

**THE INCORPORATION OF LYSINE INTO THE PEROXIDASE PEPTIDE REACTIVITY
ASSAY FOR SKIN SENSITIZATION ASSESSMENTS**

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ABBREVIATED TITLE: Peptide reactivity of pro-haptens

SUGGESTED SECTION: *In Vitro* Toxicology and Alternative Testing

ABBREVIATIONS:

ACD, Allergic Contact dermatitis; DPRA, Direct Peptide Reactivity Assay; HPLC/MS/MS, high-performance liquid chromatography and tandem mass spectrometry; HRP/P, horseradish peroxidase/hydrogen peroxide.

ABSTRACT

To establish further a practical quantitative *in chemico* reactivity assay for screening contact allergens, lysine peptide was incorporated into an LC/MS/MS-based assay for reactivity assessments of hapten and pre-/pro-hapten chemical sensitizers. Loss of peptide was determined following 24 h co-incubation with test chemical using a concentration-response study design. A total of 70 chemicals were tested in discrete reactions with cysteine or lysine peptide, in the presence and absence of horseradish peroxidase-hydrogen peroxide (HRP/P) oxidation system. An empirically derived prediction model for discriminating sensitizers from non-sensitizers resulted in an accuracy of 83% for 26 haptens, 19 pre-/pro-haptens and 25 non-sensitizers. Four sensitizers were shown to selectively react with lysine including two strong/extreme and two weak sensitizers. In addition, seven sensitizers were identified as having higher reactivity towards lysine compared to cysteine. The majority of sensitizing chemicals (27/45) were reactive towards both cysteine and lysine peptides. An estimate of the relative reactivity potency was determined based on the concentration of test chemical that depletes peptide at or above a threshold positive value. Here, we report the use of EC15 as one example to illustrate the use of the model for screening the skin sensitization potential of novel chemicals. Results from this initial assessment highlight the utility of lysine for assessing a chemical's potential to elicit sensitization reactions or induce hypersensitivity. This approach has the potential to qualitatively and quantitatively evaluate an important mechanism in contact allergy for hazard and quantitative risk assessments without animal testing.

KEYWORDS: Allergens; alternatives; skin sensitization; peptide reactivity;

INTRODUCTION

Allergic contact dermatitis (ACD) is the clinical manifestation of skin sensitization and an important toxicological endpoint for occupational and consumer health risk assessments. *In vivo* models for evaluating the potential of novel materials to act as skin allergens are used routinely and globally accepted for use in identifying hazard. Based on the murine Local Lymph Node Assay (LLNA), both hazard and potency information is used for quantitative risk assessments via determination of the concentration that produces a threshold positive response and classification of the relative skin sensitizing potency (Kimber et al., 1994; Dearman et al., 1999; Gerberick et al., 2000; Kimber et al., 2002; Basketter et al., 2002). The forthcoming ban on animal testing of cosmetic and toiletry ingredients in Western Europe will essentially make *in vivo* models obsolete, however, and require the cosmetic industry to rely solely on alternatives for assessing risk in contact allergy. Non-animal-based prediction models have therefore been a major focus of the toxicological community for more than a decade.

The ability of a chemical to react with skin proteins is thought to play a key role in the development of skin sensitization (Dupuis and Benezra, 1982; Gerberick et al, 2008; Landsteiner and Jacobs, 1936; Lepoittevin et al, 1998). Contact allergens are generally low molecular weight molecules that have the ability to penetrate the stratum corneum and covalently modify skin proteins through a variety of reactivity mechanisms. Reaction products are immunogenic and stimulate the migration and maturation of Langerhans cells from the epidermis to local skin-draining lymph nodes for antigen presentation to naive T cells, resulting in the clonal expansion of antigen-specific memory T cells (Ryan et al., 2001).

Sensitizers (or their metabolites) are generally electrophilic and reactive towards nucleophilic sites on proteins. This feature has been exploited by investigators that are interested in developing non-animal approaches for evaluating the skin sensitization potential of novel materials based on reactivity with nucleophile-containing peptides. Although there are other nucleophilic amino acids, the ϵ -NH₂ group of lysine and the -SH group of cysteine are relatively strong nucleophiles and often cited in the literature for assessing hapten reactivity (Ahlfors et al, 2003; Alvarez-Sanchez et al, 2003; Dupuis and Benezra, 1982; Lepoittevin et al, 1998; Meschkat et al, 2001).

In the Direct Peptide Reactivity Assay (DPRA), peptide depletion at a single peptide and test chemical concentration following 24 h co-incubation is determined (Gerberick et al., 2004). A prediction accuracy of 89% was demonstrated with a data set that included 82 chemicals (Gerberick et al., 2007). Some sensitizers are non-electrophilic and require activation to protein reactive intermediates (Gerberick et al., 2009; Lepoittevin, 2006; Smith and Hotchkiss, 2001). To expand the applicability domain of the DPRA, designed specifically for directly reactive haptens, we recently reported the development of a modified version with horseradish peroxidase-hydrogen peroxide (HRP/P) for the enzymatic activation of pro-hapten sensitizers with cysteine (Gerberick et al., 2009). Although thiol reactivity is very important, other nucleophiles could be useful for identifying sensitizers that are less promiscuous and preferentially react to specific amino acids other than cysteine.

Reactivity with lysine has been suggested as an important mechanism for generating modified protein targets in ACD (Alvarez-Sanchez et al., 2003, Eilstein et al., 2008, Gerberick et al., 2004,

2008). Although many chemical sensitizers are highly reactive towards thiol-containing molecules, formation of antigenic structures resulting from lysine reactivity may have synergistic effects on the amount and diversity of modified protein products. In addition, haptens covalently linked to lysine residues have been shown to play a role in MHC recognition and activation of T lymphocytes (Weltzien et al., 1996). Characterizing the potential for chemical-induced modification of lysine *in chemico* may contribute to the development of an integrated testing strategy for risk assessments.

In this report, we describe the incorporation of lysine peptide into the Peroxidase Peptide Reactivity Assay (PPRA). Discrete reactivity determinations for cysteine and lysine $\pm$ HRP/P are used to assess peptide reactivity of hapten and pre-/pro-hapten sensitizers. The assay utilizes HPLC/MS/MS detection for monitoring peptide disappearance following 24 h co-incubation with test chemicals. In addition, peptide reactivity is characterized using a concentration-response study design, such that the concentration that depletes peptide by 15% (EC15) is determined. Reactivity of 25 non-sensitizers, 26 haptens and 19 pre-/pro-haptens of diverse chemical structure and sensitization potency were examined. The PPRA screening approach described here has the potential to contribute to a more comprehensive assessment of skin sensitization potential of all possible allergens (i.e., haptens, pre-haptens and pro-haptens).

MATERIALS AND METHODS

Test chemicals and reagents

The following test chemicals, with accompanying purity and CAS numbers, were purchased from Aldrich Chemical Company (Milwaukee, WI): 5-amino-2-methylphenol, 97% [2835-95-2], aniline, 99.5% [62-53-3], 2-aminophenol, 99% [95-55-6], benzaldehyde, 95% [100-52-7], benzylidene acetone, 99% [122-57-6], 1-butanol, 99.5% [71-36-3], 2,3-butanedione, 97% [431-03-8], cinnamyl alcohol, 98% [104-54-1], diethyl acetaldehyde, 92% [97-96-1], diethyl maleate, 97% [141-05-9], dihydroeugenol, 99% [2785-87-7], eugenol, 99% [97-53-0], hexahydrophthalic anhydride, 95A% [85-42-7], n-hexane, $\geq 99\%$ [110-54-3] and hexyl cinnamaldehyde, 85% [101-86-0], isoeugenol, 98% [97-54-1], 2-methoxy-4-methylphenol, 99% [93-51-6], methyleugenol, 98% [93-15-2], 2-methylundecanal, 94% [110-41-8], 1-naphthol, 99% [90-15-3], pentachlorophenol, 98% [87-86-5], phenylacetaldehyde, 90% [122-78-1], phenyl isocyanate, 98% [103-71-9], 2-phenylpropionaldehyde, 98% [93-53-8], propyl paraben, 99% [94-13-3], tartaric acid, 99.5% [87-69-4]. The following test chemicals with accompanying purity and CAS numbers were purchased from Alfa Aesar (Pelham, NH): lauryl gallate, 98% [1166-52-5], propyl gallate, 98% [121-79-9]. The following test chemicals with accompanying purity and CAS numbers were purchased from Fluka (Milwaukee, WI): 1-bromobutane, 99% [109-65-9], fluorescein-5-isothiocyanate, 98% [3326-32-7], Glyoxal [107-22-2], 4-methoxyacetophenone, 99% [100-06-1], propylene glycol, 99.7% [57-55-6]. Bandrowski's base (N,N-bis(4-aminophenyl)-2,5-diamino-1,4-quinone-diimine), 99%, [20048-27-5] was purchased from ICN (Costa Mesa, CA). Glycerol, 99% [56-81-5] was purchased from J.T. Baker (Phillpsburg, NJ). The following test chemicals with accompanying purity and CAS numbers were purchased from Sigma Chemical Company (St. Louis, MO): 3-aminophenol, 98% [591-27-5], p-benzoquinone, 98% [106-51-4], chlorobenzene, 99% [108-907], cinnamaldehyde, 93% [104-55-2], citral, 95% [5392-40-5], coumarin, $\geq 98\%$ [91-64-5], diethyl phthalate, 99.5% [84-66-2], 3,4-

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3 dihydrocoumarin, 99% [119-84-6], ethyl acrylate, 99% [140-88-5], 4-(N-ethyl-N-2-methan-
4 sulphonamido-ethyl)-2-methyl-1,4-phenylenediamine (CD3), 100% [25646-71-3], farnesal,
5 $\geq 85\%$ [19317-11-4], glutaraldehyde [111-30-8], hydroquinone, 99% [123-31-9], 4-
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10 hydroxybenzoic acid, 99% [99-96-7], 7-hydroxycitronellal, $\geq 95\%$ [107-75-5], 2-hydroxyethyl
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12 acrylate, 96% [818-61-1], isopropanol, 99% [67-63-0], lactic acid, 85% [50-21-5], methyl
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14 acrylate, 99% [96-33-3], 3-methylcatechol, 98% [488-17-5], 6-methylcoumarin, $>99\%$ [92-48-
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17 8], methyl paraben, 99% [99-76-3], methyl salicylate, 99% [119-36-8], 2-nitro-1,4-
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19 phenylenediamine, 95% [5307-14-2], nonanoic acid, 97% [112-05-0], octanenitrile (heptyl
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21 cyanide), 97% [124-12-9], octanoic acid, 98% [124-07-2], 3-phenoxypropiononitrile, 98%
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23 [3055-86-5], 1,4-phenylenediamine, 100% [106-50-3], pyrogallol [87-66-1], salicylic acid, 99%
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25 [69-72-7], sulphanilamide, 99% [63-74-1], 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one
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27 (oxazolone), $\geq 90\%$ [15646-46-5], streptomycin sulfate [3810-74-0], α -amyl cinnamaldehyde,
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29 97% [122-40-7], β -propiolactone, $\geq 90\%$ [57-57-8]. Desferroxamine (catalog number D9533),
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31 horseradish peroxidase (catalog number P6140), hydrogen peroxide (catalog number 216763)
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33 and DL-dithiothreitol (catalog number D5545-16) were purchased from Aldrich Chemical
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35 Company (Milwaukee, WI). The cysteine peptide (Ac-RFAACAA-COOH) and lysine peptide
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37 (Ac-RFAAKAA-COOH) were prepared and purified by the SynBioSci (Livermore, CA, USA).
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39 Leucine enkephalin internal standard (catalog number L9133) was purchased from Sigma
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41 Aldrich Chemical Company (St. Louis, MO). N^{α} -acetyl-leucine enkephalin internal standard
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43 was purchased from RS Synthesis (Louisville, KY).
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53 *Direct lysine- and cysteine-peptide incubation conditions*

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Direct reactivity of test chemical to lysine-based synthetic peptide was determined in reactions containing 0.1 M ammonium acetate buffer (pH 10.2). Unless otherwise indicated, the final concentration of lysine peptide was 5 μ M in a final reaction volume of 0.3 mL. Direct reactivity of test chemicals to cysteine-based synthetic peptide was determined as previously described (Gerberick et al., 2009). Incubations with lysine or cysteine peptide and no test chemical, in the absence of HRP/P, served as zero-depletion reference controls for comparisons to direct reactivity determinations. Sample reactions were initiated by adding 3 μ L of a test chemical stock solution. The final organic content in each reaction was $\leq$ 1%. Samples were incubated under ambient lab conditions for 24 h.

Peptide incubation with HRP/P

Enzyme-mediated reactivity determinations were performed in sample reactions that were prepared as described above, but with addition of horseradish peroxidase and hydrogen peroxide (HRP/P) at a final concentration of 3 U/mL and 100 μ M, respectively. Incubations with either lysine or cysteine peptide and no test chemical, in the presence of HRP/P, served as zero-depletion reference controls for comparisons to enzyme-mediated test chemical reactivity determinations.

HRP activity characterization using pyrogallol

Enzymatic activity of horseradish peroxidase (HRP) was determined spectrophotometrically by measuring (at 420 nm) the formation rate of purpurogallin from pyrogallol. Sample reactions were initiated by addition of HRP (0.0035 units) to individual wells of a microtiter plate

containing potassium phosphate buffer (0.1 M, pH 7.4 and 10), hydrogen peroxide (0.027%, v/v) and pyrogallol (0.525%, w/v). Incubations were performed in triplicate at a final reaction volume of 0.2 mL. Sample reactions without HRP served as blanks and were used to subtract background absorbance. The change in optical density was recorded at 20 s intervals at 420 nm for 5 min (i.e., the linear portion of the kinetic curve) using a SpectraMax M5 (Molecular Devices) microplate reader. The extinction coefficient of 1 mg/mL of purpurogallin is 12 as reported by the supplier of the compound standard.

Sample processing and analysis by HPLC/MS/MS

Following the incubation period, samples were diluted with 0.3 mL of internal standard solution (3 µg/mL N^α-acetyl-leucine enkephalin in 95/5 acetonitrile/water) and 20 µL of each sample was diluted with 780 µL of 2/98 acetonitrile/water. In sample reactions containing cysteine, a 20 µL aliquot of a 16 mM dithiothreitol solution (prepared in water) was then added and samples were heated in a 45 °C oven for 30 min, then allowed to cool to room temperature prior to analysis.

Analysis was primarily designed to selectively monitor two analytes: the cysteine peptide (monomer) and N^α-acetyl-leucine enkephalin internal standard (IS), and in a separate method, the lysine peptide and IS. The HPLC/MS/MS methodology employed a CTC HTS-PAL autosampler (Leap Technologies, Carrboro, NC) for sample introduction. Chromatographic separation was carried out using a 10ADvp pumping system (Shimadzu, Columbia, MD). Injection volumes of 20 µL were made onto a Gemini-NX C18 (2.1 x 20 mm, 3 µm) analytical column (Phenomenex, Torrance, CA). The mobile phase, at a flow rate of 0.7 mL/min,

consisted of water with 1 mM ammonium bicarbonate (A) and 99/1 methanol/water, 1 mM ammonium bicarbonate (B). Separation was achieved by holding at initial conditions (99% A) for 1 min, then a 1.0 min linear gradient to 100 % B for 1 min then step down to initial conditions for a total analysis time of 6 min per sample.

The chromatographic effluent is interfaced to a Sciex API 3000 triple quadrupole mass spectrometer (Applies Biosystems, Foster City, CA), equipped with TurboIonSpray and operated in positive electrospray ionization mode. To achieve high selectivity and sensitivity, the following selected reaction monitoring (SRM) schemes were optimized for detection: cysteine (m/z 751-to-120); lysine (m/z 389-to-129) and N^α-acetyl leucine enkephalin IS (m/z 598-to-120). Note that for cysteine and IS, MH⁺ is monitored, while [M+2H]²⁺ is monitored for lysine. In addition, the thiol-dimer form of the cysteine peptide was chromatographically resolved from, and monitored in the SRM channel of the targeted monomer (due to its [M'+2H]²⁺ form).

For typical batch analyses, the cysteine samples are analyzed first to minimize the potential for subsequent dimer formation, followed by the lysine samples. For batches involving multiple test compounds, the direct and enzyme-mediated reactivity sample sets were analyzed in alternating fashion and each of the test chemical sample sets were bracketed by a pair of the respective zero-depletion control samples. This allowed for a more-precise comparison of reactivity between direct and enzyme-mediated conditions for each test compound. Also, by using the mean of the two bracketing control sample results (i.e., analyzed in closest temporal proximity to the test samples) optimal accuracy in depletion measurement was achieved.

Data reduction for peptide depletion measurements

SRM peak areas and area ratios (cysteine or lysine peptide-to-internal standard) were determined for each sample, using the Sciex Analyst 1.4.1 quantitation software package. Peptide depletion in samples with test chemical was calculated by comparing the peak area or peak area ratio in samples containing test chemical to the average of the peak area ratio that was calculated from the corresponding pair of bracketing control samples. In sample reactions that were evaluated in replicates, peptide depletion values for individual replicates were determined and then used to calculate mean (N=2) or mean and standard deviation (N=3) for both the direct (no HRP/P) and enzyme-mediated (with HRP/P) reactivity determinations. During preliminary method development work, loss of signal for the leucine enkephalin internal standard (LE IS) was observed for certain chemicals (data not shown). These results indicated an apparent reactivity between the test chemical and the internal standard, which later led to the replacement of LE IS with the current N^α-acetyl-leucine enkephalin as part of the final methodology.

EC15 Estimation

Peptide reactivity, expressed in terms of EC15, is an estimate of the test chemical concentration that depletes the peptide by 15% under the experimental conditions described above. EC15 values were determined by fitting a three-parameter log-logistic model to peptide depletion data using RExcel (www.statconn.com). Prior to analysis, all negative depletion values were set to zero. Reactivity was reported as 'NC' (not calculated) if depletion at all concentrations were at or below threshold values of 10% for cysteine without HRP/P, 40% for cysteine with HRP/P and 15% for lysine with or without HRP/P. These cut-off values were determined empirically (as

described in the Discussion section) based on direct observation of overall trends in reactivity for sensitizers and non-sensitizers with and without HRP/P. If peptide depletion was threshold positive but low or insufficient for estimating the EC15 (i.e., no concentration-response), reactivity was designated as NR (not reported). For Cooper statistics, test chemicals that were not threshold positive (i.e., NC) were considered non-sensitizers and those with EC15 values or designated as 'NR' were considered sensitizers.

RESULTS

Recovery of Lysine Peptide following Incubation with and without HRP/P

Prior to performing experiments with chemical sensitizers, a preliminary study was conducted to monitor the selective loss of lysine peptide by HPLC/MS/MS, following incubation in the presence and absence of HRP (3 U/mL) and hydrogen peroxide (100 μ M). Sample reactions were performed in triplicate as described above. In comparison to reactions that were quenched immediately following addition of lysine (5 μ M), mean ($\pm$ SD) depletion of lysine at 2 h was 7.6 $\pm$ 1.9% (without HRP/P) and -3.1 $\pm$ 5.9% (with HRP/P). Following 24 h incubation, mean lysine depletion was 6.4 $\pm$ 4.9% (without HRP/P) and 7.6 $\pm$ 2.5% (with HRP/P). These findings suggest essentially complete recovery of lysine peptide in reactions with and without HRP/P and substantiate their use as zero-depletion reference controls for calculating lysine peptide depletion in reactions containing test chemical.

HRP Activity Determinations as a function of pH

To ensure adequate enzymatic activity of HRP at pH 10, formation of purpurogallin from the HRP substrate pyrogallol was evaluated at pH 7.4 and 10. Mean HRP activity at pH 7.4 and 10 was 0.133 (3.0 %CV) and 0.130 (2.0 %CV) U/mL of enzyme, respectively, indicating no significant differences in HRP activity were observed. This finding has significance for identifying potential lysine-reactive pro-haptens in the presence of the HRP/P oxidation system. The ϵ -amino group of lysine has a pKa of 10.5. Lysine peptide reactions are performed at pH 10 to ensure that the amine is sufficiently deprotonated and available for reactivity.

Test Chemical Concentration-Dependency in Direct Lysine Peptide Reactivity

Direct lysine peptide reactivity with p-benzoquinone was determined at initial peptide concentrations of 0.005, 0.05 and 0.5 mM in triplicate reactions containing 25% ACN. For each peptide concentration examined, p-benzoquinone was co-incubated at a final concentration of 0.25, 2.5 and 25 mM in a final reaction volume of 0.3 mL. Following 24 h incubation, sample reactions at initial lysine peptide concentrations of 0.05 and 0.5 mM were diluted 1:9 and 1:99, respectively, in 0.1 M ammonium acetate buffer (pH 10.2) containing 25% ACN. The samples were then processed along with the 0.005 mM lysine peptide incubates and analyzed by LC/MS/MS as described in the materials and methods section. At concentrations of 0.25, 2.5 and 25 mM p-benzoquinone, direct lysine peptide depletion was approximately 15%, 50% and 85%, respectively, and appeared to be independent of the initial concentration of lysine peptide and corresponding peptide:chemical molar ratio (Figure 1A).

To directly compare depletion data from this modified LC/MS/MS-based reactivity assessment to historical values from the LC/UV-based DPRA method, a set of control reactions were prepared according to Gerberick et al. (2004). Briefly, triplicate reactions were prepared with a 1.0 mL reaction volume containing lysine peptide (0.5 mM) and p-benzoquinone (25 mM) in clear glass. Immediately following addition of test chemical, a 0.3 mL aliquot was removed from each replicate and transferred to individual wells of a 96-well plate. The samples were then incubated for 24 h under ambient lab conditions. As shown in Figure 1B, peptide reactivity in these split samples that were either incubated in glass or plastic, were equally high (85% depletion) and consistent with those in Figure 1A at the 25 mM test chemical concentration. Depletion of lysine peptide in reactions with p-benzoquinone corroborates DPRA depletion data (Gerberick et al., 2004) and supports the use of LC/MS/MS as an appropriate detection method for peptide reactivity assessments.

To demonstrate that the initial study results with p-benzoquinone were reproducible and to determine whether similar trends would be observed with other known lysine-reactive sensitizers, the experiment was repeated with p-benzoquinone, 3-methylcatechol and glutaraldehyde. Sample conditions were identical to those described above except for the concentration of glutaraldehyde, which was tested at final concentrations of 0.125, 1.25 and 12.5 mM. Due to the differential loss in signal of the non-acetylated internal standard (LE IS) for certain test chemical incubations, at the higher concentrations (data not shown), peptide depletion for this data set was calculated using lysine peptide peak area values rather than peak area ratios. As shown in Table 1, trends in reactivity were consistent with previous findings with p-benzoquinone. For 3-methylcatechol and glutaraldehyde, reactivity also appeared to be test

chemical concentration-dependent with maximum depletion values being observed at the highest concentrations tested, regardless of the initial concentration of peptide. Comparisons between each of the three peptide concentrations at each test chemical concentration showed that the highest depletion value typically corresponded to reactions containing either 0.005 or 0.05 mM lysine peptide. Based on these findings, the concentration of lysine peptide chosen for subsequent method development was 0.005 mM.

Concentration-response Studies in Direct Lysine Peptide Reactivity Determinations

Concentration-response studies were conducted to characterize direct reactivity profiles for 10 chemicals at final test chemical concentrations ranging from 0.008 to 25 mM in duplicate reactions containing 1% organic. Test chemicals were selected based upon historical lysine reactivity data in the DPRA and represent low, moderate and high reactivity categories. As shown in Figure 2, direct lysine peptide depletion generally increased with an increase in test chemical concentration for p-benzoquinone, glutaraldehyde, 3,4-dihydrocoumarin, 3-methylcatechol, and propyl gallate, all of which exhibited relatively high reactivity towards lysine. Although depletion increased with an increase in concentration for oxazolone, reactivity was generally low with approximately 20% depletion being observed at the two highest concentrations tested. Reactivity between lysine and cinnamaldehyde or 2-phenylpropionaldehyde was negligible at all concentrations tested except at 25 mM in which ~25% depletion was observed. Citral and n-hexane reactivity towards lysine under direct (-HRP/P) conditions was low/negligible.

In an effort to enhance lysine peptide reactivity and to minimize the need for testing at relatively high (and often solubility-limiting) test chemical concentrations, the effect of incubation temperature on direct peptide depletion was examined following 24 h incubation. Incubations were performed under ambient lab conditions or in a water bath at 30°C. As shown in Figure 3, no significant increase in depletion was noted for p-benzoquinone and 3-methylcatechol at elevated temperature. Only subtle increases in depletion were observed for α -methyl cinnamaldehyde and glutaraldehyde, which may be attributed to an increase in the rate of reaction and/or solubility of the test chemical. The finding that incubation temperature had little effect on depletion is highly unusual and suggests that the reactions have already gone to completion prior to the 24 h time point. Due to the lack of significant increases in depletion following 24 h incubation at higher temperature, sample reactions in subsequent studies were performed under ambient lab conditions.

Concentration-response testing with Lysine and Cysteine Peptides $\pm$ HRP/P

Concentration-response studies were conducted to characterize reactivity profiles for an expanded set of chemical allergens and non-allergens. Final test chemical concentrations were targeted at 0.003, 0.03, 0.3, 3 and 30 mM in reactions containing lysine (5 μ M) or cysteine (20 μ M) peptides in the presence and absence of HRP/P. Test chemical concentration ranges were selected based on preliminary method development work. In some cases, solubility limited the final concentration of test chemical in the reactions as noted. The chemicals included 16 extreme/strong, 14 moderate, 15 weak allergens and 25 non-sensitizers. Twenty-six hapten and 19 pro-/pre-hapten chemical sensitizers, likely to represent a variety of reaction mechanisms for

modifying proteins were included. Direct and HRP/P-mediated reactivity assessments for non-sensitizers and hapten chemical sensitizers are shown in Table 2. Peptide depletion data for pre-haptens and pro-haptens are presented in Table 3. The chemicals are listed according to sensitization category (as defined by published LLNA data) to facilitate better comparisons of peptide depletion across categories.

Peptide Depletion Results for Haptens, Pre-haptens, Pro-haptens and Non-sensitizers

As shown in Table 2, peptide depletion for the majority of hapten chemical sensitizers was >10% for cysteine and >15% for lysine. With exception to methyl acrylate and ethyl acrylate, maximum peptide depletion for weak haptens generally ranged from 20 to 40% for cysteine and 20 to 50% for lysine. Maximum depletion values for moderate, strong and extreme haptens typically ranged from 30 to 100% for cysteine or lysine peptides. Hapten chemical allergens that showed little to no reactivity towards both peptides were α -methyl cinnamaldehyde, hexyl cinnamaldehyde and phenylacetaldehyde. In contrast, peptide depletion in the absence of HRP/P for the majority of non-sensitizers did not exceed 10% for cysteine and 15% for lysine. In the presence of HRP/P, peptide depletion was similarly low except for a few non-sensitizers that showed >10% depletion at the 30 mM test chemical concentration with cysteine. 4-Hydroxybenzoic acid, methyl paraben and propyl paraben were reactive at lower concentrations with cysteine + HRP/P but depletion profiles generally lacked a concentration-response.

Enzyme-mediated activation of pro-haptens resulted in an increase in depletion with cysteine and HRP/P with maximum depletion values ranging from 20% to nearly 100% (Table 3). With

exception to 5-amino-2-methylphenol, 1-naphthol and lauryl gallate, no apparent reactivity was observed for pro-haptens with lysine and HRP/P. For pre-haptens, subtle differences in cysteine depletion were observed in the presence and absence of HRP/P. Hydroquinone and 3-methylcatechol were the only pre-haptens that reacted with lysine-containing peptide.

Cooper statistics were calculated to compare reactivity results from this screening assay to hazard information obtained in the LLNA. Test chemicals were classified empirically as positive (sensitizers) if depletion at any test chemical concentration was >10% for cysteine without HRP/P, >40% for cysteine with HRP/P or >15% for lysine with or without HRP/P. The test chemicals that did not meet these criteria were classified as non-sensitizers. As shown in Table 4, the accuracy, or proportion of true sensitizers and non-sensitizers predicted correctly, was 83%. Values for sensitivity and specificity were 93% and 64%, indicating the proportion of all correctly identified sensitizers and non-sensitizers, respectively. Positive predictivity (the proportion of chemicals positive in the PPRA that are sensitizers in the LLNA) was 82% and negative predictivity (the proportion of chemicals negative in the PPRA that are non-sensitizers in the LLNA) was 84%.

Finally, the estimated test chemical concentration that depletes peptide by 15% (EC15) was determined for peptide-reactive chemicals that showed a concentration-response. Test materials that were reactive but did not show a reasonable concentration-response for deriving an EC15 were designated as NR (not reported) but considered positive (for Cooper statistics). These data are presented in Table 5 along with the nucleophile and reaction condition ($\pm$ HRP/P) corresponding to the overall lowest EC15. Median EC15 values for each category, excluding

outliers, were 4.5 mM (non-sensitizers), 0.55 mM (weak), 0.042 mM (Moderate) and 0.030 mM (Strong/Extreme). Figure 4 presents these data graphically to illustrate the distribution of peptide reactivity for each sensitization category.

DISCUSSION

Advancements in understanding the pathogenesis of allergic contact dermatitis (ACD) have challenged investigators to develop and refine hypothesis-driven alternatives to ultimately inform whether a chemical has the potential to act as a contact allergen in the absence of *in vivo* data. Unlike rodent models which are intact dynamic systems and possess many important anatomical and physiological features that are similar to those in human, alternative approaches are relatively static and focus on characterizing discrete cellular, biochemical and/or chemical events for subsequent use in developing risk assessment paradigms. Although the relative importance of each test and the specifics on how the data will ultimately be integrated into a quantitative risk assessment is under evaluation, it is generally accepted that in order for a chemical to cause skin sensitization, it must react with protein within the viable epidermis to form a complete antigen. Since protein reactivity is a key step in the induction of ACD, investigators have reported the use of a number of model nucleophiles in an effort to develop *in chemico* approaches for predicting the skin sensitization potential of new chemicals based on reactivity. Published reports have included the use of synthetic peptides containing single or multiple nucleophilic amino acids, glutathione, human serum albumin and 4-nitrobenzenethiol (Aleksic et al, 2009; Alvarez-Sanchez et al., 2004a, 2004b; Aptula et al, 2006; Chipinda et al., 2010; Enoch et al., 2009; Gerberick et al, 2004, 2007, 2008; Kato et al, 2003; Natsch et al, 2007, 2008; Roberts and Natsch, 2009; Schultz et al, 2005).

As the majority of model nucleophiles are thiol-based, it is essential to also develop methods that allow for routine reactivity determinations with other nucleophiles, particularly those that have been shown to form antigenic products. It has been hypothesized that the immunogenicity of contact allergens is directly related to their ability to modify a few specific amino acids (Alvarez-Sanchez et al., 2003; Eilstein et al., 2008; Meschkat et al., 2001). Lysine is clearly an important amino acid and a good representative of hard nucleophiles that are expected to selectively react with hard electrophiles (Gerberick et al., 2004). In addition to reactivity with soft nucleophiles such as cysteine or tyrosine, the diversity of hapten-protein interactions and the selective modification of lysine residues have been demonstrated with 5-chloro-2-methylisothiazol-3-one (MCI) and *p*-amino aromatic and sultone derivatives (Alvarez-Sanchez et al., 2003; Eilstein et al., 2008; Meschkat et al., 2001). Reactivity in the DPRA further supports the utility for measuring lysine reactivity, particularly for identifying extreme and strong chemical allergens (Gerberick et al., 2004, 2007).

To establish further a practical quantitative *in chemico* peptide-based reactivity assay for screening contact allergens in our laboratory, we have recently completed the initial work of incorporating lysine peptide into an LC/MS/MS-based assay for discrete reactivity assessments of hapten, pre-hapten and pro-hapten chemical sensitizers. The approach is based on a refined version of the DPRA in which loss of peptide is determined following 24 h co-incubation with test chemical under ambient lab conditions in the presence and absence of horseradish peroxidase-hydrogen peroxide (HRP/P) oxidation system. The DPRA, which is currently in Phase III pre-validation by the European Centre for the Validation of Alternative Methods

(ECVAM), has demonstrated a strong correlation between *in chemico* reactivity of hapten chemical sensitizers to both a cysteine- or lysine-containing synthetic peptide and skin sensitization potential. A next generation assay was developed to include an activation step using a peroxidase-peroxide oxidation system for identifying potential pro-hapten chemical sensitizers using a cysteine-based synthetic peptide (Gerberick, et al 2009). The incorporation of lysine as another relevant nucleophile in this model will allow for a more comprehensive assessment of chemical reactivity for screening purposes and expands the portfolio of reactivity-based alternatives for skin sensitization testing.

In this present study, 70 allergens and non-allergens of diverse chemical structure and sensitization potency (weak-extreme) were assessed for direct and HRP/P-mediated reactivity with cysteine and lysine using a concentration-response study design. The chemicals cover a variety of reaction mechanistic domains and include 26 haptens that are comprised of acyl transfer agents, Schiff base formers, Michael acceptors and S_N2 electrophiles (Roberts et al., 2007).

The chemical set also includes 19 pro-haptens and pre-haptens for evaluating the use of a HRP/P oxidation system for activating non-electrophilic chemical sensitizers. In comparison to direct reactivity measurements, co-incubation of HRP/P with pre-/pro-haptens and cysteine resulted in significant peptide depletion that was typically observed at lower concentrations. Non-enzymatic (direct) reactivity for pre-hapten sensitizers generally resulted in similar depletion profiles compared to those with HRP/P. The activation of exclusively lysine-reactive pro-haptens with HRP/P has not been demonstrated in the present study. Although the majority of

pre-/pro-haptens were reactive with cysteine, additional testing with a more representative set of pro-haptens is needed to judge whether reactions with lysine in the presence of HRP/P is required for standard testing. For testing unknowns, the most conservative approach is including lysine +HRP/P which may identify potential lysine-selective pro-haptens.

By comparing the extent of reactivity between non-sensitizers and sensitizers, threshold positive depletion values were determined to examine the potential use of this model for basic hazard assessments. Peptide depletion for the majority of non-sensitizers was generally <10% for cysteine and <15% for lysine. In the presence of HRP/P, however, cysteine depletion for a few non-sensitizers was >10%. By comparison, cysteine depletion for pre-/pro-haptens with HRP/P was typically $\geq 40\%$. Based on these observations, threshold positive depletion values were identified empirically for any reaction with >10% or >40% for cysteine in the absence and presence of HRP/P, respectively, or >15% for lysine with or without HRP/P. A prediction accuracy of 83% was demonstrated for the 70 chemicals examined in this study. Although slightly lower than the 89% accuracy reported previously for the DPRA (Gerberick, et. al., 2007), the chemical set used to evaluate the DPRA prediction model was essentially devoid of pre-/pro-haptens. In this study, 42 of the 45 sensitizers were identified correctly. Nine of the 12 misclassifications were false-positives and 3 were false-negatives. The high sensitivity (93%) is encouraging for a provisional model that included 19 pre-/pro-haptens and 26 haptens. In addition, the relatively low specificity (64%) highlights further the need for additional test information (i.e., skin penetration) to potentially reduce the number of false-positives expected from weakly reactive non-sensitizers (i.e., methyl paraben, propyl paraben and their hydrolysis reaction product 4-hydroxybenzoic acid). The weak pro-haptens aniline and cinnamic alcohol

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3 were activated in the presence of HRP/P but cysteine depletion did not exceed the empirical
4 threshold positive value for this reaction (i.e., >40%).
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10 General trends in peptide depletion appear to coincide well with skin sensitization potential
11 (Table 2 and 3). In contrast to the majority of non-sensitizers that showed little to no peptide
12 reactivity, the majority of sensitizers, including haptens and pre-/pro-haptens, demonstrated
13 substantial reactivity. Weak sensitizers were distinguished from moderate, strong and extreme
14 sensitizers by exhibiting no measurable depletion ($\leq 10\%$) at concentrations < 3 mM. Moderate,
15 strong and extreme haptens were generally distinguished from weak haptens by higher depletion
16 values. In addition, the majority of moderate, strong and extreme sensitizers were reactive
17 towards both cysteine and lysine nucleophiles. Moreover, Figure 4 shows the median EC15
18 value for moderate and strong/extreme sensitizers were lower than those observed for weak and
19 non-sensitizers.
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36 One advantage with including lysine in reactivity assessments is to identify sensitizers that are
37 selective to ϵ -NH₂ groups. In this study, four sensitizers showed selective reactivity towards
38 lysine including two strong/extreme (hexaphthalic anhydride, phenyl isocyanate) and two weak
39 sensitizers (7-hydroxycitronellal and 4-methylundecanal). Glyoxal and 2-hydroxyethyl acrylate,
40 both moderate sensitizers, exhibited high depletion of cysteine at relatively low concentrations
41 (i.e., 0.03 or 3 mM), while depletion of lysine, although extensive (>50%), was only observed at
42 the 30 mM concentration. In addition, seven sensitizers were identified as having higher
43 reactivity towards lysine compared to cysteine. In contrast, 11 sensitizers were exclusively
44 reactive towards cysteine peptide and nearly all of them were pre-/pro-haptens. The majority of
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sensitizing chemicals examined (27/45) were reactive towards both cysteine and lysine peptides. Although oxazolone, 3,4-dihydrocoumarin and farnesal were slightly reactive to cysteine, higher depletion was observed with lysine and the depletion profiles were amenable for determining an EC15 values.

Trends in reactivity for weak allergens examined in this study were less consistent than moderate, strong and extremes, demonstrating further the need for considering other variables (e.g., bioavailability, dendritic cell activation) for accurate predictions of skin sensitization potency. In general, however, negligible loss of peptide was typically observed at concentrations ≤ 3 mM for at least one nucleophile. It is interesting to note that moderate sensitizers were generally more reactive than structural analogs that are classified as weak or non-allergens and less reactive than strong/extremes (i.e., cinnamaldehyde, acrylates, coumarins, dialdehydes/diketones).

For the terpene aldehydes (7-hydroxycitronellal, citral and farnesal), reactivity towards cysteine was typically low and lacked a concentration-response. Depletion of lysine, however, was $\geq 30\%$ but only at the highest concentration tested (i.e., 30 mM). Since the reactive species for these materials may be attributed to hydroperoxide formation via auto-oxidation in storage, the observed depletion might be due to reactivity of low-level hydroperoxide degradants. One issue for *in chemico* assays is test chemical solubility, particularly in reactions with lipophilic molecules that are performed with a low organic content (Merckel et al., 2010). The use of microemulsions has been shown recently to overcome compatibility problems with 7-hydroxycitronellal and citral reactivity towards glutathione (GSH) (Merckel et al., 2010). These

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3 materials were reactive to the thiol group of GSH, and for 7-hydroxycitronellal, secondary
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5 reactions at the α -NH₂ group to form a cyclic hemithioacetal structure was observed (Merckel et
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7 al., 2010). Although lysine and cysteine reactions reported here are performed in separate
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9 incubations, these findings suggest the potential for reactivity of these materials to nucleophiles
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11 which contain -SH and -NH₂ groups.
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18 The target test chemical concentration range selected in this study (i.e., 0.003-30 mM) was based
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20 on preliminary studies that indicated higher concentrations of chemical were needed for
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22 detecting reactivity with lysine or chemicals that are weakly reactive towards cysteine. Although
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24 the final organic content is low (i.e., 1%), the procedure for selecting a compatible solvent begins
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26 with solubility testing in various solvents prior to performing the actual experiment. One
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28 potential limitation of the assay is limited or unknown aqueous solubility of the test chemical.
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30 Despite the potential solubility issues for certain chemicals, this may be overcome by reducing
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32 the final test chemical concentration or increasing the amount of co-solvent to ensure that the test
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34 chemical is fully soluble. The primary reason for reducing the % organic in the PPRA is to
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36 minimize the potential for reduced HRP-mediated activation via inhibition and/or protein
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38 denaturation. This has not been examined experimentally with HRP but significant solvent
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40 effects have been demonstrated for P450-mediated metabolism studies with small organic
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42 molecules. Another potential challenge is selecting an appropriate test chemical concentration
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44 for unknowns, thus, repeat experiments may be needed to adjust the concentration to an optimal
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Although peptide reactivity is an important criterion for evaluating the potential of novel materials to initiate skin sensitization, it is not expected to correlate completely with skin sensitization potency as defined by existing animal and human data. Skin penetration, dendritic cell activation and immune recognition by T cells should also be considered for a more holistic assessment of skin sensitization potential. For routine screening purposes during early product development, however, the prospective use of peptide reactivity data may involve comparisons of reactivity profiles between novel materials and structural analogs that are known sensitizers. In this way, it may be useful to examine the data set between structural analogs to correlate peptide reactivity to skin sensitization potency. For example, 6-methylcoumarin and coumarin, both non-sensitizers, exhibited little to no reactivity towards lysine or cysteine, while reactivity of 3,4-dihydrocoumarin towards lysine was substantial and showed a clear concentration-response. Similarly, direct (-HRP/P) lysine depletion at the 30 mM test chemical concentration was equally extensive for moderate and strong dialdehyde/diketone and acrylate sensitizers in comparison to weak sensitizing analogs which appeared to exhibit similar reactivity profiles towards cysteine.

In summary, reactivity data presented here highlight the utility for testing lysine as a relevant nucleophile for screening contact allergens. The majority of sensitizers examined (27 of 45) were lysine reactive and 4 showed preferential reactivity towards this nucleophile. Characterizing the potential for chemical-induced modification of lysine residues *in chemico* could contribute significantly to the development of integrated testing strategies in the near-term and reduce the number of false negatives with sensitizers that are exclusively reactive to $-NH_2$ groups in proteins. An assessment of the amino acid composition in pig skin revealed that the

amount of lysine is greater than 10-fold higher than cysteine, and nearly 3-fold higher than histidine (Nguyen et al., 1986). Changes in protein structure at lysine and cysteine residues may create new epitopes and increase the risk in immunogenicity for contact allergens. Reactivity to lysine may also be relevant for chemicals that are highly reactive to cysteine, which may result in a decrease in the antioxidant protection via glutathione depletion *in vivo*. Similar to the LLNA, the approach involves the generation of concentration-response data such that an estimate of the relative reactivity potency can be made based on the concentration of test chemical that depletes peptide at or above a threshold positive value. Here, we report the use of EC15 as one example to illustrate the use of the model as a screening approach for assessing the peptide reactivity potential of novel chemicals. Quantifying reactivity based on concentration rather than % depletion may be useful for extrapolating *in chemico* data to the *in vivo* situation and may involve the use of modeling approaches for internal dosimetry determinations. In this way, basic hazard information from non-animal models can be integrated for comparison to human exposure data for quantitative risk assessments.

FUNDING

This work is funded in part by The European Cosmetics Association.

ACKNOWLEDGEMENTS

The authors wish to acknowledge George Daston and Cindy Ryan for their excellent review of the manuscript. We also acknowledge Joel Chaney for his assistance on the statistical procedures

used in this manuscript. None of the P&G authors will benefit financially from this work other than their salaries. Dr. Lepoittevin and Jon Lalko work in collaboration with the P&G scientists and are not receiving any financial support from P&G for this work.

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TABLES

Table 1 Direct Lysine Peptide Reactivity with p-benzoquinone, 3-methylcatechol and glutaraldehyde following 24 h incubation at test chemical and peptide concentrations indicated

Chemical Name	Test Chemical Conc. (mM)	Lysine Peptide Conc. (mM)	Peptide:Chemical Molar Ratio	Mean ($\pm$ SD) Lysine Peptide Depletion (%)
p-Benzoquinone	0.25	0.005	1:50	15.4 $\pm$ 1.2
		0.05	1:5	17.1 $\pm$ 2.8
		0.5	1:0.5	12.2 $\pm$ 3.3
	2.5	0.005	1:500	53.4 $\pm$ 1.0
		0.05	1:50	55.2 $\pm$ 1.2
		0.5	1:5	48.3 $\pm$ 3.1
	25	0.005	1:5000	96.7 $\pm$ 0.7
		0.05	1:500	94.3 $\pm$ 0.8
		0.5	1:50	91.8 $\pm$ 0.4
3-Methylcatechol	0.25	0.005	1:50	10.5 $\pm$ 3.7
		0.05	1:5	9.3 $\pm$ 3.6
		0.5	1:0.5	3.5 $\pm$ 0.6
	2.5	0.005	1:500	38.2 $\pm$ 1.0
		0.05	1:50	43.7 $\pm$ 2.5
		0.5	1:5	36.9 $\pm$ 0.8
	25	0.005	1:5000	67.7 $\pm$ 0.6
		0.05	1:500	58.3 $\pm$ 1.0
		0.5	1:50	51.4 $\pm$ 3.9
Glutaraldehyde	0.125	0.005	1:25	14.5 $\pm$ 1.1
		0.05	1:2.5	10.5 $\pm$ 2.1
		0.5	1:0.25	10.5 $\pm$ 1.7
	1.25	0.005	1:250	40.5 $\pm$ 0.4
		0.05	1:25	32.7 $\pm$ 1.8
		0.5	1:2.5	31.3 $\pm$ 2.2
	12.5	0.005	1:2500	76.2 $\pm$ 1.9
		0.05	1:250	59.0 $\pm$ 1.4
		0.5	1:25	56.1 $\pm$ 1.3

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TABLE 2

Peptide Depletion Data for Hapten and Non-sensitizers

Test Chemical Name	CAS No.	Test Chemical Conc	Cysteine Peptide Depletion (%) ^a										Lysine Peptide Depletion (%) ^a									
			(-HRP/P)					(+HRP/P)					(-HRP/P)					(+HRP/P)				
			C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5
Non-sensitizer																						
1-Bromobutane	109-65-9	A	6.8	4.0	1.2	19.0	79.8	6.3	10.0	12.8	18.9	46.7	4.4	0.0	3.9	0.0	0.0	2.0	12.1	16.1	8.2	1.8
1-Butanol	71-36-3	A	2.4	2.9	4.2	0.9	0.8	2.4	2.5	5.5	6.2	11.7	6.5	3.0	1.5	0.0	5.1	5.7	0.5	3.5	8.8	7.5
3-Phenoxypropiononitrile	3055-86-5	A	1.9	1.9	4.6	3.2	1.5	0.0	0.0	0.7	5.9	1.0	0.0	0.0	1.1	0.0	1.9	0.2	3.3	0.0	0.0	4.3
4-Hydroxybenzoic acid	99-96-7	A	4.6	4.3	1.4	3.4	3.7	33.4	44.2	44.8	47.2	54.1	0.0	0.0	0.0	0.0	0.0	2.9	8.8	26.1	0.0	0.0
4-Methoxyacetophenone	100-06-1	A	0.0	0.0	0.0	5.5	4.4	2.7	0.0	3.6	10.4	17.2	0.5	2.2	0.7	0.0	0.0	0.0	2.0	0.0	13.6	1.3
6-Methylcoumarin	92-48-8	A	3.5	0.0	7.3	0.8	0.0	0.0	0.0	0.5	0.0	4.9	0.0	0.0	0.1	0.0	10.9	11.2	4.1	6.5	14.5	12.1
Benzaldehyde	100-52-7	A	1.9	0.0	0.0	0.0	0.0	8.3	0.0	3.3	9.1	21.3	0.0	2.0	0.6	0.0	13.3	0.6	0.0	8.1	0.3	3.9
Chlorobenzene	108-90-7	A	0.0	4.1	0.0	3.3	1.1	0.0	0.0	5.1	5.6	10.2	0.0	1.3	8.4	0.0	3.9	0.0	2.5	0.0	12.8	6.0
Coumarin	91-64-5	A	6.6	6.6	0.1	10.1	15.1	3.9	6.1	0.0	4.6	17.9	10.8	6.4	3.4	13.3	27.7	10.0	0.0	0.9	3.6	16.2
Diethyl phthalate	84-66-2	A	2.2	0.0	0.0	0.0	0.0	0.0	1.0	2.1	0.8	0.0	1.8	5.7	0.0	0.0	4.6	0.0	0.0	0.0	0.0	0.0
Glycerol	56-81-5	A	2.3	0.0	0.0	2.3	2.7	3.2	0.1	0.1	4.8	5.4	0.0	0.0	1.4	5.6	0.0	4.0	0.0	13.1	8.1	9.7
Hexane	110-54-3	A	0.0	22.8	0.0	0.0	0.0	0.7	0.0	5.0	5.0	2.9	4.9	0.3	0.0	0.0	6.4	0.0	0.0	0.0	0.0	0.0
Isopropanol	67-63-0	A	0.0	1.3	6.3	13.8	22.7	9.7	0.0	0.0	0.0	4.3	0.0	0.0	0.0	0.0	0.0	1.7	6.6	6.8	5.5	0.0
Lactic acid	50-21-5	A	0.0	0.0	2.2	3.6	4.6	0.0	2.0	0.0	4.5	19.4	2.8	1.6	0.0	2.3	2.2	4.4	0.0	5.4	0.0	4.1
Methyl paraben	99-76-3	A	2.0	2.0	4.4	7.4	0.0	16.9	25.1	31.8	28.9	36.3	4.1	0.0	0.0	0.0	0.0	0.4	0.0	0.0	4.5	0.0
Methyl salicylate	119-36-8	A	2.7	2.7	3.5	0.0	9.0	1.4	0.0	0.0	7.5	35.0	2.9	6.0	4.7	10.0	2.4	0.0	0.0	1.4	0.0	0.0
Nonanoic acid	112-05-0	A	4.4	11.9	17.5	21.1	0.0	9.1	0.9	0.0	0.0	18.5	0.0	0.1	1.5	4.6	2.8	0.0	0.0	4.3	0.0	0.0
Octanenitrile	124-12-9	A	0.0	0.3	2.9	0.0	7.0	0.0	0.0	0.0	0.0	10.1	6.0	0.0	4.5	4.7	6.4	0.0	0.0	0.0	2.4	0.0
Octanoic acid (Caprylic acid)	124-07-2	A	5.7	5.5	1.8	4.6	0.0	0.0	0.0	0.0	8.0	16.3	10.3	0.0	0.0	0.0	0.0	2.9	0.7	0.0	0.0	0.0
Propyl paraben	94-13-3	A	4.3	10.0	0.4	6.6	3.0	21.0	38.7	39.9	43.0	41.0	0.0	0.9	0.0	4.3	0.0	0.0	0.0	0.0	0.0	0.0
Propylene glycol	57-55-6	A	2.6	2.0	0.0	3.4	0.8	0.0	0.0	1.3	12.2	14.4	0.0	5.4	0.0	0.0	0.3	2.1	8.6	4.9	7.2	13.4
Salicylic acid	69-72-7	A	7.2	0.0	4.0	8.9	19.0	0.1	2.6	0.0	6.2	23.4	7.0	7.8	9.7	0.0	7.4	0.0	1.0	0.0	1.5	0.0

Streptomycin sulfate	3810-74-0	A	0.0	0.0	0.0	1.3	4.4	1.0	0.0	0.0	0.0	0.0	4.0	0.0	0.5	10.5	0.0	1.2	0.0	0.5	0.0	0.0
Sulphanilamide	63-74-1	A	3.4	1.0	0.0	1.5	0.5	4.4	6.8	8.6	9.4	5.5	0.0	0.0	5.7	0.4	2.6	5.6	3.1	3.5	0.0	1.0
Tartaric acid	87-69-4	A	0.0	1.0	0.0	2.6	5.1	1.0	2.3	0.0	4.4	41.7	10.7	7.4	11.4	0.0	4.9	1.6	3.2	2.4	0.0	1.2
Weak																						
Diethyl acetaldehyde	97-96-1	A	0.0	0.0	7.4	9.3	30.8	0.0	0.0	13.5	8.0	35.5	0.0	2.7	1.0	0.1	20.6	2.8	0.0	4.8	4.2	16.7
7-Hydroxycitronellal	107-75-5	A	0.0	0.0	0.0	0.0	2.3	1.9	0.0	0.0	0.0	0.0	5.0	0.0	5.1	5.0	17.9	6.8	8.4	11.1	25.8	53.3
Ethyl acrylate	140-88-5	A	6.2	7.9	35.9	77.4	78.2	0.0	5.8	14.6	46.9	59.9	0.8	0.0	2.1	15.5	29.6	0.0	0.0	0.0	0.0	26.1
Methyl acrylate	96-33-3	A	7.4	17.6	51.8	79.3	84.9	0.0	0.0	11.7	50.7	70.0	0.0	0.0	4.6	8.5	60.3	2.1	ND	3.8	4.3	59.3
Citral	5392-40-5	A	1.3	10.1	3.4	9.7	25.3	0.0	0.0	10.6	37.8	17.0	10.0	0.0	5.2	12.5	31.4	8.4	7.8	0.5	5.2	15.4
Farnesal	19317-11-4	A	8.7	0.0	1.1	11.2	5.0	0.0	0.0	0.0	9.0	20.8	6.3	6.8	0.9	4.3	31.8	0.0	7.0	0.0	8.0	28.7
2,3-Butanedione	431-03-8	A	0.8	3.8	10.1	29.3	41.8	0.0	0.0	3.7	3.5	23.6	5.4	0.0	5.2	11.9	32.4	0.0	0.0	0.0	8.8	53.7
Hexyl cinnamic aldehyde	101-86-0	A	0.0	0.0	6.5	3.1	3.4	0.5	0.0	4.6	7.9	3.9	0.0	0.0	0.0	0.0	0.0	0.7	9.3	4.2	0.0	0.0
α-Amyl cinnamic aldehyde	122-40-7	A	0.0	3.1	2.0	4.2	11.0	0.0	0.0	8.8	13.2	14.6	3.0	0.0	0.0	8.1	0.0	11.3	2.8	0.0	3.4	0.0
2-Methylundecanal	110-41-8	A	5.1	5.2	3.8	6.5	2.8	1.7	2.1	2.8	0.0	0.0	2.2	7.1	6.7	4.5	22.0	0.0	0.3	3.5	5.8	37.4
Moderate																						
2-Phenylpropionaldehyde	93-53-8	A	2.3	0.0	0.0	0.0	11.2	2.6	0.5	10.3	27.1	22.1	0.0	0.0	0.0	0.0	0.4	4.7	0.0	0.0	15.3	31.3
Diethyl maleate	141-05-9	A	8.0	14.6	39.4	88.3	92.5	0.0	0.0	6.9	8.6	38.7	0.0	0.0	8.3	5.0	38.1	0.0	28.0	4.9	0.0	43.7
3,4-Dihydrocoumarin	119-84-6	A	0.0	0.0	14.6	26.1	19.7	0.0	0.0	0.0	0.0	0.0	1.5	10.6	32.7	51.9	55.6	0.0	0.0	24.3	45.3	56.9
Benzylidene acetone (4-phenyl-3-buten-2-one)	122-57-6	A	7.3	9.4	52.8	79.5	85.2	3.1	3.8	9.7	32.6	46.4	2.7	12.8	0.0	16.8	27.6	0.0	2.6	2.7	8.9	25.9
Cinnamic aldehyde	104-55-2	A	0.0	5.3	12.0	53.8	68.2	0.0	0.0	2.6	36.7	47.4	2.1	4.0	0.0	0.0	0.0	1.4	1.0	0.0	0.0	0.0
Phenylacetaldehyde	122-78-1	A	1.9	1.7	0.0	0.2	5.8	0.3	0.0	9.8	14.8	21.6	0.0	0.0	1.5	3.9	0.0	2.4	0.0	9.5	10.2	5.2
Glyoxal	107-22-2	A	0.0	3.6	23.7	79.2	93.7	0.0	0.9	4.4	26.5	86.2	4.6	0.0	0.0	18.5	67.0	0.0	1.1	0.5	15.8	62.0
2-Hydroxyethyl acrylate	818-61-1	A	10.1	56.7	82.7	83.9	92.3	6.9	7.0	29.2	65.7	91.1	0.0	0.0	4.1	12.6	78.0	0.2	2.8	4.2	24.7	81.3
Strong/Extreme																						
Hexahydrophthalic anhydride	85-42-7	B	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.5	0.0	29.5	4.1	2.0	6.7	14.2	29.4	0.0	2.0	7.3	14.5	22.8
Propyl gallate	121-79-9	A	34.8	66.2	82.5	96.8	98.4	58.9	65.1	74.0	76.4	96.1	0.0	0.0	11.3	28.1	68.1	0.0	4.9	18.4	52.1	86.6
β-Propiolactone	57-57-8	A	0.0	11.0	33.6	50.3	91.2	1.1	6.0	11.2	58.0	91.4	2.3	16.1	5.9	18.5	49.8	8.5	2.3	3.7	15.5	48.2
Fluorescein-5-isothiocyanate	3326-32-7	C	0.0	1.5	0.0	13.1	33.8	0.0	0.0	16.9	35.0	44.1	0.0	0.1	3.3	0.9	37.7	0.0	1.7	0.0	10.7	41.8
Glutaraldehyde	111-30-8	D	0.0	0.0	11.6	71.2	88.0	0.0	0.0	0.0	18.8	59.7	0.9	8.9	23.9	38.8	63.4	0.0	0.0	8.8	40.8	72.1
Phenyl isocyanate	103-71-9	A	0.0	0.3	0.0	0.0	1.9	7.6	29.6	13.0	18.7	17.5	0.0	0.0	0.0	9.6	47.6	1.5	0.0	0.0	19.9	49.2

p-Benzoquinone	106-51-4	A	0.0	0.0	0.0	24.1	100	2.6	0.0	11.5	72.0	100	1.4	7.7	15.0	68.5	92.8	0.0	0.0	14.4	74.8	100
4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone)	15646-46-5	A	3.9	4.2	0.0	13.9	8.9	6.0	16.5	11.8	27.6	34.3	0.6	10.8	9.2	29.3	49.1	0.0	1.0	12.0	26.3	50.0

Test Chemical Concentrations:
A: C1 = 0.003 mM, C2 = 0.03 mM, C3 = 0.3 mM, C4 = 3.0 mM, C5 = 30 mM
B: C1 = 0.001 mM, C2 = 0.01 mM, C3 = 0.1 mM, C4 = 1.0 mM, C5 = 10 mM
C: C1 = 0.0001 mM, C2 = 0.001 mM, C3 = 0.01 mM, C4 = 0.1 mM, C5 = 1.0 mM
D: C1 = 0.0015 mM, C2 = 0.015 mM, C3 = 0.15 mM, C4 = 1.5 mM, C5 = 15 mM
E: C1 = 0.000275 mM, C2 = 0.00275 mM, C3 = 0.0275 mM, C4 = 0.275 mM, C5 = 2.75 mM
F: C1 = 0.002 mM, C2 = 0.02 mM, C3 = 0.2 mM, C4 = 2.0 mM, C5 = 20 mM
G: C1 = 0.000937 mM, C2 = 0.00937 mM, C3 = 0.0937 mM, C4 = 0.937 mM, C5 = 9.37 mM
H: C1 = 0.00095 mM, C2 = 0.0095 mM, C3 = 0.095 mM, C4 = 0.95 mM, C5 = 9.5 mM

^aReactivity was determined following 24 hr incubation under ambient conditions in reactions containing cysteine (20 μM) or lysine (5 μM) peptide in the presence and absence of HRP (3.0 U/mL) and hydrogen peroxide (100 μM) in 0.1 M potassium phosphate buffer (pH 7.4) or 0.1 M ammonium acetate buffer (pH 10.2), respectively.

LLNA potency data was from Gerberick GF., et al, (2005), except for 3-methylcatechol (unpublished data). NS = non-sensitizer. NC = not calculated. ND = Not determined.

TABLE 3

Peptide Depletion Data for Pre-/Pro-haptens

Cysteine Peptide Depletion (%)													Lysine Peptide Depletion (%)									
(-HRP/P)							(+HRP/P)						(-HRP/P)					(+HRP/P)				
Test Chemical Name	CAS No.	Test Chemical Conc	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5
Weak																						
Aniline	62-53-3	A	0.0	0.0	2.0	7.0	9.9	0.0	11.0	7.5	21.5	31.9	6.2	0.0	0.0	9.9	0.0	0.0	0.0	2.9	0.0	0.1
Methyleugenol	93-15-2	A	4.5	6.7	7.9	6.0	10.0	0.0	1.1	23.0	60.4	45.7	0.0	1.1	0.0	0.0	0.0	0.0	5.0	0.0	3.3	0.0
Cinnamyl Alcohol	104-54-1	A	0.0	0.3	0.0	3.0	13.8	0.0	0.0	8.1	11.0	23.8	0.5	11.6	4.6	3.4	6.1	2.2	0.0	0.0	0.0	1.0
Pentachlorophenol	87-86-5	A	0.0	0.0	0.0	0.0	0.0	2.9	5.1	9.8	29.9	42.9	1.9	15.3	12.2	10.7	13.5	3.1	1.3	0.0	0.0	0.8
Eugenol	97-53-0	A	3.5	5.1	6.3	65.1	67.5	10.9	41.0	51.3	48.8	99.4	2.2	5.6	0.3	6.3	0.0	7.0	0.0	1.8	0.0	0.9
Moderate																						
Dihydroeugenol (2-methoxy-4-propylphenol)	2785-87-7	A	0.0	0.0	0.0	2.9	9.7	16.9	46.1	64.8	66.3	99.0	0.0	0.0	0.0	0.0	7.4	4.2	0.0	2.3	0.0	2.6
2-Methoxy-4-methylphenol	93-51-6	A	1.2	0.0	0.0	8.2	34.2	20.7	53.8	71.0	75.4	99.0	5.7	0.0	0.0	5.4	3.3	0.0	0.0	1.2	0.0	0.0
5-Amino-2-methylphenol; 4-Amino-2-hydroxytoluene	2835-95-2	A	7.4	1.6	21.6	59.6	69.7	6.2	16.9	24.0	42.8	84.0	0.0	4.5	4.9	25.1	39.2	1.7	0.0	13.0	33.0	44.3
3-Aminophenol	591-27-5	E	0.0	0.0	0.5	3.7	2.5	0.0	27.7	31.5	69.1	97.7	1.1	0.0	0.0	16.6	18.6	0.0	0.2	12.1	2.8	0.0
1-Naphthol	90-15-3	A	0.0	0.0	9.1	27.6	0.0	0.0	15.2	60.2	98.9	98.1	0.0	8.8	4.8	14.9	35.7	2.0	9.4	33.7	25.2	31.4
Isoeugenol	97-54-1	C	0.0	1.3	15.4	59.1	90.5	0.0	2.6	33.8	71.0	67.6	4.3	7.7	6.9	0.0	0.0	1.9	1.7	3.6	0.0	0.0
Strong/Extreme																						
4-(N-Ethyl-N-2-methan-sulphonamido-ethyl)-2-methyl-1,4-phenylenediamine (CD3)	25646-71-3	A	0.0	0.0	9.2	50.6	95.2	0.0	17.2	43.5	64.7	98.9	0.0	1.8	0.0	0.0	0.0	11.3	9.4	10.4	6.6	0.0
2-Aminophenol	95-55-6	A	0.7	0.0	0.0	8.6	29.9	0.0	0.0	45.0	39.1	99.1	0.0	0.0	0.4	8.3	0.0	0.0	4.1	0.0	9.0	0.0
2-Nitro-1,4-	5307-	F	0.0	13.2	29.7	65.1	93.7	17.1	41.6	45.5	99.6	100	0.6	0.0	1.3	8.3	5.0	0.0	0.0	0.0	9.8	9.9

phenylendiamine	14-2																					
Lauryl gallate	1166-52-5	G	0.0	0.0	4.7	25.2	71.2	65.3	76.0	82.7	88.4	80.2	0.0	0.0	7.8	7.7	3.9	0.0	0.3	35.8	41.2	35.3
1,4-Phenylenediamine	106-50-3	H	7.6	13.0	43.3	79.7	98.0	11.6	59.0	51.3	88.9	100	6.5	1.7	13.4	13.2	5.0	4.4	3.7	3.0	16.7	16.3
Hydroquinone	123-31-9	A	5.2	21.2	43.5	45.7	56.7	28.6	34.8	34.3	63.5	100	5.8	5.9	19.0	46.0	100	6.1	10.2	30.1	85.2	100
Bandrowski's Base (N,N-bis(4-aminophenyl)-2,5-diamino-1,4-quinone-diimine)	20048-27-5	A	16.1	58.0	69.0	98.6	98.2	20.6	48.8	62.2	95.3	100	0.0	5.8	3.9	6.3	35.1	1.4	10.4	13.5	0.0	24.6
3-Methylcatechol	488-17-5	A	4.0	72.4	86.0	91.3	98.0	32.7	73.2	70.7	100	100	5.6	3.7	11.7	34.0	74.9	0.0	0.0	3.4	46.3	100

Test Chemical Concentrations:
A: C1 = 0.003 mM, C2 = 0.03 mM, C3 = 0.3 mM, C4 = 3.0 mM, C5 = 30 mM
B: C1 = 0.001 mM, C2 = 0.01 mM, C3 = 0.1 mM, C4 = 1.0 mM, C5 = 10 mM
C: C1 = 0.0001 mM, C2 = 0.001 mM, C3 = 0.01 mM, C4 = 0.1 mM, C5 = 1.0 mM
D: C1 = 0.0015 mM, C2 = 0.015 mM, C3 = 0.15 mM, C4 = 1.5 mM, C5 = 15 mM
E: C1 = 0.000275 mM, C2 = 0.00275 mM, C3 = 0.0275 mM, C4 = 0.275 mM, C5 = 2.75 mM
F: C1 = 0.002 mM, C2 = 0.02 mM, C3 = 0.2 mM, C4 = 2.0 mM, C5 = 20 mM
G: C1 = 0.000937 mM, C2 = 0.00937 mM, C3 = 0.0937 mM, C4 = 0.937 mM, C5 = 9.37 mM
H: C1 = 0.00095 mM, C2 = 0.0095 mM, C3 = 0.095 mM, C4 = 0.95 mM, C5 = 9.5 mM

TABLE 4

Cooper Statistics

**Chemical
Classification
(based on LLNA)**

	Non-Sensitizer	Sensitizer ^a	total
Non-Sensitizer	16	9	25
Sensitizer	3	42	45
total	19	51	70

table statistics for the shadowed 2 x 2 table

<i>sensitivity:</i>	93%
<i>specificity:</i>	64%
<i>positive predictivity:</i>	82%
<i>negative predictivity:</i>	84%
<i>accuracy:</i>	83%

^a Test chemicals with depletion >10% for cysteine (-HRP/P), >40% for cysteine (+HRP/P), >15% for lysine (-HRP/P) or >15% for lysine (+HRP/P) were classified as sensitizers.

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TABLE 5

EC15 Values for Peptide-Reactive Test Chemicals

Test Chemical Name	CAS No.		EC15 (mM) ^a					Corresponding Peptide (+HRP/P)
			Cysteine (-HRP/P)	Cysteine (+HRP/P)	Lysine (-HRP/P)	Lysine (+HRP/P)	Overall Lowest	
Non-Sensitizer								
1-Bromobutane	109-65-9	Reactive-NS	2.3	0.55	NC	NR	0.55	Cys +HRP/P
4-Hydroxybenzoic acid	99-96-7	Reactive-NS	NC	NR	NC	NR	NR	Cys and Lys +HRP/P
Coumarin	91-64-5	Reactive-NS	60	NC	2.6	10	2.6	Lysine -HRP/P
Hexane	110-54-3	Reactive-NS	NR	NC	NC	NC	NR	Cysteine -HRP/P
Isopropanol	67-63-0	Reactive-NS	3.7	NC	NC	NC	3.7	Cysteine -HRP/P
Nonanoic acid	112-05-0	Reactive-NS	NR	NC	NC	NC	NR	Cysteine -HRP/P
Propyl paraben	94-13-3	Reactive-NS	NC	NR	NC	NC	NR	Cysteine +HRP/P
Salicylic acid	69-72-7	Reactive-NS	13	NC	NC	NC	13	Cysteine -HRP/P
Tartaric acid	87-69-4	Reactive-NS	NC	5.3	NC	NC	5.3	Cysteine +HRP/P
Weak								
Aniline	62-53-3	Pre-/pro-hapten	NC	NC	NC	NC	NC	N/A
Diethyl acetaldehyde	97-96-1	Hapten	5.5	NC	15	24	5.5	Cysteine –HRP/P
7-Hydroxycitronellal	107-75-5	Hapten	NC	NC	20	0.47	0.47	Lysine +HRP/P
Methyleugenol	93-15-2	Pre-/pro-hapten	NC	0.26	NC	NC	0.26	Cysteine +HRP/P
Ethyl acrylate	140-88-5	Hapten	0.088	0.27	2.8	21	0.088	Cysteine –HRP/P
Cinnamyl Alcohol	104-54-1	Pre-/pro-hapten	NR	NC	NC	NC	NR	Cysteine –HRP/P
Methyl acrylate	96-33-3	Hapten	0.021	0.40	5.2	8.3	0.021	Cysteine –HRP/P
Pentachlorophenol	87-86-5	Pre-/pro-hapten	NC	0.50	NR	NC	0.50	Cysteine +HRP/P
Citral	5392-40-5	Hapten	5.5	NC	3.6	NR	3.6	Lysine –HRP/P
Eugenol	97-53-0	Pre-/pro-hapten	0.46	0.0011	NC	NC	0.0011	Cysteine +HRP/P
Farnesal	19317-11-4	Hapten	NR	NC	5.2	4.3	4.3	Lysine +HRP/P
2,3-Butanedione	431-03-8	Hapten	0.55	NC	4.3	4.2	0.55	Cysteine –HRP/P
Hexyl cinnamic aldehyde	101-86-0	Hapten	NC	NC	NC	NC	NC	N/A
α-Amyl cinnamic aldehyde	122-40-7	Hapten	NR	NC	NC	NC	NR	Cysteine –HRP/P
2-Methylundecanal	110-41-8	Hapten	NC	NC	11	8.7	8.7	Lysine +HRP/P
Moderate								

Dihydroeugenol (2-methoxy-4-propyl-phenol)	2785-87-7	Pre-/pro-hapten	NC	0.00038	NC	NC	0.00038	Cysteine +HRP/P
2-Phenylpropionaldehyde	93-53-8	Hapten	40	NC	NC	3.0	3.0	Lysine +HRP/P
Diethyl maleate	141-05-9	Hapten	0.046	NC	7.9	15	0.046	Cysteine –HRP/P
2-methoxy-4-methylphenol	93-51-6	Pre-/pro-hapten	4.0	0.00057	NC	NC	0.00057	Cysteine +HRP/P
3,4-Dihydrocoumarin	119-84-6	Hapten	NR	NC	0.056	0.16	0.056	Lysine –HRP/P
Benzylidene acetone (4-phenyl-3-buten-2-one)	122-57-6	Hapten	0.042	0.52	2.4	8.2	0.042	Cysteine –HRP/P
5-Amino-2-methylphenol; 4-Amino-2-hydroxytoluene	2835-95-2	Pre-/pro-hapten	0.19	0.066	1.1	0.42	0.066	Cysteine +HRP/P
3-Aminophenol	591-27-5	Pre-/pro-hapten	NC	0.0018	0.24	NC	0.0018	Cysteine +HRP/P
Cinnamic aldehyde	104-55-2	Hapten	0.35	0.97	NC	NC	0.35	Cysteine –HRP/P
Phenylacetaldehyde	122-78-1	Hapten	NC	NC	NC	NC	NC	N/A
Glyoxal	107-22-2	Hapten	0.18	1.4	2.7	2.9	0.18	Cysteine –HRP/P
2-Hydroxyethyl acrylate	818-61-1	Hapten	0.0043	0.078	3.5	27	0.0043	Cysteine –HRP/P
1-Naphthol	90-15-3	Pre-/pro-hapten	NR	0.034	2.4	NR	0.034	Cysteine +HRP/P
Isoeugenol	97-54-1	Pre-/pro-hapten	0.0099	0.0056	NC	NC	0.0056	Cysteine +HRP/P
Strong/Extreme								
Hexahydrophthalic anhydride	85-42-7	Hapten	NC	NC	1.1	1.0	1.0	Lysine +HRP/P
4-(N-Ethyl-N-2-methan-sulphonamido-ethyl)-2-methyl-1,4-phenylenediamine (CD3)	25646-71-3	Pre-/pro-hapten	0.54	0.022	NC	NC	0.022	Cysteine +HRP/P
2-Aminophenol	95-55-6	Pre-/pro-hapten	3.8	0.079	NC	NC	0.079	Cysteine +HRP/P
2-Nitro-1,4-phenylenediamine	5307-14-2	Pre-/pro-hapten	0.040	0.0014	NC	NC	0.0014	Cysteine +HRP/P
Propyl gallate	121-79-9	Hapten	0.00032	NR	0.78	0.21	0.00032	Cysteine –HRP/P
Lauryl gallate	1166-52-5	Pre-/pro-hapten	0.44	NR	NC	0.037	0.037	Lysine +HRP/P
1,4-Phenylenediamine	106-50-3	Pre-/pro-hapten	0.0098	0.00024	NC	0.31	0.00024	Cysteine +HRP/P
β-Propiolactone	57-57-8	Hapten	0.052	0.37	1.1	2.7	0.052	Cysteine –HRP/P
Fluorescein-5-isothiocyanate	3326-32-7	Hapten	0.11	0.0095	0.53	0.12	0.0095	Cysteine +HRP/P
Hydroquinone	123-31-9	Pre-/pro-hapten	0.014	0.0010	0.18	0.10	0.0010	Cysteine +HRP/P
Glutaraldehyde	111-30-8	Hapten	0.19	1.3	0.056	0.30	0.056	Lysine –HRP/P
Phenyl isocyanate	103-71-9	Hapten	NC	NC	4.1	2.7	2.7	Lysine +HRP/P
Bandrowski's Base (N,N-bis(4-aminophenyl)-2,5-diamino-1,4-quinone-diimine)	20048-27-5	Pre-/pro-hapten	0.0013	0.00086	7.8	6.7	0.00086	Cysteine +HRP/P
3-Methylcatechol	488-17-5	Pre-/pro-hapten	0.0062	0.00017	0.44	0.97	0.00017	Cysteine +HRP/P
p-Benzoquinone	106-51-4	Hapten	2.4	0.38	0.27	0.32	0.27	Lysine –HRP/P
4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone)	15646-46-5	Hapten	NR	NC	0.43	0.70	0.43	Lysine –HRP/P

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^aPeptide reactivity, expressed in terms of EC15, is an estimate of the test chemical concentration that depletes the peptide by 15% under the conditions described. EC15 was estimated by fitting a three-parameter log-logistic model to peptide depletion data using RExcel. If peptide depletion did not exceed 10% for cysteine without HRP/P, 40% for cysteine with HRP/P, >15% for lysine with or without HRP/P, reactivity was reported as ‘NC’ (not calculated). If peptide depletion exceeded a threshold value (above) but was generally low or insufficient for estimating the EC15, reactivity was designated as NR (not reported). Non-sensitizers that were NC for cysteine and lysine $\pm$ HRP/P were excluded from this table. N/A = not applicable.

FIGURE LEGENDS

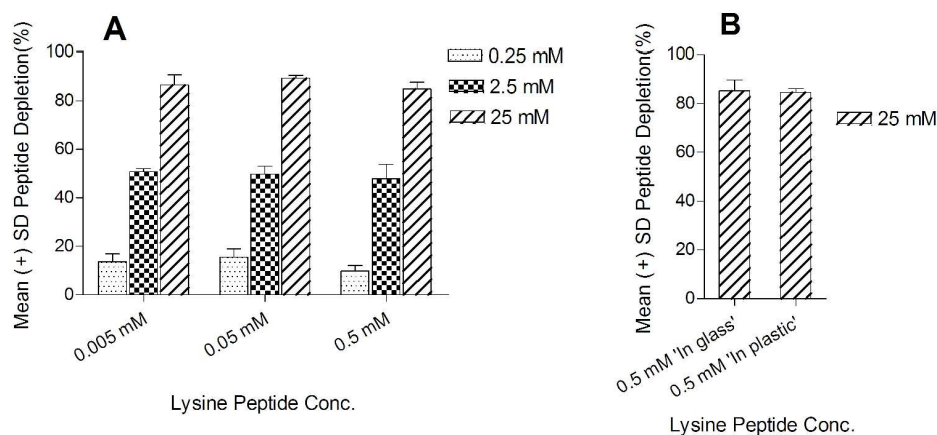
Figure 1. Direct lysine peptide reactivity with p-benzoquinone at peptide and chemical concentrations of (A) 0.005, 0.05 and 0.5 mM and 0.25, 2.5 and 25 mM, respectively. Incubations were performed in a 96-well plate at a final reaction volume of 0.3 mL (in triplicate). (B) Comparator reactions were prepared in glass (in triplicate) to mimic incubation conditions in the DPRA (i.e., final reaction volume of 1.0 mL at peptide and chemical concentrations of 0.5 mM and 25 mM, respectively). Immediately following addition of test chemical to these samples, an aliquot of the reaction was transferred to a 96-well plate (in plastic) and incubated for 24 hr. The figures show the percentage of lysine peptide depletion, expressed in terms of mean ($\pm$ SD) of triplicate incubations.

Figure 2. Direct lysine peptide reactivity with 0.005 mM lysine peptide and test chemical concentrations indicated. Incubations were performed in a 96-well plate at a final reaction volume of 0.3 mL (in duplicate). The figure shows the percentage of lysine peptide depletion, expressed in terms of the average of duplicate incubations.

Figure 3. Effect of incubation temperature on direct lysine peptide depletion. Incubations were performed in a 96-well plate at a final reaction volume of 0.3 mL. The figure shows the percentage of lysine peptide depletion with p-benzoquinone (p-Benz), 3-methylcatechol (3-MC), α -methyl cinnamaldehyde (α -methyl CA) and glutaraldehyde. RT = room temperature.

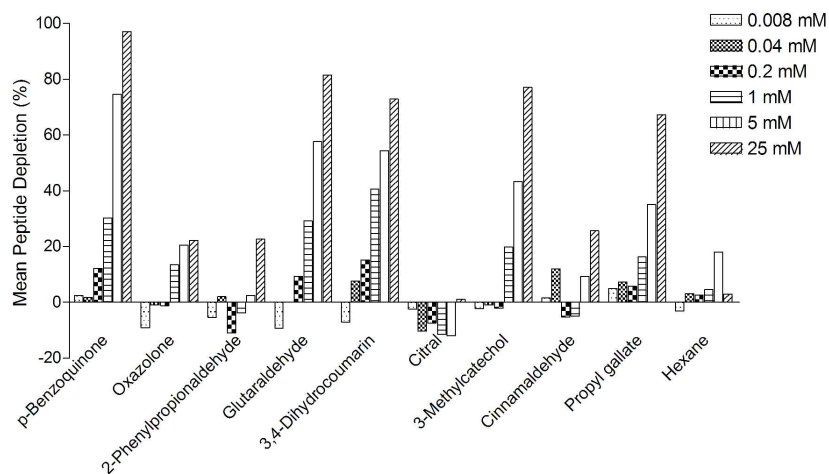
Figure 4. Box plots of the lowest EC15 values for each peptide-reactive test chemical and sensitization category. The box gives the distribution of EC15 values between the first and third quartiles; the median value (second quartile) is indicated by a black horizontal line within each

box. The whiskers indicate the greatest and least EC15 values, excluding outliers which are identified by an open circle. Outliers are determined based on the interquartile range (IQR); observations less than the first quartile – 1.5*IQR or greater than the third quartile + 1.5*IQR are declared outliers. The two outliers shown are 1-bromobutane (NS) and eugenol (weak sensitizer).



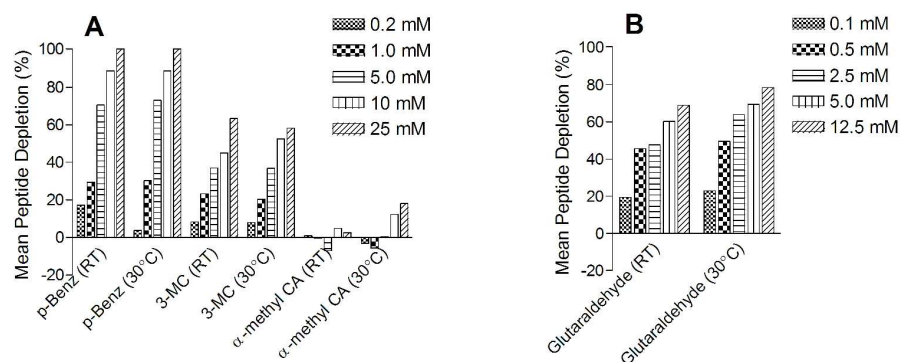
Direct lysine peptide reactivity with p-benzoquinone at peptide and chemical concentrations of (A) 0.005, 0.05 and 0.5 mM and 0.25, 2.5 and 25 mM, respectively. Incubations were performed in a 96-well plate at a final reaction volume of 0.3 mL (in triplicate). (B) Comparator reactions were prepared in glass (in triplicate) to mimic incubation conditions in the DPRA (i.e., final reaction volume of 1.0 mL at peptide and chemical concentrations of 0.5 mM and 25 mM, respectively). Immediately following addition of test chemical to these samples, an aliquot of the reaction was transferred to a 96-well plate (in plastic) and incubated for 24 hr. The figures show the percentage of lysine peptide depletion, expressed in terms of mean (+ SD) of triplicate incubations.

137x69mm (600 x 600 DPI)



Direct lysine peptide reactivity with 0.005 mM lysine peptide and test chemical concentrations indicated. Incubations were performed in a 96-well plate at a final reaction volume of 0.3 mL (in duplicate). The figure shows the percentage of lysine peptide depletion, expressed in terms of the average of duplicate incubations.

181x96mm (600 x 600 DPI)



Effect of incubation temperature on direct lysine peptide depletion. Incubations were performed in a 96-well plate at a final reaction volume of 0.3 mL. The figure shows the percentage of lysine peptide depletion with p-benzoquinone (p-Benz), 3-methylcatechol (3-MC), α -methyl cinnamaldehyde (α -methyl CA) and glutaraldehyde. RT = room temperature.
143x62mm (600 x 600 DPI)

