOP for skin sensitization as adapted from the OECD document is shown in Figure 1.

Bioavailability, metabolism, and reactivity are relevant considerations for respiratory sensitization. Thus, elements from tools such as TIMES-SS could form a pragmatic basis for developing a predictive toolbox for respiratory sensitization. Rather than modify TIMES-SS itself, a new pipeline architecture as encoded within OASIS technology was developed to model these initial events. The pipeline comprises a set of components which address each of these events in turn. The actual pipeline implementation together with the basis of each of the components is discussed in more detail later in this article.

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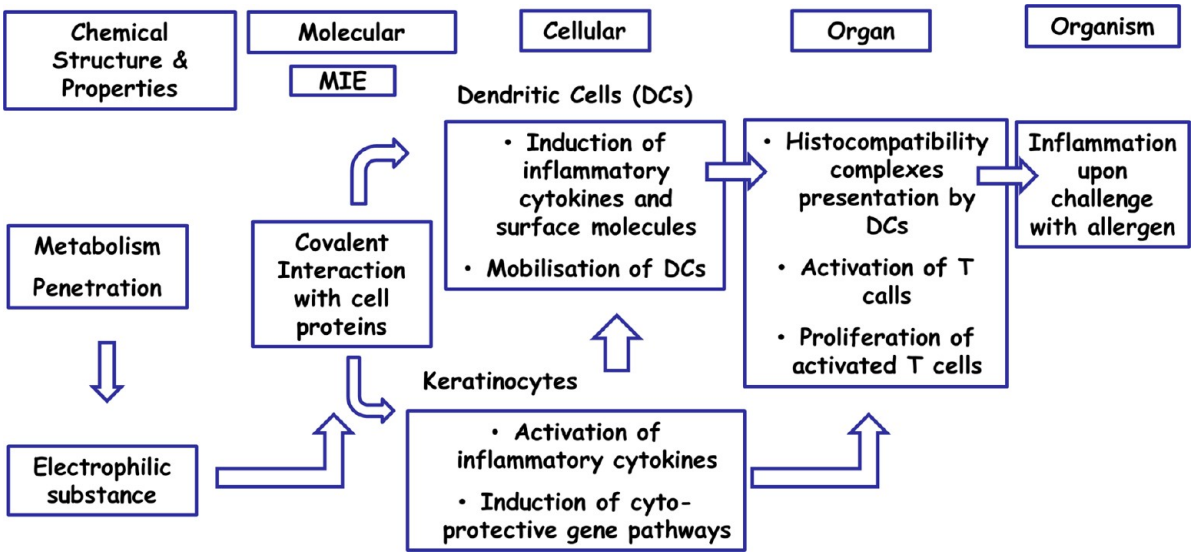

Figure 1. AOP for skin sensitization adapted from that published by the OECD. Adapted with permission from ref 24. Copyright 2012 OECD.

Initial Events within an AOP for Respiratory Sensitization. Factors which characterize early steps in an AOP for respiratory sensitization can be defined as bioavailability, reactivity, and electrophilic activation. Bioavailability in the context of respiratory sensitization could be defined as partitioning through the nasal cavity or tracheal membrane, and molecular features such as size, hydrophobicity, and aqueous solubility will therefore play a role in modulating partitioning. Reactivity (electrophilic) may be delineated using existing structure−activity rules as developed for skin sensitization with appropriate adaptations given the predominance of lysine, the key biological nucleophile in the lung. The concept of hard and soft electrophiles therefore plays a role in helping to identify those skin sensitizers that have respiratory sensitization potential. As noted in Enoch et al.[13,14] a cross-linking mechanism could also play a role in driving respiratory sensitization. Adapting existing SARs to account for cross-linking was explored as part of this study. A number of substances require electrophilic activation, either chemical or metabolic: the existing metabolism and autoxidation simulators developed for TIMES-SS[25] were adapted and incorporated as separate components in the overall OASIS pipeline that was developed. It is known that other mechanisms such as free radical mechanisms can also play a role in inducing sensitization. Components to encode alerting groups for such mechanisms were incorporated into the pipeline.

The novelty in the OASIS pipeline lies in the fact that no training set was used to derive a classical QSAR model, unlike the other *in silico* studies cited. Instead, the approach was built on an understanding of the mechanistic domain of alerts and estimating the hardness/softness of reaction centers of both parent and simulated metabolites using quantum chemical calculations.

EXPERIMENTAL PROCEDURES

Data Availability. No defined test guideline exists for the prospective identification of chemical respiratory sensitizers. The majority of data available are typically associated with low molecular weight (LMW) chemicals such as anhydrides and isocyanates for which there is good evidence of respiratory sensitization. Positive respiratory sensitization data were identified for 120 chemicals which had been collected from different sources; 57 positives were found in the following references[10,26−30] on the basis of clinical case reports (for human) and animal test results (the guiding principles as outlined in Karol et al.[27] were used). Eighteen of these 57 chemicals were taken from Annex VI of the European Commission Directive 93/21/EEC.[30] In this case, these chemicals had been previously classified as R42 or R42/43 on the basis of this regulation. The remaining positives were taken from the publication of Enoch et al.[15] The 82 negatives in the data set were also taken from Enoch et al.[15]

Such a heterogeneous data set is well recognized as not a preferred approach for developing QSARs, but the absence of a test protocol does render the collection of quality data for this end point a practical challenge. In this study and unlike typical QSAR studies, the data set that was ultimately collected did not form the basis of a "training set" per se; rather, the data set collected served as the test set to evaluate the predictivity of the overall pipeline developed. Each of the individual components were derived on the basis of different information sources, experimental or theoretical, and the prediction model is effectively an integration of these components, akin to a semi-qualitative integrated testing strategy (ITS). This type of approach meant that the underlying drivers for respiratory sensitization were modeled and ensured that the chemical applicability domain was not necessarily restricted by the scope of this test data set of 202 chemicals.

Bioavailability: Modeling Nasal Penetration. Some authors[27] have inferred that respiratory sensitizers tend to possess higher molecular weights, lower log K_{ow} values, and greater water solubility than nonsensitizers. No explicit boundaries or thresholds have been established as such for respiratory sensitization. Factors modulating nasal penetration have been analyzed with respect to acute inhalation toxicity. It has been found that acute inhalation toxicity is associated with the following physicochemical thresholds which chemicals need to meet in order to have nasal penetration:[31] aerodynamic D, d_a< 100 μm; vapor pressure, VP > 0.5 kPa or boiling point, BP < 150 °C; logP > 0; and water solubility, WS > 1 mg/L. Inhalation toxicity is also associated with low molecular weight (LMW) chemicals (having MW < 1 kDa).[7,32,33]

The ranges of these physicochemical parameters could be assumed to provide an indication of the likely bioavailability to the sites of action, at least for volatile substances. Given that no specific physicochemical thresholds have been defined for respiratory sensitizers as such and more experimental data would need to be produced and analyzed to propose even tentative boundaries, the only characterization of bioavailability that was undertaken here was to consider a molecular weight boundary of 1000 Da.

Pre- and Pro-electrophilic Activation. *Lung Metabolism Simulator.* Many respiratory sensitizers require metabolic activation in order to elicit their effect. In order to reproduce the effect of these chemicals, a metabolic simulator of lung metabolism using the probabilistic approach for developing metabolic simulators was created.[34−36] The simulator comprises a list of hierarchically ordered transformations

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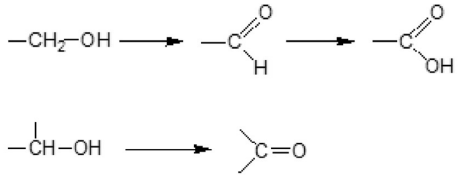
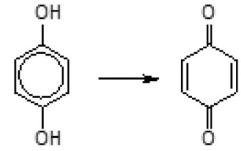
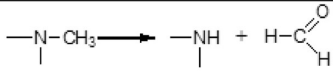
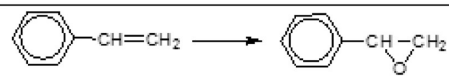
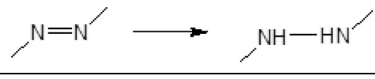
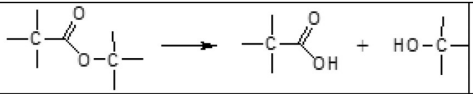
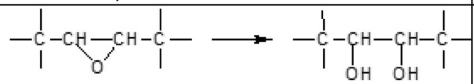
Table 1. Comparison of the Metabolic Transformations in Liver/Skin and Lung

Type of reaction	Molecular transformation	Liver/skin	Lung	Comment
Oxidation reactions				
Hydroxylation		+	+	
Oxidation		+	- Alcohol and aldehyde dehydrogenases are in small quantities[65]	Note: In case of hydroquinones, the following abiotic oxidation to quinones is proposed by some authors[14]: 
N-,O-oxidative dealkylation		+	+	
Epoxidation		+	+	
Reduction reactions				
NO_2-reduction		+	+	
Azo reduction		+	+	According to Enoch et al.[14] given the oxidizing nature in lung, reduction of azo dyes in appears unlikely. According to Autrup and Warwick[66], azoreductase activity in lung depends on the parent structure.
Hydrolysis reactions				
Ester hydrolysis		+	+	
Epoxy hydrolysis		+	+	

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and a substructure matching engine for their implementation. The hierarchy of transformations is defined by the probabilities of transformations determined in such a way as to reproduce a database of documented metabolic transformations or rate of disappearance data. The transformation probabilities are related to the rate constants being associated with the feasibility of occurrence of reactions within the time frame of metabolism tests. The simulator is based on the assumption that transformations are independent and performed sequentially. An analysis was performed to identify the key differences in the enzymatic systems available in the lung as compared to those in the skin and liver. The results are listed in Table 1. For example, alcohol and aldehyde dehydrogenases are not at appreciably high levels in the lung[37] to detoxify low molecular weight aldehydes generated upon metabolic transformation. Given the lung is a very oxidizing environment,[12] the rate of the oxidation reactions should be higher compared with the skin. Thus, chemicals that are not able or are hardly oxidized in skin for instance would be oxidized far more readily in the lung. For example, styrene has negative skin sensitization data, and this is probably due to its high volatility.[38] It is oxidized in the lung[7,12] to the corresponding epoxide and then binds to lung proteins (Figure 2), whereas in the skin,

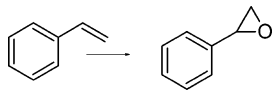

Figure 2. Epoxidation of styrene followed by protein binding.

this process is hampered given that styrene needs to penetrate into the skin. The oxidizing nature of the lung hampers reduction reactions; thus, the rate of reduction reactions should be decreased in the lung as compared with the skin or the liver. Chemicals such as azo dyes, for example, that undergo reduction reactions in the skin/liver (the reductase mechanism has been reported to explain the genotoxicity of azo-dyes[39]), are unlikely to undergo such transformations in the lung. Overall, compared with the liver, the lung possesses a more limited metabolic capacity,[40−42] although oxidative enzymes are particularly well expressed.[15]

The starting point for building a lung metabolic simulator relied upon the existing rodent liver microsomal/S9 metabolic simulator for predicting *in vitro* genotoxicity effects after metabolic activation that exists within TIMES.[43] The development of the lung metabolic simulator was based on a training set of 150 chemicals with *in vitro* metabolic information derived in lung subcellular fractions and whole tissues that was observed experimentally. The majority of the experimental data collected were taken from subcellular fractions (microsomes, S9 fraction, etc.) derived from rat and mouse lung tissues (65−70%). Data from tissue slices and perfused lung in rats and mice as well as other species (e.g., rabbit, human, etc.) were also collected to broaden the scope of molecular transformations that could be simulated. This training set of chemicals is provided as Supporting Information, which provides details on the CAS number, name, and full literature reference.

Some of the existing molecular transformations were modified, some were deleted, and some new transformations were added. The modified transformations were mainly associated with the inhibiting "masks" of certain oxidative metabolic transformations such as aromatic hydroxylation, aliphatic epoxidation, S-oxidation, oxidative desulfuration, N-hydroxylation, oxidative deamination, formation of PAH epoxides, etc. New transformations included examples such as oxidative deamination and formaldehyde release, whereas transformations that were deleted were in cases where the likelihood of metabolism in the lung relative to the liver was much lower, e.g., β-oxidation. The metabolism simulator predicts the transformation pathways (the metabolic map) as well as the probability and reliability of generated transformation products.

Autoxidation. Autoxidation is a spontaneous abiotic process of oxidation of chemicals exposed to air at room temperature. It is a free-radical chain reaction of a chemical with oxygen, resulting in the formation of oxidation products such as hydroperoxides.[44] The autoxidation simulator, recently developed for TIMES-SS on the basis of data extracted from the literature for 138 chemicals [including terpenes, simple aliphatic and polyethylene glycol ethers, aldehydes, and aminophenols] was implemented into the OASIS pipeline framework. A simplified version of this simulator has also been made available within the OECD Toolbox, v3.1. The set of chemicals, their names, CAS numbers, and full literature citation is provided as Supporting Information.

Electrophilic Reactivity. *Hard/Soft Interactions between Electrophiles and Nucleophiles.* As noted earlier, while the mechanism of respiratory sensitization to airborne chemical substances is not as well elucidated as that for skin sensitization, the initial events as depicted in the AOP for skin sensitization are common to both end points.[7,32,45−47] Work already explored by Enoch et al.[13,14] has alluded to the types of reaction domains that are more prevalent for respiratory sensitizers, which include acylating agents and Schiff base formers. These could be categorized as hard electrophiles. Definitions exist for hard and soft electrophiles based on the theory of hard and soft acids and bases (HSAB).[48,49] By definition, electrophiles react preferentially with nucleophiles of similar hardness or softness. Parameters can also be calculated to quantify the extent of hardness or softness as explained below. According to the HSAB definitions,[50,51] soft electrophilic sites are atoms with large atomic radii that contain unshared electron density in their valence shells and have low local orbital electronegativity. They form predominantly covalent bonds with interacting nucleophilic sites. In contrast, hard electrophilic sites are atoms with small atomic radii that do not contain unshared electron density in their valence shells and have high local orbital electronegativity. They form predominantly ionic bonds with interacting nucleophilic sites. The donor atoms of the soft nucleophiles are of low electronegativity, of high polarizability, and easy to oxidize, whereas the donor atoms of the hard nucleophiles are those which are of high electronegativity, of low polarizability, and difficult to oxidize.

Since electrophiles tend to accept electron density during the interaction with the nucleophiles and this depends on the degree of the positive charge located at the attacked atom, convenient reactivity indices to encode hardness/softness of electrophiles include net atomic charge (eq 1) and nucleophilic (acceptor) superdelocalizability (eq 2). Net atomic charge (Q) is determined by the sum of the partial electron densities across occupied molecular orbitals (MOs). This parameter characterizes the localization of the total charge at an atomic site, across all occupied MOs. The more positive the charge, the harder is the electrophilic site. Nucleophilic superdelocalizability (S_μ^N), also known as acceptor superdelocalizability, is a measure of the ability of atoms to accept additional electron density. The larger the acceptor superdelocalizability, the softer is the electrophilic site. The mathematical formalisms of both these parameters are provided in eqs 1 and 2.

$$Q_\mu = \sum_i n_i c_{i,\mu}^2, \ [\text{a.u.}] \tag{1}$$

where $c^2_{i\mu}$ is the partial electron density of μ atoms in i molecular orbitals, and n_i is the orbital population ($0 \le n \le 2$).

$$S_\mu^N = 2 \sum_{\substack{j \\ \text{unoccupied} \\ \text{MO}}} n_i \frac{c_{j\mu}^2}{E_j}, \ [(\text{a.u.})^2/\text{ev}] \tag{2}$$

where $c^2_{j\mu}$ is the partial electron density of μ atoms in j molecular orbitals, and E_j is the energy of the j molecular orbital.

For nucleophiles which donate electron pairs (or electron density) to an electrophile to form a chemical bond, the net atomic charge (eq 1) and electrophilic superdelocalizability (eq 3) can be used to assess their hardness/softness. Electrophilic superdelocalizability (S_μ^E), also known as donor superdelocalizability, is a measure of the availability of electron density on an atom.

$$S_\mu^E = 2 \sum_{\substack{j \\ \text{occupied} \\ \text{MOs}}} n_i \frac{c_{j\mu}^2}{E_j}, \ [(\text{a.u.})^2/\text{ev}] \tag{3}$$

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where $c^2_{j\mu}$ is the partial electron density of μ atoms at j molecular orbitals, and E_j is the energy of the j molecular orbital.

Thus, acceptor and donor superdelocalizabilities should be correlated with the extent of covalent bond formation, whereas the net atomic charges should be related with the extent of ionic bond formation.

The HSAB concept was exploited to create a scale of hard and soft electrophiles. The net atomic charge and donor superdelocalizability of the reaction site of the amino acids cysteine, histidine, lysine, tyrosine and arginine (Figure 3) were calculated within the AM1 Hamiltonian

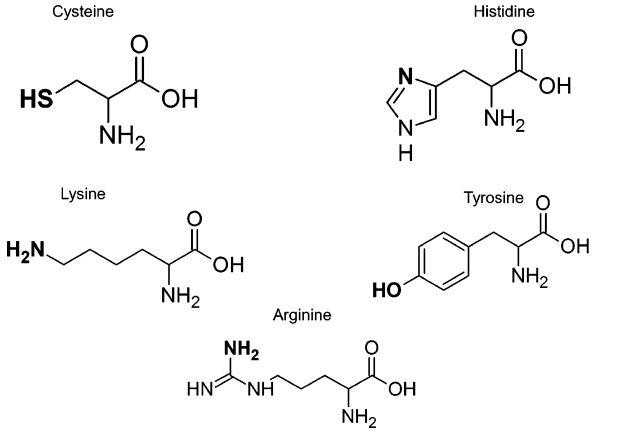

Figure 3. Cysteine, histidine, lysine, tyrosine and arginine. The reaction group involved in the reactions with the electrophiles are in bold font.

using MOPAC 93 (Table 2). The hardest nucleophilic site was found to be the nitrogen atom in the amino group of lysine having the most negative net atomic charge.

An analysis of the hardness/softness of electrophiles by consideration of the reaction mechanistic domains as defined by Aptula et al.[16] (Table 3) was undertaken. The net atomic charge and the acceptor superdelocalizability of the reaction center of electrophiles of known protein binding alerts[25,35,52] were calculated by applying AM1 Hamiltonian, MOPAC 93. Six categories of hardness and softness based on the net atomic charge of the electrophilic sites were defined (Table 4). Representative structures having protein binding alerts belonging to specific hard/soft ranges of electrophilicity are illustrated in Figure 4.

***In Chemico* Models for Evaluating Reactivity.** Recently, peptide reactivity tests have been exploited as a means of screening for potential skin sensitization. One such assay is the DPRA,[19,20] which evaluates the ability of chemicals to react with glutathione or synthetic peptides. The chemical reactivity toward each model peptide is expressed as a percentage of peptide depletion. The model nucleophiles glutathione, cysteine, or lysine are mixed with the test chemical at a ratio of 1:100, 1:10, and 1:50, respectively. Sodium phosphate buffer is added to the solution for both the cysteine and glutathione assays (pH 7.4−7.5), and ammonium acetate buffer is added to the lysine assay (pH 10.2). The samples are incubated for 15 min of reaction time for GSH and 24 h of reaction time for cysteine and lysine. The samples are analyzed by HPLC-UV, and peptide depletion is evaluated. One hundred three chemicals with DPRA lysine depletion data were found in the literature.[19,20,53] A component was developed to encode semiquantitative rules for evaluating the reactivity of chemicals toward lysine given that NH2-Lys amino acid residues are predominantly available in the lung.[13,14,17] The lysine profiler developed contains 24 structural alerts. Depending on the percentage depletion, the chemicals were divided into four categories (Table 5): nonreactive (lysine depletion ≤5) (58 chemicals); low reactivity (lysine depletion = 5−40%) (33 chemicals); moderate reactivity (lysine depletion = 40−80%) (8 chemicals); and high reactivity (lysine depletion >80%) (6 chemicals). The profiling component has since been implemented into the OECD Toolbox, v3.1, together with the associated lysine depletion data. Chemicals belonging to each of the 4 categories were used to derive structural boundaries. Mechanistic justification of the reactivity of the chemical classes were developed using expert knowledge and literature data. An illustration of the model outcome is presented in Figure 5.

Table 3. Hard and Soft Electrophiles as found in the Literature

hard electrophiles	soft electrophiles
aldehydes (Schiff base formers)	α,β-unsaturated carbonyl compounds (Michael acceptors)
anhydrides (acylating agents)	quinones (Michael acceptors)
acid chlorides (acylating agents)	alkyl halides (SN2 electrophiles)
isocyanates (acylating agents)	alkyl sulfates, phosphates (SN2 electrophiles)
carbonyl group with a suitable leaving group (acylating agents)	epoxides (SN2 electrophiles)
strained 4-membered ring (acylating agents)	carbon in strained ring lactones

Table 4. Hard/Soft Categories based on Net Atomic Charges

category	Q_r [a.u.]
very soft electrophiles	< −0.20
soft electrophiles	−0.20 ÷ −0.10
weak soft electrophiles	−0.10 ÷ 0.10
weak hard electrophiles	0.10 ÷ 0.20
hard electrophiles	0.20 ÷ 0.30
very hard electrophiles	>0.30

Modeling Cross-Linking. The cross-linking mechanism of respiratory sensitization has been discussed (see refs 7, 8, 13, 14, and 18). The ability to cross-link proteins is not a prerequisite for a chemical to cause respiratory sensitization but can augment the potency of compounds that without cross-linking would not be sufficiently reactive.[14] A filter to help identify those substances with two alerting groups of which one needs to possess a hard electrophilic center was derived.

Other Interactions. Rules to account for other mechanisms such as free radical interactions were also encoded into a component. Only a handful of substances within the training set were impacted. The rules and example chemicals will be discussed in the next section.

RESULTS AND DISCUSSION

Overall Structure of the Pipeline Model. A pipeline was developed using OASIS pipeline technology to encode relevant information characterizing the initial events pertinent for respiratory sensitization as part of a generalized model. Two versions of this pipeline are available, one that includes a DPRA component and one that excludes it. The test set of chemicals with respiratory sensitization information were processed through these pipelines, and their performances were compared between each other and in conjunction with the available experimental data.

The scheme including the DPRA component is illustrated in Figure 6. The specific components represented are extreme physicochemical properties, autoxidation simulator, DPRA-lysine reactivity, cross-linking, hard electrophilicity, other types of interactions, and lung metabolism simulator.

Table 2. Calculated Q and S_μ^E of the Reactive Site of the Amino Acids

	cysteine (Cys) SH-Cys	histidine (His) Cycl-3N-His	lysine (Lys) NH2-Lys	tyrosine (Tyr) OH-Tyr	arginine (Arg) NH2-Arg
Q_r [a.u.]	0.00 ÷ 0.01	−0.16 ÷ −0.13	−0.37 ÷ −0.34	−0.25	−0.43 ÷ −0.37
S_μ^E, [(a.u.)^2 /eV]	0.39 ÷ 0.40	0.25	0.25 ÷ 0.28	0.25	0.24 ÷ 0.25

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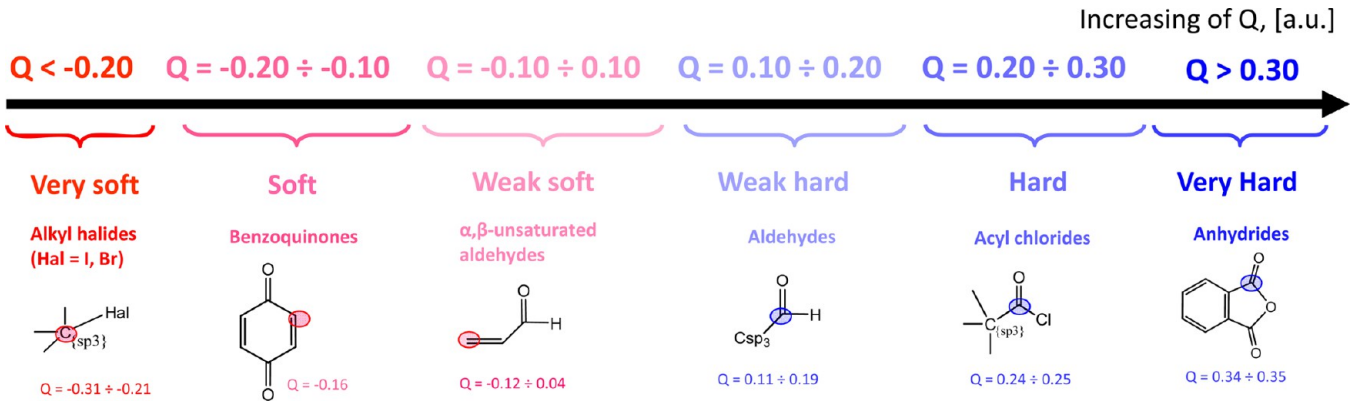

Figure 4. Distribution of protein binding alerts across the scale.

Table 5. Potency Categories based on Lysine and Cysteine Depletion (DPRA) Experimental Data

potency category	lysine depletion, %
high reactivity	>80.0
moderate reactivity	(40.0; 80.0)
low reactivity	(5.0; 40.0)
nonreactive	≤5.0

The basis of each of these components has been discussed in the Experimental Procedures section. Here, we outline how they are integrated together to facilitate an assessment of potential respiratory sensitization.

The pipeline begins with a filter to exclude anything other than discrete organic chemicals. The next step is a component which identifies those chemicals having extreme physicochemical properties. Currently, the only threshold applied is for molecular weight, which effectively excludes the assessment of polymers. If the target chemical has MW < 1000 Da, it continues to the subsequent components. The pipeline technology is sufficiently flexible to allow for future physicochemical threshold refinements as and when they are established. Work is ongoing to investigate whether tentative thresholds could be established for physicochemical parameters such as vapor pressure, log K_{ow}, etc. as described in the Experimental Procedures section.

The next component invokes the application of the autoxidation simulator where oxidation products are generated for the target chemical. The sensitivity and predictivity of the autoxidation model to reproduce the observed autoxidation pathways of the 138 chemicals in the training set were 88.9% and 84.9%, respectively. The simulation of the autoxidation pathway for limonene as an example is illustrated in Figure 7. All of the

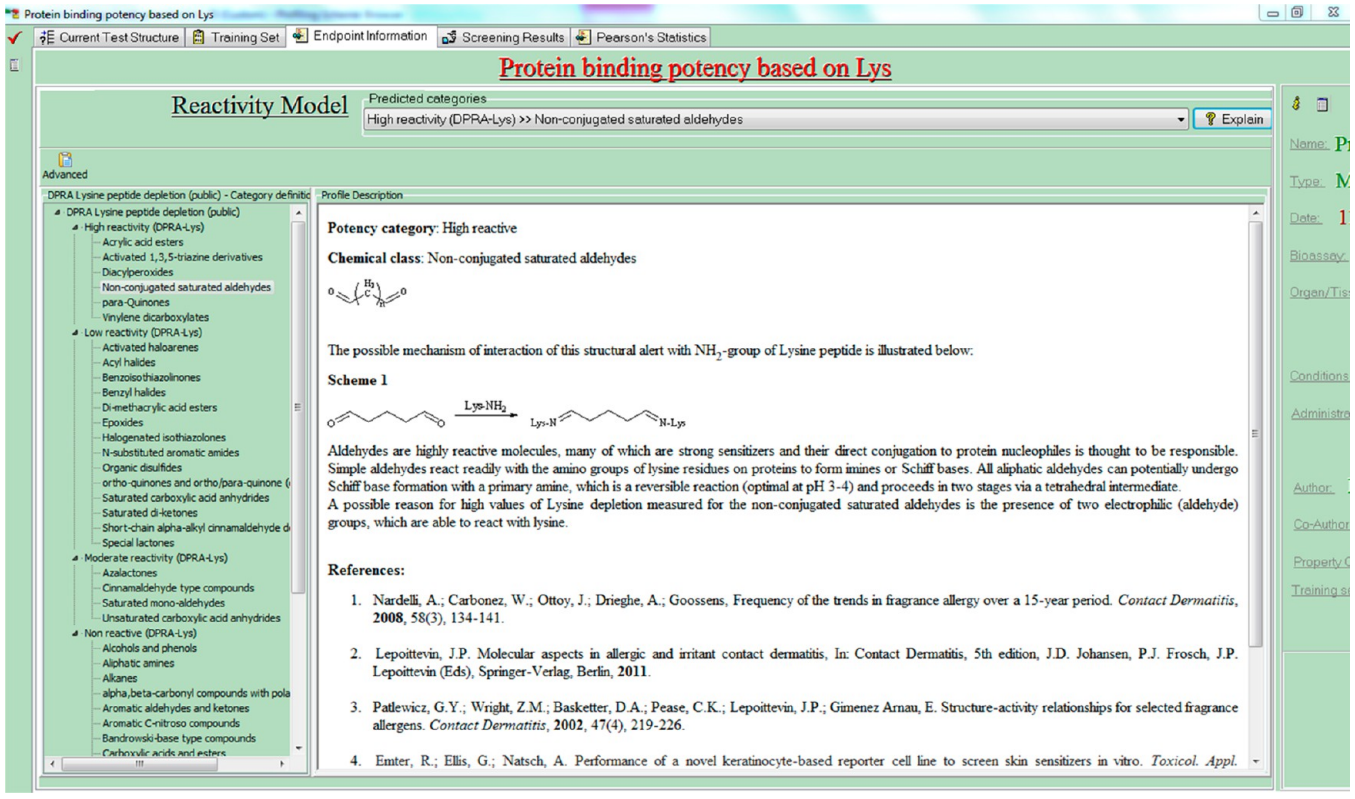

Figure 5. Illustration of the outcome of the DPRA-Lys reactivity module.

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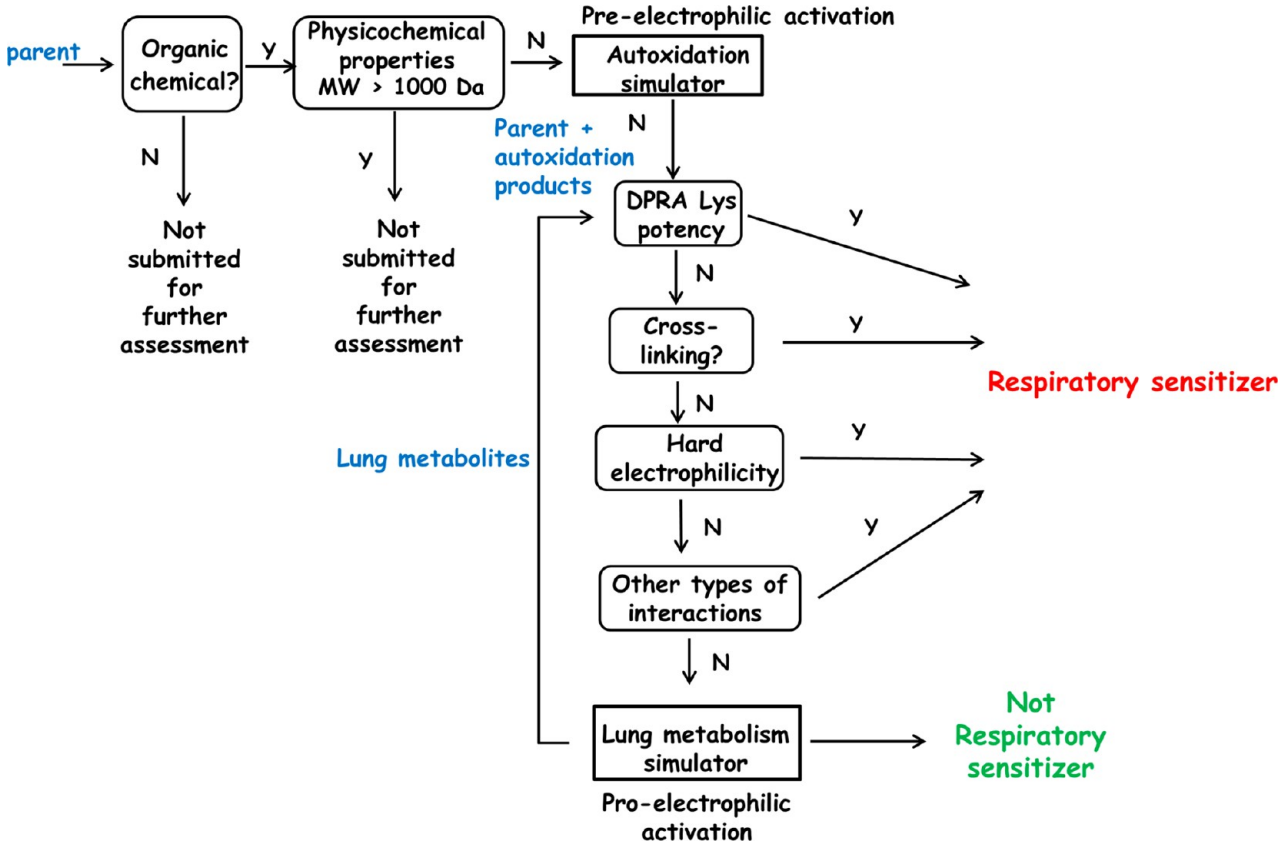

Figure 6. Pipeline workflow for respiratory sensitization including the DPRA-lysine reactivity component.

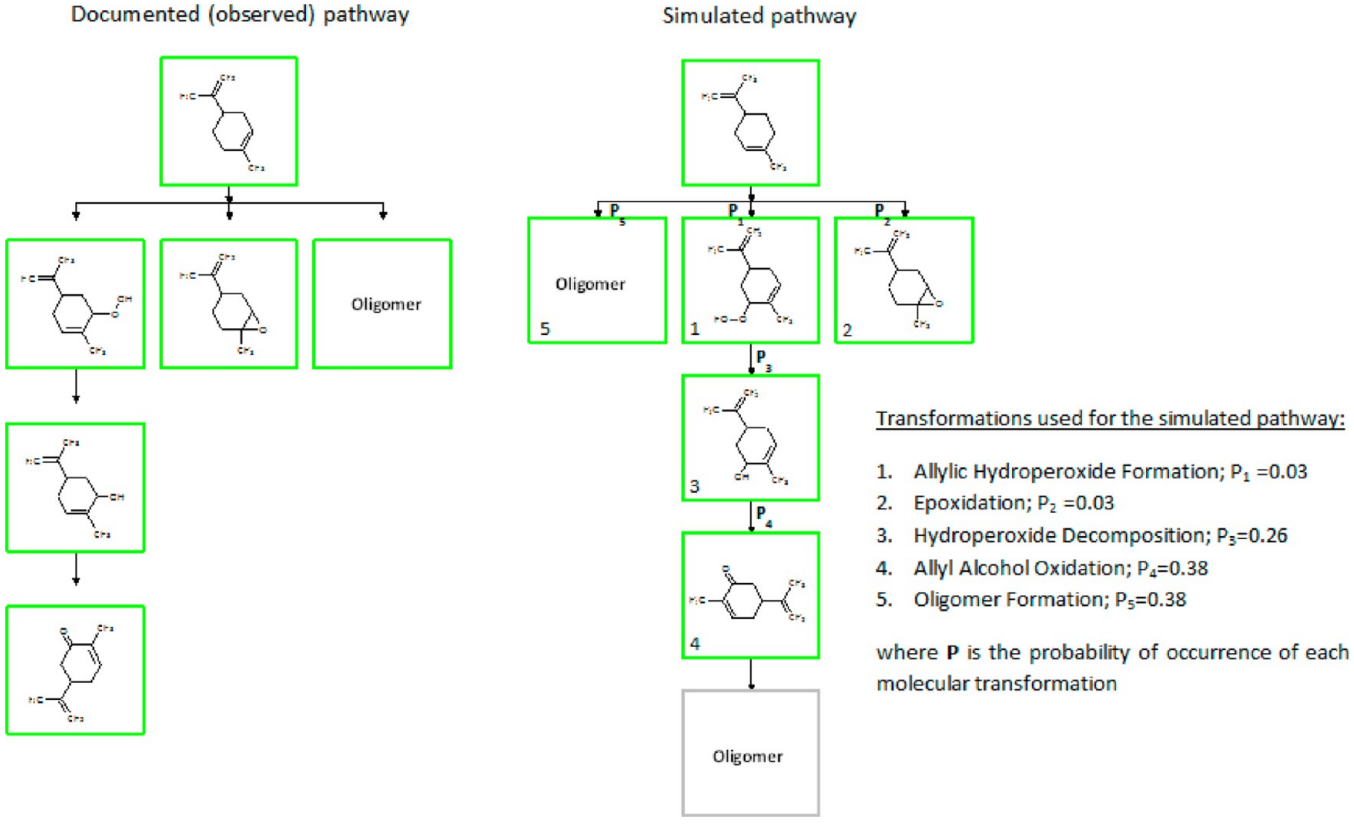

Figure 7. Simulation of the autoxidation pathway of limonene.

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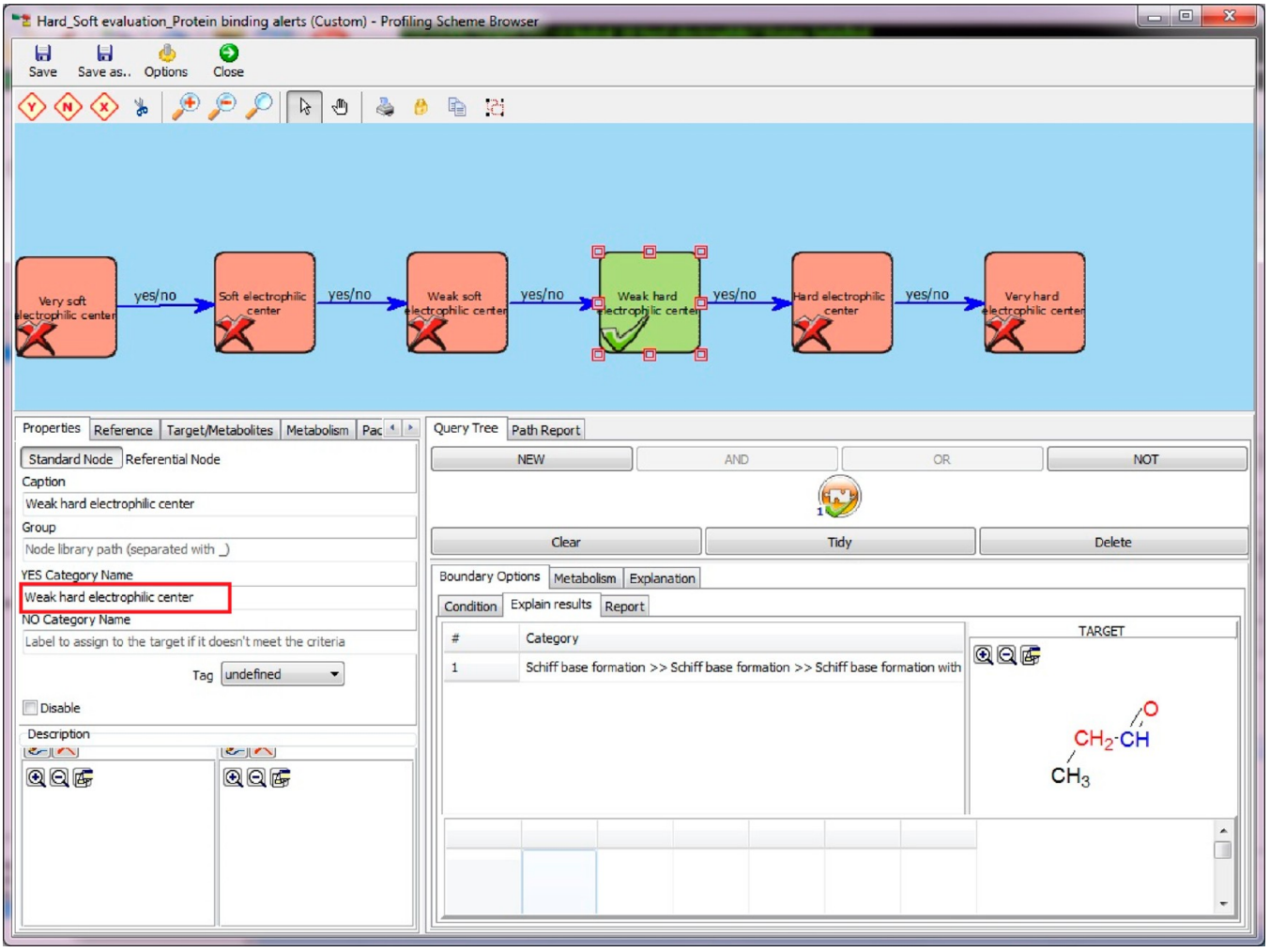

Figure 8. Illustration of the outcome of the hard electrophilicity module.

documented (observed) autoxidation products were actually simulated in this case.

In the pipeline, the autoxidation simulator is applied before the DPRA-lysine reactivity or the hard/soft electrophilicity components. Parent and autoxidation products (if any) are then assessed for their DPRA-lysine reactivity. If the parent or its autoxidation products meet the structural boundaries for high, moderate, or low reactivity with lysine, the pipeline concludes with a potential respiratory sensitizer categorization as its prediction. Autoxidation is to an extent inherently captured within the DPRA assay since the chemical samples are incubated for 24 h with either lysine or cysteine with air exposure. This could explain the reactivity of chemicals such as 1,4-benzenediol (with lysine depletion of 51.1%).[19] 1,4-Benzenediol does not possess an alerting group for protein binding but upon oxidation, it is transformed into its respective quinone which can then bind covalently with protein. The associated mechanistic justification is also described in Roberts et al.[54] who reasoned the behavior on account of the quinoid structure, rather than the parent diol.

The next component identifies any cross-linking functionality. If the parent or its autoxidation products satisfy one of the criteria, either to have reactivity toward lysine or to be a cross-linker with at least one hard electrophilic center, this is assumed to be sufficient to assign a respiratory sensitization prediction.

If no positive effect is predicted for the target chemical or its autoxidation products, the hard electrophilicity component is then triggered. Here, a comparison is made against a library of protein binding alerts for skin sensitization. The net atomic charge of the reaction center of the alert is calculated quantum-chemically to determine the degree of hardness/softness based on the ranges outlined in Table 4. An illustration for the outcome of this component is presented in Figure 8. If no electrophilic center is identified, no hard/soft electrophilicity is assigned for the chemical under investigation, and the next component "other types of interaction" is invoked. A positive identification of an alert could result in a potential respiratory sensitizer prediction.

The lung metabolism model is next to be triggered to simulate potential metabolites of the starting parent and any autoxidation products. The performance of the lung metabolic simulator was fair given the limited data set of 150 chemicals, with an average sensitivity and predictivity of 85.2% and 39.2%, respectively. The low predictivity is mainly due to the way the experimental metabolism studies that were identified in the literature were reported. In many of the publications, the objective of the studies was to focus on specific metabolites rather than elucidating an entire metabolic map. In many cases, phase II transformations were not documented. In contrast, the metabolic pathways simulated are complete. Hence, metabolites which are not documented but generated are counted as false positives though of course they could be real. This could explain the low predictivity of the metabolic models derived. Work will be ongoing to extend

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the scope of the lung metabolism simulator as new data becomes available or is harvested.

Finally, the resulting metabolites of target chemical and its autoxidation products are fed back into the components for assessing lysine reactivity, cross-linking, hard electrophilicity, etc. If no reactive metabolites are generated, the target chemical is concluded to be an unlikely respiratory sensitizer.

Overall Summary Performance. The full test set of 202 chemicals, 120 respiratory sensitizers and 82 nonsensitizers, was profiled through the two pipeline models. The summary of performance statistics is presented in Table 6. Both models are reasonable at identifying likely respiratory sensitizers based on the test set profiled as evident from the high sensitivities 84–89%, but both suffer from a high percentage of false positives (specificities were between 52 and 54%). The similar performance of the two pipelines does call into question the added information that the DPRA component for lysine reactivity brings to bear in terms of predicting likely respiratory sensitization. Identifying potential respiratory sensitizers using reactivity as quantified by quantum chemical calculations alone results in very similar performance statistics. So far, the body of DPRA data is still quite limited, which is reflective of its recent uptake. The component derived was based on a data set of 103 chemicals resulting in 24 alerts. Going forward, the number of alerts and the underlying data set could be expected to broaden in scope, and with it, a shift in performance between the two pipelines might result. Having said that, reactivity as measured in the DPRA-lysine assay is often not well modeled for certain hard electrophiles as a result of the dilute aqueous conditions such that a lack of lysine depletion may not be necessarily due to nonreactivity but to the limitations of the test protocol.[55] Work will be ongoing to determine the value of the DPRA-lysine assay information to the overall pipeline.

Table 6. Summary of Performance Characteristics of the two Pipeline Models

	pipeline 1: with DPRA-lysine reactivity	pipeline 2 without DPRA-lysine reactivity
sensitivity	89%	84%
specificity	52%	54%
accuracy	74%	72%
false positive rate	48%	46%
false negative rate	11%	16%
positive predicted value	73%	73%
negative predicted value	77%	70%

A more detailed evaluation of performance was also summarized for the two pipelines in Tables 7 and 8. These show the breakdown of predictions across the different components to provide further information of the extent to which chemicals were predicted to be respiratory sensitizers on account of the parent materials or whether they required activation (either metabolic or chemical) to be active. The statistics also highlight whether the likely mode of action could be attributed to a covalent mechanism and therefore characterized by the DPRA-lysine, cross-linking, or hard–soft components or whether the likely mode of action was driven by another mechanism (e.g., free radical). The distribution of predictions across the common components was practically the same between the two pipelines.

Performance of the Hard/Soft Electrophilicity Component. To analyze the performance of the HSAB approach, the 120 chemicals with positive respiratory sensitization data from the test set were profiled using the pipeline model with DPRA-lysine reactivity. Ninety-one of the chemicals were categorized as active due to the HSAB approach. Of these 91 active chemicals, 58 had protein binding alerts in their parent structures; hence, no metabolism was needed for their activation. The assessment of hard/soft electrophilicity of these chemicals showed that 33 of them belonged to the very hard range of electrophilicity and 5 to the hard electrophilicity range; 6 were weak hard electrophiles; and the remainder (14 chemicals) belonged to the weak soft electrophilicity range. For chemicals that required metabolic or chemical activation, the profile showed that 1 chemical was a very hard electrophile, 5 were hard/soft electrophiles, 15 were weak hard electrophiles, and the remainder (12) were weak soft electrophiles. The distribution of the positive respiratory sensitizers across this "hard/soft" axis is illustrated in Figure 9. The results show how positive respiratory sensitizers tend to occupy the "harder" part of the scale for hardness/softness. The distribution of protein binding alerts for skin and respiratory sensitization across the hard/soft scale could be explained by the nucleophilic diversity of protein amino acids in the skin (Figure 10a) and lung (Figure 10b). As can be seen, there is a relatively even distribution of the amino acids in skin,[14,56,57] and this explains why the protein binding alerts for skin sensitization tend to cover the entire range of hard/soft electrophilicity. In other words, there is no requirement for hard/soft discrimination of the alerts in order to cause skin sensitization. In contrast, the protein binding alerts for respiratory sensitization were found to cover the "harder part" of the electrophilicity scale, and this could be reasoned as consistent with the predominant availability of

Table 7. Summary of Predictions when applying the Pipeline Model with the DPRA-Lysine Reactivity Component[a]

	predictions		total # of chemicals	% of positive predictions	active due to				
					peptide reactivity	cross-linking	hard el.	ionic interactions	radical interactions
120 RS (positives)	positive as p		67	89.2%	20	23	58	1	
	positive as m	AU	15		10	3	9		6
		lung	25		12	9	24		
	negative		13 (FN)						
82 not RS (negatives)	positive as p		13	47.6% (FP)	1	1	12		
	positive as m	AU	4		2		2		3
		lung	22		17	3	22		
	negative		43						

[a]Notes: AU = autoxidation, RS = respiratory sensitizer, Hard el. = hard electrophile, p = parent, and m = metabolism.

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Table 8. Summary Predictions when applying the Pipeline Model without the Peptide Reactivity Component[a]

					active due to			
	predictions		total # of chemicals	% of positive predictions	cross-linking	hard el.	ionic interactions	radical interactions
120 RS (positives)	positive as p		62	84.2%	24	61	1	
	positive as m	AU	12		3	9		6
		lung	27		10	27		
	negative		19 (FN)					
82 not RS (negatives)	positive as p		12	46.3% (FP)	1	12		
	positive as m	AU	4			2		3
		lung	22		3	22		
	negative		44					

[a]Notes: AU = autoxidation, RS = respiratory sensitizer, Hard el. = hard electrophile, p = parent, and m = metabolism.

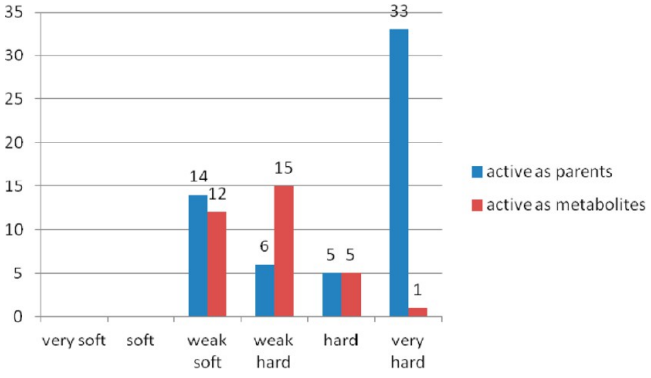

Figure 9. Distribution of 91 RS active as hard electrophiles (58 active as parents and 33 as metabolites).

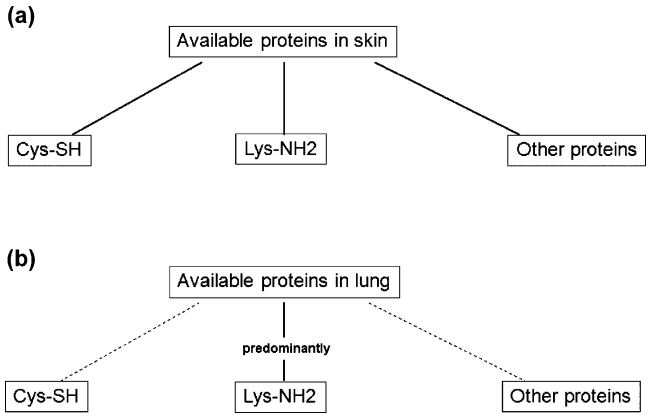

Figure 10. (a) Availability of protein amino acids in skin. (b) Availability of protein amino acids in the lung.

NH2-lysine amino acid in the lung[13,14,17] – having the hardest nucleophilic site among the other amino acids.

For example, tetrachlorophthalic anhydride has two alerting groups for protein binding, and conceivably, it could bind to protein by two different mechanisms, either by an acylation mechanism due to the anhydride group or by a SNAr due to activated haloarene functionality. Calculation of net atomic charges shows that the carbonyl atom in the acid anhydride group is a very hard electrophilic center, and the carbon atom in the haloarene group is a weak soft electrophilic center (Figure 11). Thus, the respiratory sensitization potential of tetrachlorophthalic anhydride is most likely to be driven by the presence of the anhydride group which contains a hard electrophilic center. This is plausible given the Michaelis–Menten kinetics

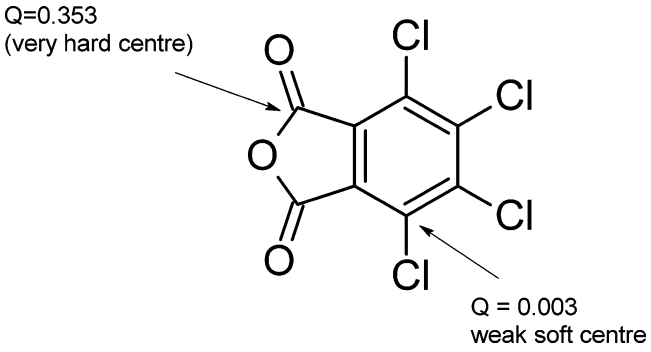

Figure 11. Calculations of net atomic charge [*Q*, a.u.] for the electrophilic centers in protein binding alerting groups of tetrachlorophthalic anhydride.

found in catalyzed solvolysis reactions of tetrachlorophthalic anhydride.[58]

Other Types of Interactions. As noted earlier, two "other types of interactions" were encoded into a component that is present in the two pipelines: ionic interaction and free radical interactions. Within the test set, 1 chemical, chlorhexidine [CAS no. 55-56-1] was identified as positive due to an ionic interaction. The alerting group and its application to chlorhexidine is shown in Figure 12.

Six chemicals were found to be correctly predicted as active and were assigned a free radical interaction as the proposed rationale. The six chemicals were eugenol [CAS no. 97-53-0], 3-carene [CAS no. 13466-78-9], abietic acid [CAS no. 514-10-3], diacetyl morphine [CAS no. 561-27-3], *N*-methylmorpholamine [CAS no. 109-02-4], and morphine hydrochloride [CAS no. 52-26-6]. Eugenol has been studied extensively for its skin sensitization potential and shown to be a weak sensitizer in the LLNA. In previous evaluations by Roberts et al.,[45,54] two explanations were proposed to rationalize its skin sensitization potential. On the one hand, eugenol could be subject to oxidation to form a quinone methide structure capable of reacting by a Michael addition reaction. On the other hand, oxidation to result in another hydroxy group ortho to the original hydroxy group could be subject to direct binding to protein via attack of a protein-centered radical. The rationales that were put forward are reflected in the outcomes of the various components for respiratory sensitization. The DPRA-lysine reactivity component triggered no alerts, and equally, there were no flags for cross-linking. The hard/soft electrophilicity component and the "other interactions" flagged activity upon oxidation (autoxidation). In view of Michael acceptors being soft electrophiles and respiratory sensitizers tending to favor hard electrophiles, it is possible that

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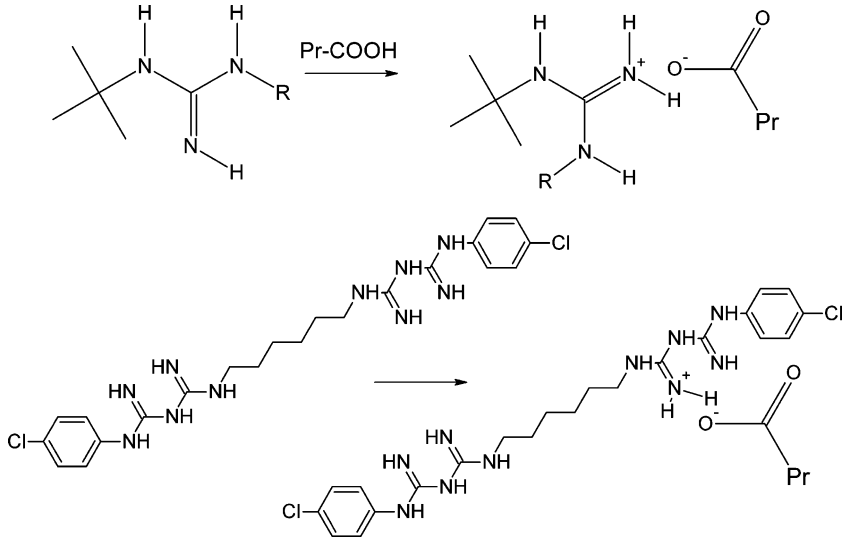

Figure 12. Ionic type of interaction and its application to chlorhexidine [CAS no. 55-56-1].

the free radical reaction mechanism could predominate in accounting for the respiratory sensitization potential of eugenol. Abietic acid [CAS no. 514-10-3], another weak sensitizer in the LLNA has also been discussed in Roberts et al.[45] Only a free radical reaction scheme was put forward to rationalize its sensitizing potential, which is also mirrored by the outcomes of the components in the pipeline here. 3-Carene [CAS no. 13466-78-9] is postulated to react by both electrophilic and free radical mechanisms based on the outcomes of the DPRA-lysine reactivity (non-low reactivity), the hard/soft electrophilicity component, and the other interactions component. Oxidation of 3-carene could result in 3 different products: an epoxide across the double bond, which could be then subjected to a ring-opening reaction, a SN2 reaction scheme, formation of an α,β-carbonyl to facilitate Michael addition, or formation of a quinone capable of Michael addition. All three of these reaction schemes are driven by soft electrophilic centers. The reaction scheme for the proposed mechanism for 3-carene [CAS no. 13466-78-9] by the free radical route may be more likely and is presented in Figure 13. By chemical inspection, diacetyl morphine [CAS no. 561-27-3] reveals reaction schemes via both electrophilic and free radical routes, the former as parent and the latter upon autoxidation. Diacetyl morphine contains 2 esters, an alkyl ester capable of reaction via SN2 and an activated aryl ester capable of an acylation reaction scheme. The acylation route is more likely to be driving the respiratory sensitization rather than the SN2 route. The free radical route might also be implicated in the response. Morphine hydrochloride [CAS no. 52-26-6] is postulated to undergo a free radical route, but an oxidation reaction to produce a cyclohexanone ring system capable of acting as an electrophile has also been proposed.[15]

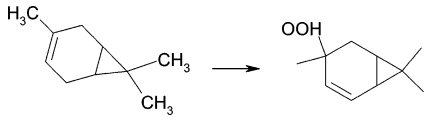

Figure 13. Radical interaction scheme as applied to 3-carene [CAS no. 13466-78-9].

Substances which are Respiratory Sensitizers but are predicted to be nonactive by the Pipeline Model with the DPRA-Lysine Component. Thirteen substances were found to be false negatives. The substances are listed in Table 9. Eleven of the substances were included in the data set evaluated by Enoch et al.[15] Acetic acid [CAS no. 64-19-7], adipic acid [CAS no. 124-04-9], and dodecanedioic acid [CAS no. 693-23-2] were considered respiratory sensitizers, but perhaps, they are in reality false positives on account of their irritation effects. Some irritants have been known to give rise to positive skin sensitization results, and the same might apply in this case. Certainly this was the rationale Enoch et al.[15] put forward to account for the unexplained behavior of these acids.

Table 9. Substances that were False Negatives by the Pipeline Model with the DPRA-Lysine Reactivity Component

CAS no.	name
64-19-7	acetic acid
124-04-9	adipic acid
117-81-7	dioctylphthalate
693-23-2	dodecanedioic acid
141-43-5	ethanolamine
98-00-0	furfuryl alcohol
860-22-0	indigotine
54-85-3	isoniazid
80-62-6	methyl methacrylate
305-80-6	Pauli's reagent (4-diazobenzenesulphonic acid)
15318-45-3	thiamphenicol
17095-24-8	Rifazol black GR (reactive black 5)
100-34-5	phenyl diazonium chloride

Dioctylphthalate [CAS no. 117-81-7] is not an electrophile. A number of phthalates were included in the set of substances that were experimentally not respiratory sensitizers. Enoch et al.[15] suggested that an impurity could be the root cause for the respiratory sensitization effect. Presence of phthalic anhydride a known respiratory sensitizer as an impurity in dioctylphthalate could be implicated in a positive call. Ethanolamine [CAS no. 141-43-5] did not trigger any alerts as such, but Roberts et al.[54] has categorized a number of aliphatic amines as pro-Schiff base formers on account of their potential to be oxidized whereby CH-N is converted into C═O. Ethanolamine could be converted to glyoxal [CAS no. 107-22-2], which is a strong skin sensitizer and may account for the positive respiratory sensitization outcome. Furfuryl alcohol [CAS no. 98-00-0] may be

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oxidized to its corresponding aldehyde, furfuryl aldehyde [CAS no. 98-01-1], which is a Schiff base former as discussed by Enoch et al.[15] Indigotine [CAS no. 860-22-0] could react via a Michael addition route on account of its α,β-unsaturated carbonyl as shown in Figure 14. Isoniazid [CAS no. 54-85-3] is a hydrazine

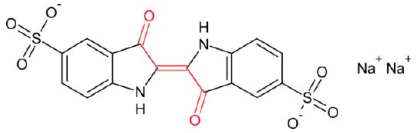

Figure 14. Electrophilic sites for Michael addition in indigotine.

derivative capable of reaction via a SN2 reaction with N2 as a leaving group as outlined in Enoch et al.[15] Methyl methacrylate [CAS no. 80-62-6] can react as a Michael acceptor due to the methacrylate moiety. For methyl methacrylate to be implicated as a respiratory sensitizer is surprising given that it is a weak skin sensitizer. In the LLNA, many acrylates and methacrylates are much less potent than would be expected from their reactivity. This is believed to be in part due to their volatility and in part due to their rapid polymerization (with loss of electrophilic reactivity) when exposed to air. In the case of respiratory sensitization, it is expected that methyl methacrylate will exist in the vapor phase and therefore will not be subjected to such extensive polymerization. That said, a critical review of methyl methacrylate in 2011[59] concluded that it was not a respiratory sensitizer on the basis of a weight of evidence approach.

Pauli's reagent (4-diazobenzenesulphonic acid) [CAS no. 305-80-6] has been proposed to react via a SNAr reaction,[15] which could account for its respiratory sensitizing effects. Thiamphenicol [CAS no. 15318-45-3] contains a dichloroacetamide moiety that can undergo hydrolysis to produce a glyoxal type species, which can react as a Schiff base former.[15]

Rifazol black GR (reactive black 5) [CAS no. 17095-24-8] can tautomerize to produce a quinone imine, which is capable of reaction via Michael addition (see Figure 15).

Phenyl diazonium chloride [CAS no. 100-34-5] is an arenediazonium cation that can react directly with cellular macromolecules via an SN2 reaction, which may explain its

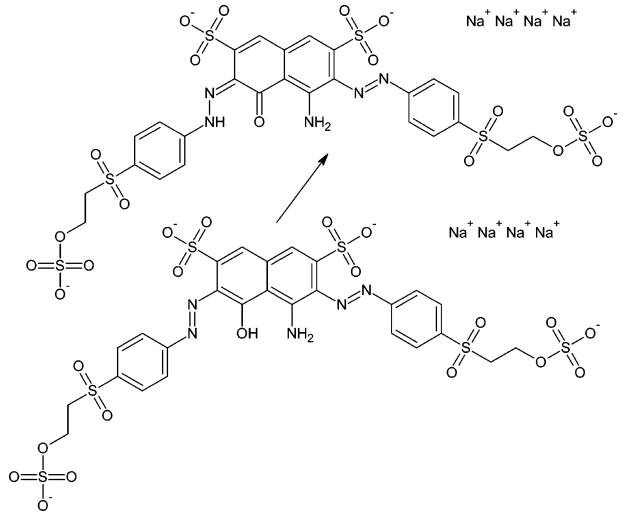

Figure 15. Tautomerization of Rifazol black GR to form a quinone-imine.

respiratory sensitization outcome. Such cations are very hard electrophiles.

Of the 13 substances that were incorrectly identified as non-sensitizing, 4 of these might be false positives. For the remaining nine substances, justifications have been proposed to explain why these substances are experimental respiratory sensitizers.

Substances which are Respiratory Sensitizers but are predicted to be nonactive by the Pipeline Model without the DPRA-Lysine Component. In addition to the 13 false negatives that were identified by the pipeline model above, 6 additional chemicals were identified that were false negatives when the DPRA-lysine component was not implemented. The six chemicals are listed in Table 10. Ethylene oxide

Table 10. Additional False Negatives as determined by the Pipeline Model without the DPRA-Lysine Reactivity Component

CAS no.	name
24447-78-7	ethoxylated bisphenol A diacrylate
75-21-8	ethylene oxide
86-54-4	hydralazine
123-31-9	hydroquinone
15625-89-5	trimethylolpropane triacrylate
1260-17-9	carminic acid

[CAS no. 75-21-8] can undergo a SN2 ring-opening reaction as has been postulated for skin sensitization. Hydralazine [CAS no. 86-54-4] acts as a hydrazine derivative in the same fashion as has already been described for isoniazid [CAS no. 54-85-3]. Hydroquinone [CAS no. 123-31-9] will be readily oxidized to its strongly reacting benzoquinone, which is an extreme skin sensitizer capable of Michael addition reactions and sufficiently reactive to target hard nucleophiles. Ethoxylated bisphenol A diacrylate [CAS no. 24447-78-7] and trimethylolpropane triacrylate [CAS no. 15625-89-5] are also capable of reacting via a Michael addition reaction. In addition, both contain multiple acrylate units allowing for cross-linking with protein chains.[15] Conceivably, the acrylates could oxidize across the double bond to form epoxides, which might also account for their sensitizing ability. Carminic acid [CAS no. 1260-17-9] could potentially oxidize to form a quinone moiety that is capable of a Michael addition reaction as shown in Figure 16.

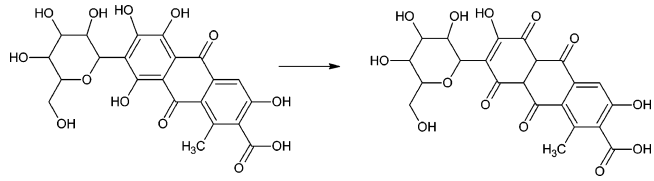

Figure 16. Oxidation of carminic acid.

Substances that were incorrectly assigned could act by reaction mechanisms more likely favored by soft electrophiles. It is worth remembering that while the basic principle of HSAB is that the reactions between partners with same type of electrophilicity (hard−hard and soft−soft) are energetically favored, soft electrophiles can still interact with hard nucleophiles and vice versa.

Substances which are not Respiratory Sensitizers but are predicted to be active by the Pipeline Models. Thirty-nine chemicals were predicted to be respiratory sensitizers by the

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pipeline model with DPRA-lysine but were categorized as nonsensitizing on the basis of no clinical reports of occupational asthma. The full data set of 82 chemicals were the same as used by Enoch et al.[15] and Jarvis et al.[11] Of the 39 chemicals, 13 were expected to be respiratory sensitizers as parents, 4 were expected to be sensitizers after autoxidation, and 22 on the basis of metabolic activation. In the pipeline without the DPRA-lysine reactivity model, 38 chemicals were found to be false positives where 12 chemicals were expected to be respiratory sensitizers as parents.

The four substances expected to be sensitizers after autoxidation are listed in Table 11 together with the oxidation products implicated and any literature references substantiating the alerts identified. The four chemicals are the same irrespective of the pipeline model applied. 2-(2-Methoxyethoxy)ethanol [CAS no. 111-77-3] and 2,6-di-*tert*-butyl-*p*-cresol [CAS no. 128-37-0] may give rise to hydroperoxides that can be implicated in sensitization. Pyrocatechol [CAS no. 120-80-9] could become oxidized to form an *ortho*-quinone capable of Michael addition similar to how 1,4-dihydroxybenzene would react. Autoxidation of nicotine [CAS no. 54-11-5] may result in the release of formaldehyde, which is a respiratory sensitizer. Plausible reaction mechanisms can be proposed for the four substances despite the fact that no cases of respiratory sensitization have been reported. While they have the potential to drive effects, it could be that the rate of oxidation is low such that autoxidation is too slow to make these substances sensitizers. Pyrocatechol's lack of respiratory sensitization is surprising given its positive skin sensitization outcome in guinea pigs (data from the OECD Toolbox, v3.1) and its similarity to hydroquinone which is categorized as a respiratory sensitizer. 2-(2-Methoxyethoxy)ethanol [CAS no. 111-77-3] and 2,6-di-*tert*-butyl-*p*-cresol [CAS no. 128-37-0] were both negative in guinea pig tests based on data reported in the OECD Toolbox.

Twenty-two chemicals were expected to be respiratory sensitizers on account of active metabolites formed as a result of oxidation. Four of these 22 compounds which are representative of the types of transformations likely are described in more detail in Table 12 in terms of proposing the metabolites that could be formed and hence could be implicated for potential respiratory sensitization. Acrylonitrile [CAS no. 107-13-1] could oxidize across its double bond to form an epoxide that is capable of a SN2 ring-opening reaction. Ethanol [CAS no. 64-17-5] and butan-1-ol [CAS no. 71-36-3] could form their respective aldehydes upon oxidation. Halothane [CAS no. 151-67-7] can be oxidized to form an acyl chloride, which is then capable of an acylation mechanism. The metabolic pathway is shown in Figure 17.[60] It is likely that the rate of oxidation is too low to render these compounds sensitizers. On the basis of indirect evidence, it has been suggested that halothane could fulfill a sensitization-facilitating role, if not for itself, at least for coadministered innocuous antigens.[61]

Table 11. Negative RS Chemicals Producing Autoxidation (AU) Activated Products (having a protein binding alert)

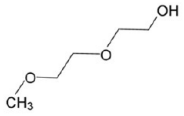
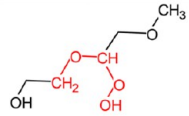
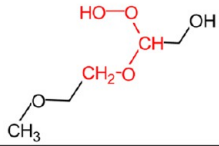
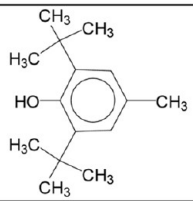
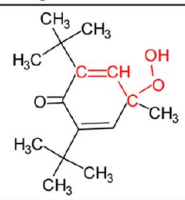
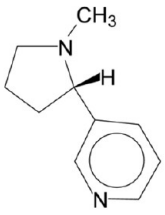
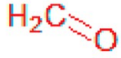
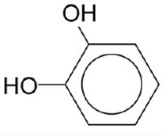
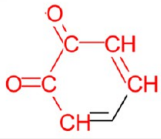
Name	parent	AU documented products having protein binding alert (red highlighted)	Literature	Alert performance (number of chemicals in local training sets)
2-(2-Methoxyethoxy)ethanol [111-77-3]			Reference: 67	100% (0 as p; 6 as AU products), High performance
				100% (0 as p; 6 as AU products), High performance
2,6-Di-tert-butyl-p-cresol [128-37-0]			References: 68-69	100% (0 as p; 6 as AU products), High performance
Nicotine [54-11-5]			Reference: 70	100 % (3 as p; 0 as AU products), Undetermined alert
Pyrocatechol [120-80-9]			References: 71-72	100% (0 as p; 12 as AU products), High performance

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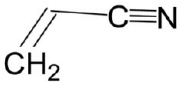
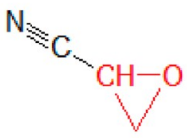
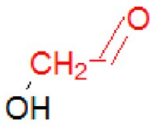
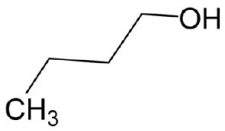
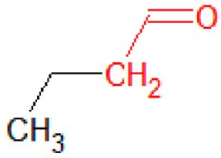
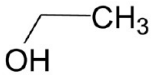
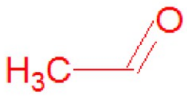
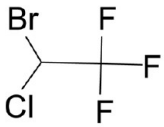
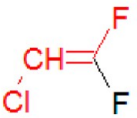
Table 12. Negative RS Chemicals activated by Lung Metabolism

Name	parent	Lung metabolism documented products having protein binding alert (red highlighted)	Literature	Alert performance (number of chemicals in local training sets)
Acrylonitrile [107-13-1]			Reference: 73	100% (3 as p; 3 as m), High performance
				100 % (3 as p; 19 as m), High performance
Butan-1-ol [71-36-3]			Reference: 74	100 % (3 as p; 19 as m), High performance
Ethanol [64-17-5]			Reference: 75	100 % (3 as p; 19 as m), High performance
Halothane [151-67-7]			Reference: 76	100 % (2 as p; 2 as m), Undetermined alert

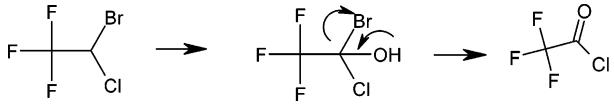


Figure 17. Metabolism of halothane [CAS no. 151-67-7].

Thirteen nonsensitizing chemicals were categorized as active as parents. They are listed in Table 13. 2-Hydroxypropyl acrylate [CAS no. 999-61-1] was identified by the pipeline with the additional lysine reactivity component only. As an acrylate, it would be expected to react by Michael addition. Chloroacetaldehyde [CAS no. 107-20-0] is an activated Schiff base former on account of the chlorine, although it is also an activated (by the carbonyl) as a polar SN2 reactor. *p*-Toluenesulphonyl chloride [CAS no. 98-59-9] can react via an acylation mechanism. Diethyl sulfate [CAS no. 64-67-5] is a moderate skin sensitizer capable of acting via a SN2 polar mechanism. Bromacil [CAS no. 314-40-9] triggers two alerts: an acylating route on account of the *N*-acylamide moiety and a Michael addition through α,β-carbonyl compounds. Propoxur [CAS no. 114-26-1] and captan [CAS no.

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Table 13. Negative RS Chemicals Active as Parents

CAS no.	name
999-61-1	2-hydroxypropyl acrylate*
78-59-1	3,5,5-trimethylcyclohex-2-enone
314-40-9	bromacil (ISO)
133-06-2	captan (ISO)
107-20-0	chloroacetaldehyde
2921-88-2	chloropyrifos (ISO)
64-67-5	diethyl sulfate
115-29-7	endosulfan
121-75-5	malathion
298−02−2	phorate
1918-02-1	picloram (ISO)
114-26-1	propoxur
98-59-9	*p*-toluenesulphonyl chloride

*Identified by the pipeline with DPRA-lysine only.

Table 14. Summary of Predictions when applying the Pipeline Model with the DPRA-Lysine Reactivity Component

		predicted positive			
original data	after QA of the data	as p	as AU	as lung m	predicted negative
120 positive RS chemicals	41 are classified as RS	32	2	6	1
	6 are classified as "potential RS"	4	2		
	73 have no RS data found	31	11	19	12
82 negative RS chemicals	2 are classified as RS			1	1
	2 are classified as "potential RS"			1	1
	78 have no RS data found	13	4	20	41

133-06-2] trigger acylation by a carbamate mechanism. 3,5,5-Trimethylcyclohex-2-enone [CAS no. 78-59-1] flags a Michael addition route on account of an α,β-unsaturated carbonyl. Chloropyrifos [CAS no. 2921-88-2], malathion [CAS no. 121-75-5], and phorate [CAS no. 298-02-2] all trigger nucleophilic substitution on the thiophosphate alert, whereas endosulfan [CAS no. 115-29-7] flags a nucleophilic substitution at the vinylic (sp2) carbon atom. Picloram [CAS no. 1918-02-1] triggers a SNAr mechanism for activated halo-pyridine derivatives.

Both pipeline models suffer from a large number of false positives. Plausible reaction mechanisms can be postulated for all these chemicals. The inactives are all control substances that Enoch et al.[15] took from workplace exposure limit tables, which exhibited no evidence of respiratory sensitization effects in workers. The lack of a definitive test method makes evaluation of these inactives a particular challenge. The quality of experimental data for the identified false positives could be questioned; alternatively, identifying secondary effects which detoxify these chemicals could be investigated further.

Quality Assurance (QA) Exercise for the Test Set. The performance characteristics, in particular the high number of false positives, prompted a re-evaluation of the data set of 202 chemicals that was used to test out the pipeline models. For each of the 202 chemicals, a more extensive search was undertaken to identify additional information that would either support the original categorizations of sensitizing or nonsensitizing or provide plausible rationales to account for the original call. This quality assurance step made use of the scientific literature,

Table 15. Summary of Predictions when applying the Pipeline Model without the DPRA-Lysine Reactivity Component

		predicted positive			
original data	after QA of the data	as p	as AU	as lung m	predicted negative
120 positive RS chemicals	41 are classified as RS	32	2	6	1
	6 are classified as "potential RS"	4		1	1
	73 have no RS data found	26	10	20	17
82 negative RS chemicals	2 are classified as RS			1	1
	2 are classified as "potential RS"			1	1
	78 have no RS data found	12	4	20	42

publically available databases such as those offered by the US National Library of Medicine, and material safety datasheets (MSDSs) from sources such as Sigma Aldrich. For the 120 substances that were originally assigned as positive respiratory sensitizers, 41 substances were found to be classified within the EU as respiratory sensitizers and associated with a risk phrase of R42, and 6 were categorized as "potential respiratory sensitizers" on account of information provided in a MSDS. For the remaining 73 substances, no additional respiratory sensitization information was found, but of those, 59 were either classified as respiratory irritants, skin irritants, or sensitizers, or were flagged as potential respiratory irritants. For the 82 negative respiratory sensitizers, 2 were found to be classified in the EU as R42, and 2 were categorized as potential respiratory sensitizers by MSDSs, and for the remaining 78 substances, no additional respiratory sensitization information could be found. Fifty-nine of these 78 were either classified as respiratory irritants, potential respiratory irritants, or skin irritants/sensitizers. For the 21 substances left, no sensitization or irritation data could be found. Thus, as a result of this review, 51 substances of the original test set of 202 were confirmed as "true" respiratory sensitizers. The performance of the pipeline models to correctly identify these 51 respiratory sensitizers was evaluated. For the pipeline accounting for DPRA-lysine reactivity, 36 substances were correctly predicted as parents, 4 were correctly predicted after autoxidation, 8 were correctly predicted after metabolism, and 3 substances were predicted as false negatives, i.e., 94.1% of positive predictions. For the pipeline model without the DPRA-lysine reactivity, the performance was similar with 36 substances correctly predicted as parents, 2 correctly predicted after autoxidation, and 9 after metabolism, and 4 substances were predicted as false negatives, i.e., 92.2% of positive predictions. Tables 14 and 15 reflect the impact the QA of the data has made on the prediction profiles. Table 16 shows the summary of performance characteristics. The performance characteristics are improved if only the "confirmed" respiratory sensitizers are considered; otherwise, there is little apparent difference between that reported in Tables 6 and 7, though some interesting insights were revealed as a result of the QA exercise.

Chlorhexidine was identified as positive due to an ionic interaction; no additional respiratory sensitization was found during the QA review though a classification for respiratory irritation was noted. Eugenol [CAS no. 97-53-0], 3-carene [CAS no. 13466-78-9], abietic acid [CAS no. 514-10-3], diacetyl morphine [CAS no. 561-27-3], *N*-methylmorpholamine [CAS no. 109-02-4], and morphine hydrochloride [CAS no. 52-26-6] were

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Table 16. Summary of Predictions when applying Both Pipeline Models

revised data	predictions		with peptide reactivity component: total # of chemicals	% of positive predictions	without peptide reactivity component: total # of chemicals	% of positive predictions
124 RS (positives)	positive as p		67	87.9%	62	83.1%
	positive as m	AU	15		12	
		lung	27		29	
	negative		15 (FN)		21	
78 not RS (negatives)	positive as p		13	47.4% (FP)	12	46.1% (FP)
	positive as m	AU	4		4	
		lung	20		20	
	negative		41		42	

Table 17. Structural Boundaries associated with the Respiratory Sensitizing effect exerted by the Epoxides, Aziridines, and Sulfuranes Alert

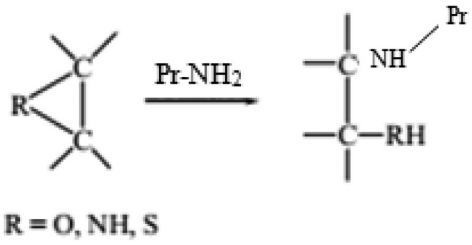
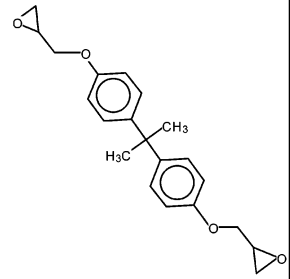
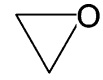
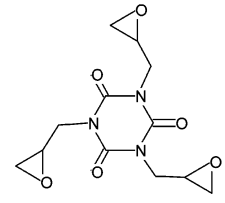
Structural boundary	
	

Table 18. Epoxides with Positive RS Effect as Parents

#	CAS	Name	2D
1	1675-54-3	Diglycidyl ether of bisphenol A	
2	75-21-8	Ethylene oxide	
3	2451-62-9	Triglycidyl isocyanurate	

predicted as positive where a free radical mechanism was primarily proposed to account for their activity. Eugenol has been confirmed to be a respiratory sensitizer as well as a skin sensitizer based on its classifications within the EU following the QA review. No information could be found to further substantiate the activity of morphine hydrochloride. While 3-carene and abietic acid are both skin sensitizers, they both have been classified as respiratory irritants. Diacetyl morphine and *N*-methylmorpholamine have been flagged as potential respiratory irritants. Thus, 4 of the 6 substances categorized originally as respiratory sensitizers are at least known or suspected respiratory irritants.

Of the false negatives identified in Table 9, acetic acid, adipic acid and dodecanediodic acid were discussed as false positives on account of potential irritation effects. Following the QA, respiratory irritant classifications have been found for these 3 substances. Furthermore, all of the substances listed in Tables 9 and 10 (with the exception of phenyl diazonium chloride and ethoxylated bisphenol A diacrylate for which no additional information was found) were associated with respiratory irritation information.

These findings pose an interesting question: to what extent are the reported calls confounded by substances that are respiratory

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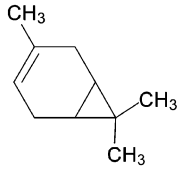
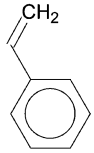
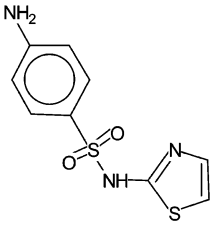
Table 19. Lung Metabolism Activated RS

#	CAS	Name	2D
1	13466-78-9	3-Carene	
2	100-42-5	Styrene	
3	72-14-0	Sulfathiazole	

irritants rather than true immunological respiratory sensitizers, or to what extent can irritation facilitate sensitization as discussed in ref 61? Despite our best efforts to substantiate the test set of positives, only 51 substances could be additionally confirmed as respiratory sensitizers, with about half of those remaining being associated with some irritant activity. Irritant activity would have likely contributed to the reported sensitizing potential in the original reported calls. Since irritation may also be a useful predictor of sensitizing capacity, it will be investigated further as a potential future component in the pipelines developed.

Despite the QA performed, the model still culminated in a high number of false positives. Further investigations were carried out to consider what potential refinements could be made to the components to improve the model specificity without compromising the sensitivity. The impact of limiting the reactivity alerts to only those supported by experimental sensitizing data was explored as well as an evaluation of what if any redundancy existed between transformations encoded within the AU and lung metabolism simulators. The results of these preliminary investigations appeared promising, and a significant reduction in the number of false positives was observed. These refinements will be factored in as part of the ongoing developments of this model.

Alert Reliability. As with any model, in accordance with the OECD Validation Principles,[62] having a means of assessing the robustness of the structural alerts that form the basis of the components within the pipeline is critical. While there has been considerable progress in defining and characterizing applicability domains for QSARs,[63,64] the same perhaps cannot be said for structural alerts. Within the study here, the concept of alert reliability was introduced to provide additional confidence for the robustness of a given prediction based on the structural alerts identified. Three key attributes were defined to help in evaluating alert reliability: (1) the number of compounds (n) in the local training set (compounds with observed data having the same structural functionality believed to cause the toxic effect); (2) alert performance, defined as the ratio between the correctly predicted compounds over the total number of compounds in the local training set; and (3) mechanistic justification of toxic end point exerted by the alert.

The specific reliability thresholds applied in the pipeline model are as follows: (1) high performance alert based on performance $\geq$60%; $n \geq 5$, mechanistic justification availability; (2) low performance alert based on performance $\leq$60%; $n \geq 5$, mechanistic justification availability; (3) undetermined alerts based on $n < 5$ mechanistic justification availability but does not take into account the performance; and (4) undetermined theoretical alerts based on mechanistic justification of the toxic end-point.

Alert reliability for the alert epoxides, aziridines, and sulfuranes as shown in Table 17 is summarized as follows by way of an illustrative example: (1) the number of compounds in the local training set is 6; (2) 3 are active as parents (Table 18), and 3 are activated as a result of lung metabolism (Table 19). Correctly predicted compounds are as follows: 3 out of 3 respiratory sensitizers are correctly predicted as parents; 3 out of 3 respiratory sensitizers are correctly predicted as metabolites; and alert performance = (*correctly predicted compounds*)/(*total number of compounds in training set*) = 6/6 = 100 %. (3) Mechanistic justification availability.

The epoxides, aziridines, and sulfuranes alert in the respiratory sensitization model would be categorized as possessing "high performance". Alert reliability will be discussed in more detail in a separate publication.

CONCLUSIONS

In the absence of a definitive test method, using an AOP type framework to characterize early events that lead to the induction of respiratory sensitization and building on existing knowledge of skin sensitization can form a pragmatic basis for screening and grouping chemicals for their respiratory sensitization potential. Here, a pipeline was developed based on the understanding of the initial events that drive respiratory sensitization rather than being parametrized by an external training set, as is typical in classical QSAR studies.

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An analysis of protein reactivity of chemicals eliciting skin and respiratory sensitization as well as the nucleophilic diversity of the skin and lung was performed, which showed that while skin sensitizers cover the entire scale of hard/soft electrophilicity, respiratory sensitizers tend to favor the hardest part of this scale. Thus, one could expect all respiratory sensitizers to be skin sensitizers but expect the converse not to be true.

This study explored the similarities between skin and respiratory sensitization in terms of reaction chemistry principles as part of a pipeline approach for modeling respiratory sensitization. Components were derived to encode knowledge about hard electrophiles based on quantum chemical calculations and *in chemico* data from peptide reactivity studies.

The pipeline approach also considered the potential of chemicals to be activated either chemically and/or metabolically to exert their effect. Components for simulating autoxidation and lung metabolism were developed to address such activation processes. Other mechanisms for inducing respiratory sensitization including cross-linking and free radical routes were also implemented as components. Two pipelines resulted, which encompassed a series of components which when integrated together provide an indicator of likely respiratory sensitization potential. The pipelines were profiled using a test set of 202 chemicals; 82 control chemicals; and 120 respiratory sensitizers. This test set was intended to provide some measure of performance and highlighted some of the shortcomings of modeling respiratory sensitization in the absence of a definite test method. That said, both pipelines were found to be very promising in terms of identifying potential respiratory sensitizers. Outliers were rationalized in terms of their likely reaction chemistry, physicochemical characteristics, or metabolism. A QA of the test set performed demonstrated the role that irritation may have played in either confounding or facilitating respiratory sensitization potential. Future work will involve refining the lysine reactivity component as new information is generated, collecting more data to improve the lung metabolism simulator, exploring physicochemical thresholds, and investigating the feasibility of a new component to identify irritant activity.

■ ASSOCIATED CONTENT

Supporting Information

Underlying training sets for the lung metabolism and autoxidation simulators. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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The authors declare no competing financial interest.

■ ABBREVIATIONS

d_a, aerodynamic diameter; AU, autoxidation; AM1, Austin model 1; AOP, adverse outcome pathway; BP, boiling point; Derek; deductive estimation of risk from existing knowledge; DPRA, direct peptide reactivity assay; EU, European Union; GSH, glutathione; HSAB, hard and soft acids and bases; HPLC-UV, ultraviolet high-performance liquid chromatography; IES, integrated evaluation strategy; ITS, integrated testing strategy; LLNA, local lymph node assay; LMW, low molecular weight; MOs, molecular orbitals; MW, molecular weight; MOPAC, molecular orbital package; MCASE, MultiCASE; Q, net atomic charge; OECD, Organisation for Economic Co-operation and Development; PAH, polyaromatic hydrocarbon; PPRA, peroxidase peptide reactivity assay; (Q)SAR, (q)uantitative structure−activity relationship; REACH, Registration Evaluation Authorisation and Restriction of Chemicals; RI, respiratory irritant; RS, respiratory sensitizer; SNAr, substitution nucleophilic aromatic; SN1, substitution nucleophilic (unimolecular); SN2, substitution nucleophilic (bimolecular); SS, skin sensitizer; S_μ^E, electrophilic superdelocalizability; S_μ^N, nucleophilic superdelocalizability; TIMES-SS, tissue metabolism simulator for skin sensitization; VP, vapor pressure; WoE, weight of evidence; WS, water solubility

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