Slides for ICCVAM Meeting

The Development and Application of Peptide Reactivity Assays for Skin Sensitization Risk Assessment

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Presented by: Darrell R. Boverhof
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January 20, 2011

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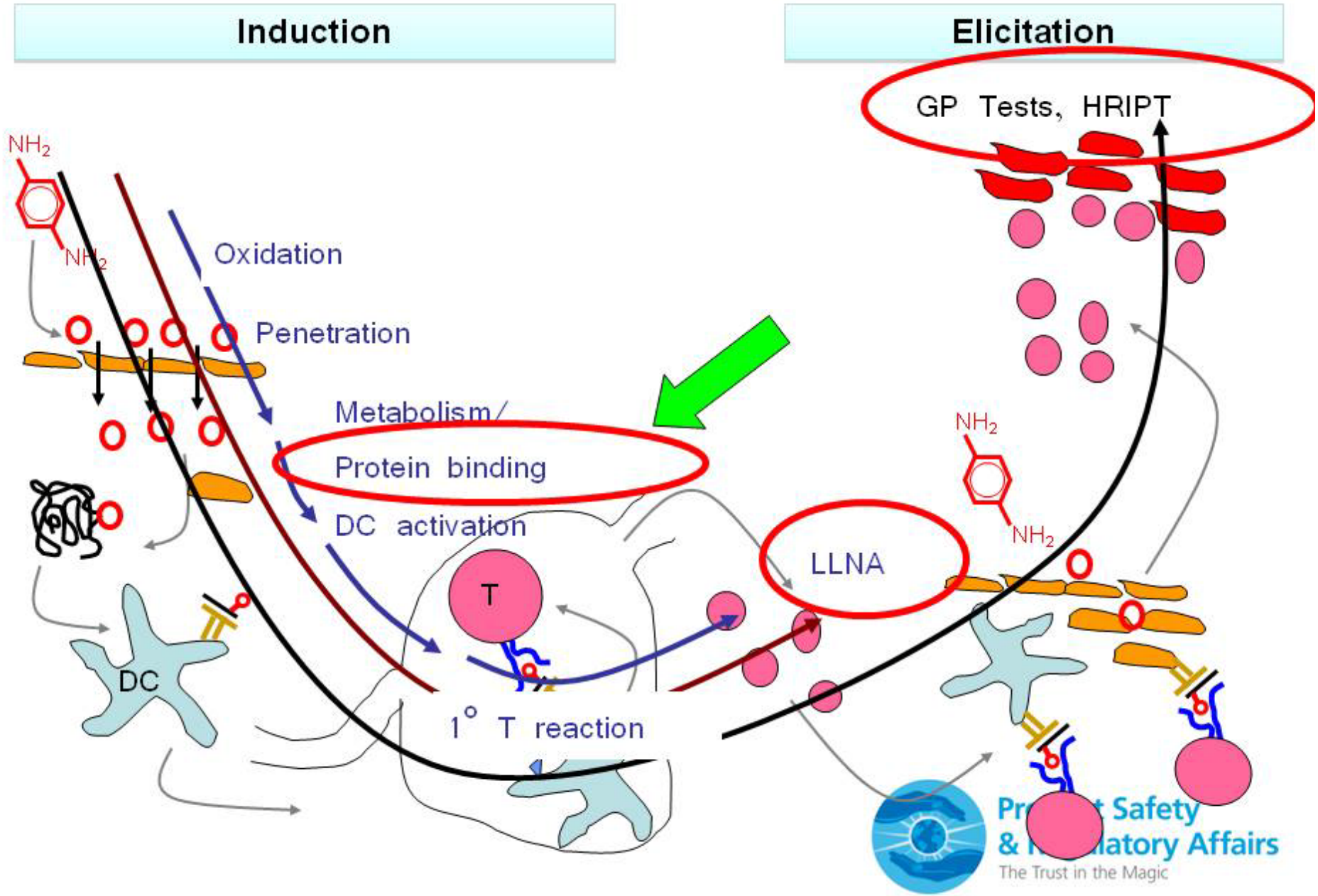
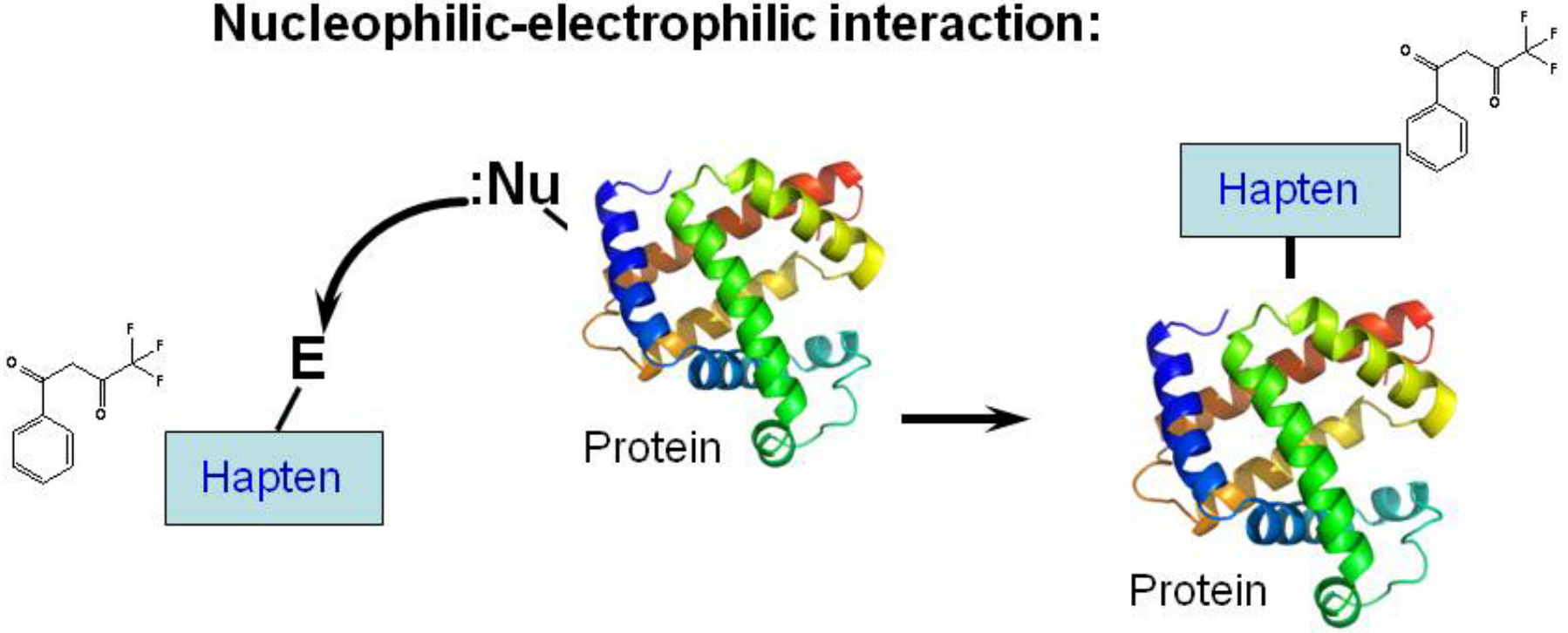
Predictive Tests for Sensitization



Chemical-Protein Reactivity, Metabolism and Skin Sensitization

Nucleophilic-electrophilic interaction:



The correlation of skin protein reactivity and skin sensitization is well established and has been known for many years.

(Landsteiner and Jacobs, 1936; Dupuis and Benezra, 1982; Lepoittevin et al, 1998)

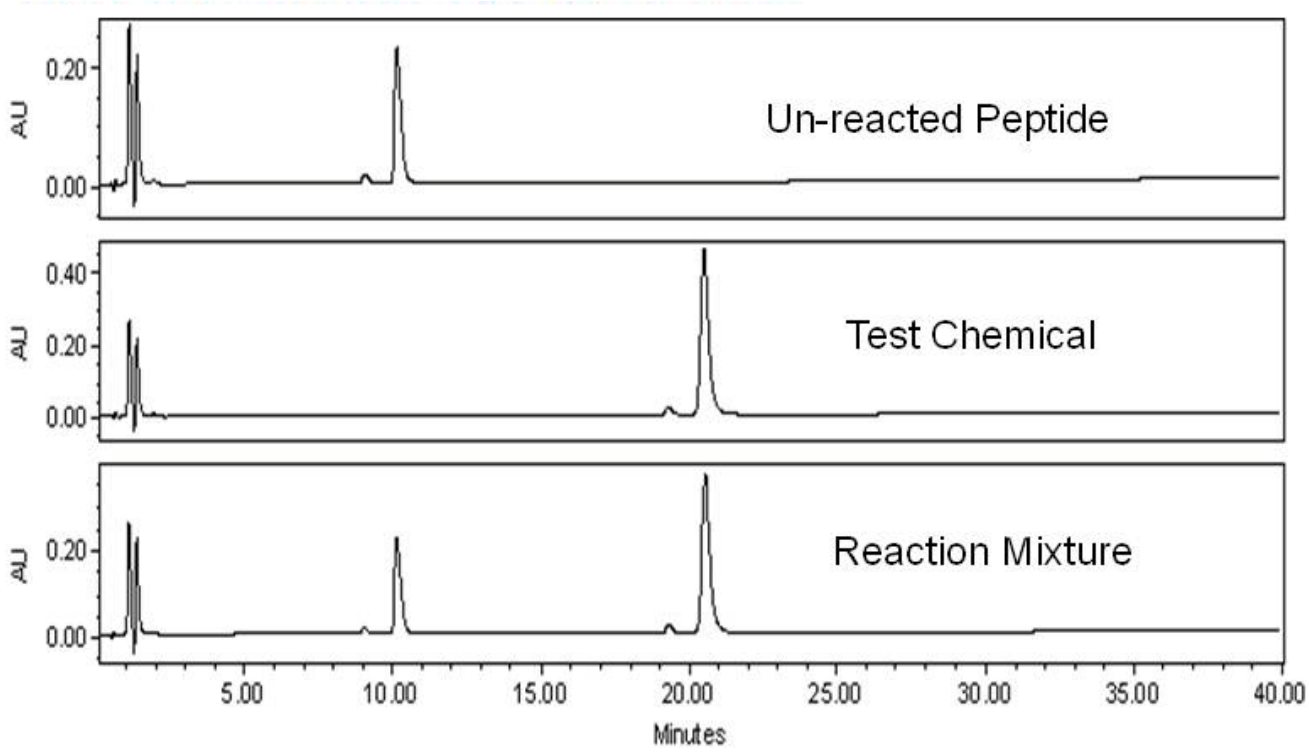
Leads to stable association with proteins, in order that an immunogenic complex is created; this requires that the chemical is inherently protein reactive, or can be transformed in a protein reactive species within the skin.

Readout for Direct Peptide Reactivity Assay (DPRA): Peptide Depletion

Test chemical dissolved in acetonitrile.

Test chemical incubated with peptide (10:1 or 50:1) for 24 hours.

Peptide depletion monitored by HPLC at 220 nm.



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Development and Optimization of the DPRA

TOXICOLOGICAL SCIENCES **81**, 332–343 (2004)
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Advance Access publication July 14, 2004

Development of a Peptide Reactivity Assay for Screening Contact Allergens

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Gerberick, *et al*. (2004) *Tox. Sci*. **81**, 332-343

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Development and Optimization of the DPRA

- Objective: Determine if chemical reactivity toward nucleophilic amino acids correlates with sensitization potential
  - Examined reactivity of 38 different chemicals with varying degrees of sensitization potency:
    - 11 non sensitizers
    - 7 weak sensitizers
    - 11 moderate sensitizers
    - 5 strong sensitizers
    - 4 extreme sensitizers
  - Evaluated reactivity toward glutathione, or 3 synthetic peptides (cysteine, lysine, histidine)
  - After the chemical:peptide incubation, samples analyzed by HPLC-UV for peptide depletion.
  - Also evaluated parameters such as kinetics and peptide:chemical concentration ratios

Gerberick, *et al*. (2004) *Tox. Sci*. **81**, 332-343

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Development and Optimization of the DPRA

	Peptide			
	Glutathione	**Lysine**	**Cysteine**	**Histidine**
Sensitivity	55.6%	53.8%	80.8%	11.5%
Specificity	90.9%	100.0%	90.9%	100.0%
Accuracy	65.8%	66.7%	83.8%	36.1%

- Results:
  - Significant correlation was identified between sensitization potency and peptide depletion to glutathione and cysteine and lysine peptides
  - Provided initial evidence for utility of assessing peptide reactivity for assessment of sensitization potential

Gerberick, *et al*. (2004) *Tox. Sci*. **81**, 332-343

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Development and Optimization of the DPRA

TOXICOLOGICAL SCIENCES **97(2)**, 417–427 (2007)
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Advance Access publication March 30, 2007

Quantification of Chemical Peptide Reactivity for Screening Contact Allergens: A Classification Tree Model Approach

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Gerberick *et al*. (2007). *Tox. Sci.*, **97**, 417-427

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Development and Optimization of the DPRA

- Test chemical set expanded to 82 (all with existing LLNA data; 38 original plus 44 new)
- 3 Nucleophiles/Peptides: Glutathione, Cysteine and Lysine
- Use two ratios of peptide: test chemical (1:10 and 1:50)
- Reaction time set to 24 hours
- Monitored peptide depletion by HPLC-UV

Gerberick *et al*. (2007). *Tox. Sci.*, **97**, 417-427

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Results based on Cys 1:10 and Lys 1:50 (n=81)

Predicted Classification (based on classification tree model)

Chemical Classification[a] LLNA data	Non-Sensitizer	Sensitizer	total
Non-Sensitizer	**26**	**3**	**29**
Sensitizer	**6**	**46**	**52**
total	**32**	**49**	**81**

sensitivity:	88%	(46/52)
specificity:	90%	(26/29)
accuracy:	**89%**	((26+46)/81)

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Use of Classification Tree Approach for Analysis of GSH, Cys and Lys Data

- A form of binary recursive partitioning
- Used when observations need to be assigned to a category based on a number of predictor variables:
  - non-sensitizer, weak, moderate, strong
- Used peptide depletion data and LLNA potency data to generate models

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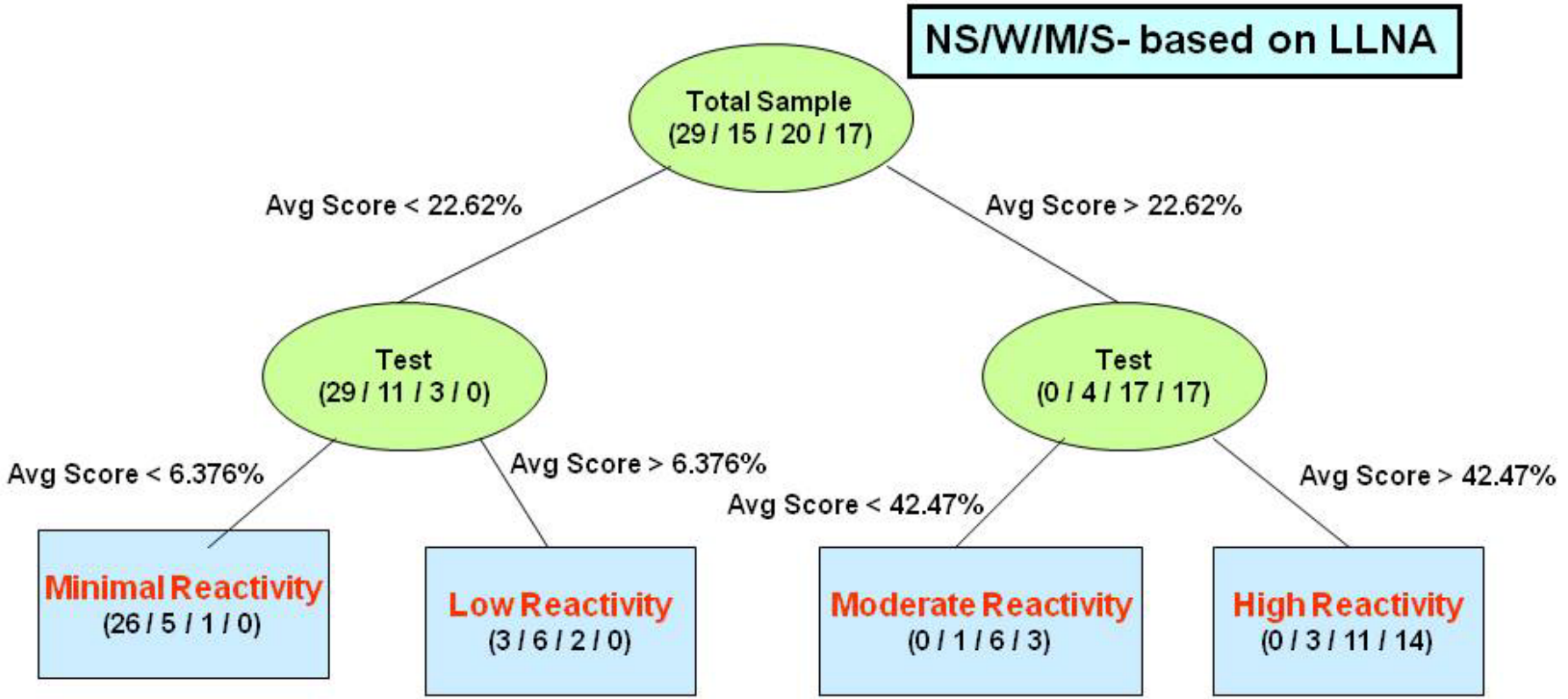
Prediction Model for predicting potency- based on Cys 1:10 and Lys 1:50 (n=81)



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Additional Analysis of Chemicals in the DPRA

- 76 new test chemicals analyzed with Cysteine and Lysine since the prediction model was developed
- Total compounds tested to date = 157
  - 38 Extreme/Strong
  - 43 Moderate
  - 38 Weak
  - 38 Non-sensitizers
- Accuracy = 85%

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Inter-laboratory Studies to evaluate Direct Peptide Reactivity Assay

- We have completed 2 Inter-laboratory studies to evaluate the transferability of the DPRA.
- Scientists from Kao, L'Oreal and Givaudan visited P&G for "hands on" training
- Ring Trial 1 consisted of 15 chemicals with very good results
- Ring Trial 2 consisted of 28 chemicals
- The chemicals of Ring Trial 2 proved to be a bit more challenging but provided us with an opportunity to improve the SOP
- The 2 successful inter-laboratory studies encouraged us to move forward with ECVAM for validation of the assay.

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ECVAM Pre-validation of DPRA

- Test Submission to ECVAM – February, 2009
- DPRA SOP finalized – December, 2009
- Participating labs for pre-validation study identified – January, 2010
- Training and Transfer plan approved – February 2010
- ECVAM Pre-validation
  - Phase A, Stage I: SOP training- March 31, 2010
  - Phase A, Stage II: SOP transfer- June 30, 2010
  - Phase B, Stage I: 9 chemicals- July 31, 2010
  - Phase B, Stage II: 15 chemicals- September 15, 2010
  - Data analysis (ECVAM biostatistician)- March 31, 2011
  - Final Pre-validation Report- May 31, 2011

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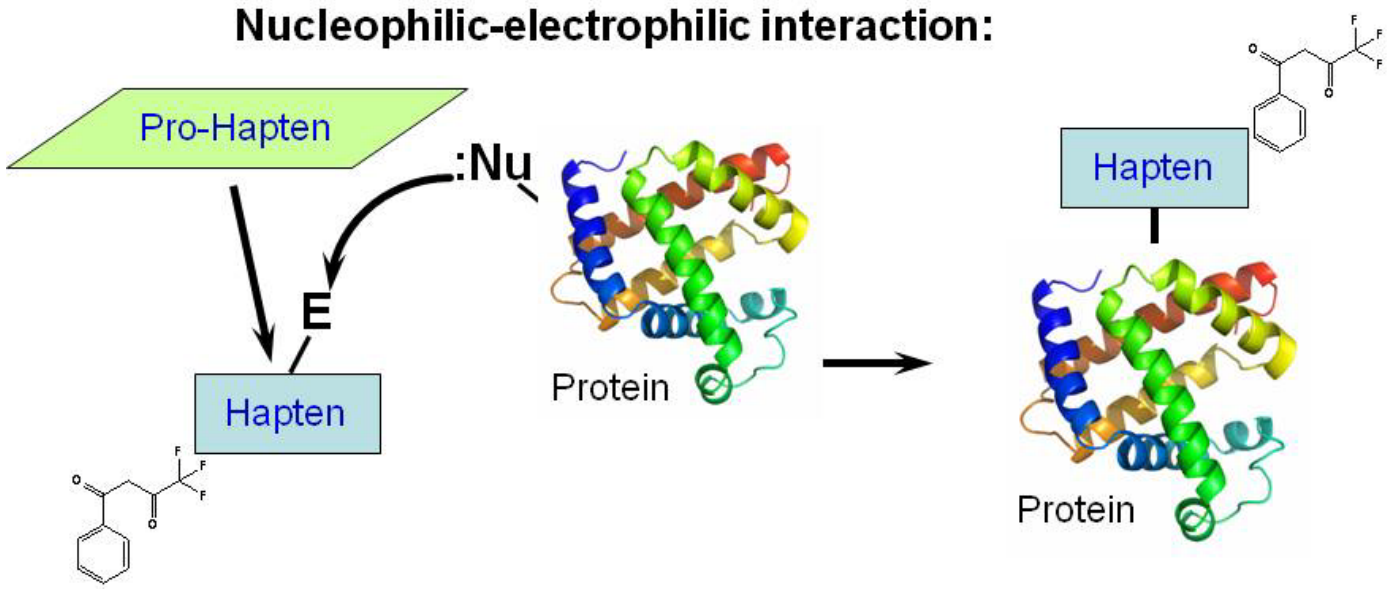
Chemical-Protein Reactivity, Metabolism and Skin Sensitization



- **Limitation of the DPRA is that it cannot readily measure the reactivity of pro-hapten chemical sensitizers. Pro-haptens are chemical sensitizers that are not directly reactive and must first be bio-activated in vivo to become reactive**

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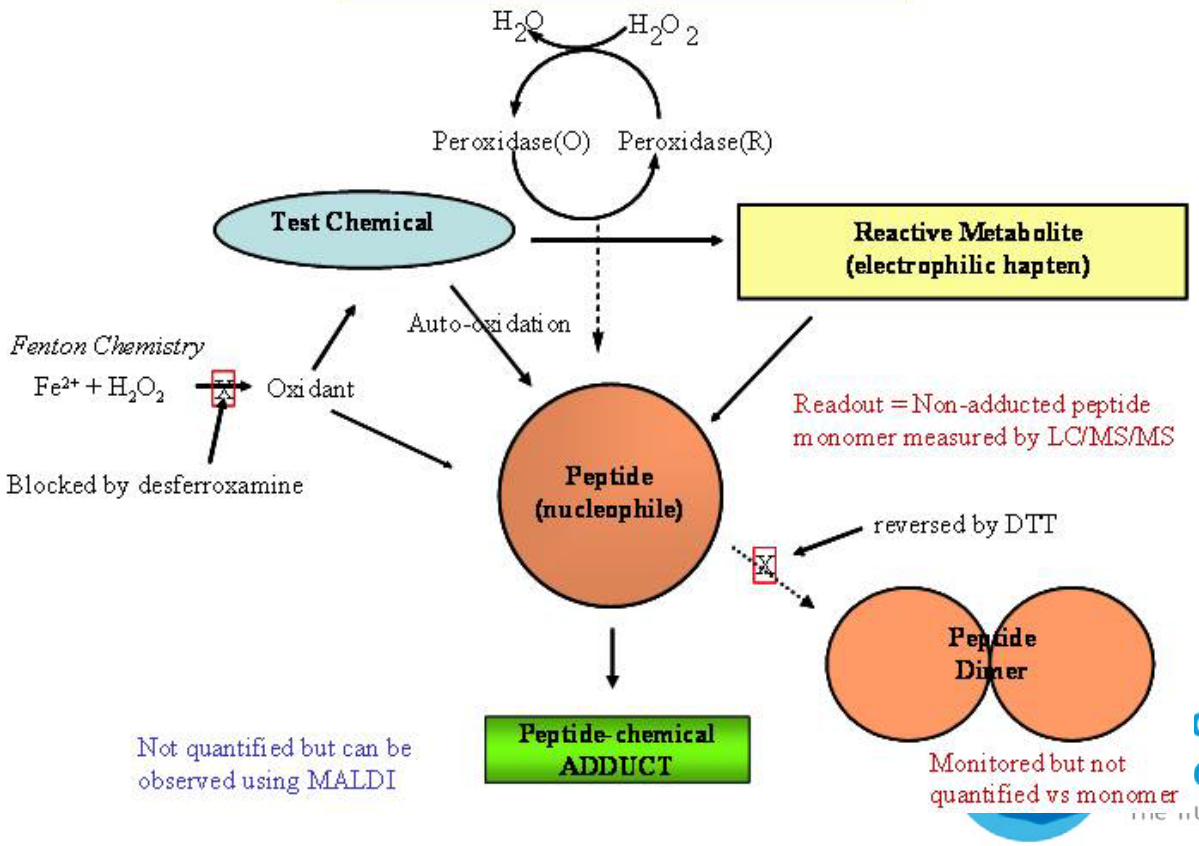
Next Generation Peptide Reactivity Assay

Objective: Develop a modified version of the DPRA to incorporate an activation step for identifying pro-hapten chemical sensitizers.

Principle of the Assay



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Optimization of Assay Conditions with Cysteine Peptide

- **Peroxide concentration**
- **Peroxidase concentration**
- **Incubation time**

- **<u>Test Chemicals</u>:**
  - **2-Aminophenol**
  - **Eugenol**
  - **1,4-Phenylenediamine**
  - **2-Methoxy-4-methylphenol**
  - **3-Methylcatechol**

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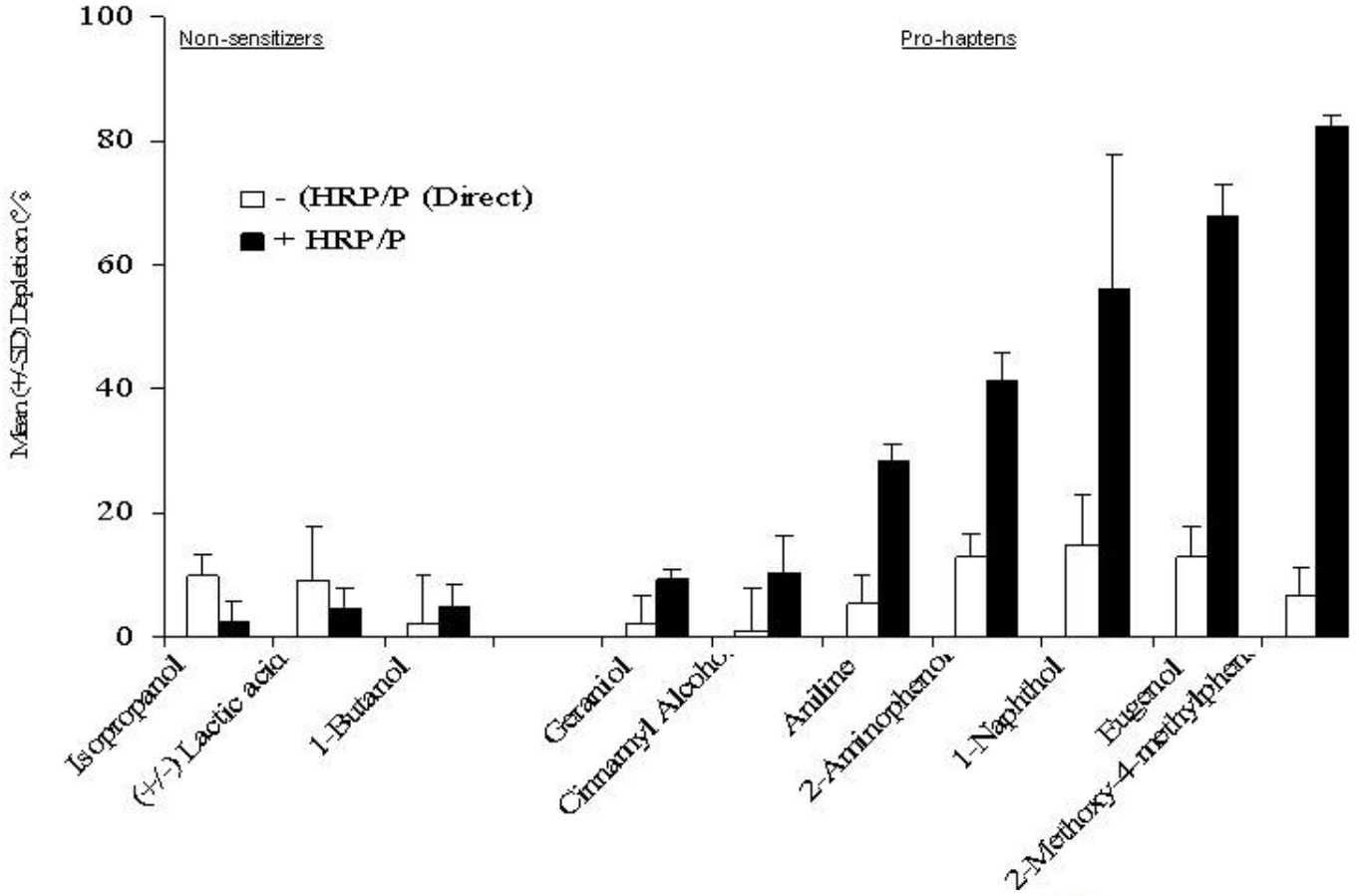
Reactivity Screen with Cysteine under optimized Conditions



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Reactivity Screen with Cysteine under optimized Conditions

TOXICOLOGICAL SCIENCES **112(1)**, 164–174 (2009)
doi:10.1093/toxsci/kfp192
Advance Access publication September 11, 2009

Investigation of Peptide Reactivity of Pro-hapten Skin Sensitizers Using a Peroxidase-Peroxide Oxidation System

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Received June 25, 2009; accepted August 4, 2009

- Peptide Reactivity Summary:
- Depletion was generally ≤ 10% for non-sensitizers with or without HRP
- Prohapten sensitizers showed minimal to no peptide depletion in the absence of HRP/P
- Addition of HRP/P resulted in statistically significant increases in peptide depletion for all pro-haptens

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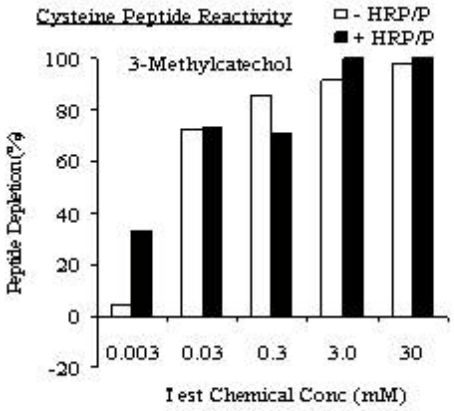
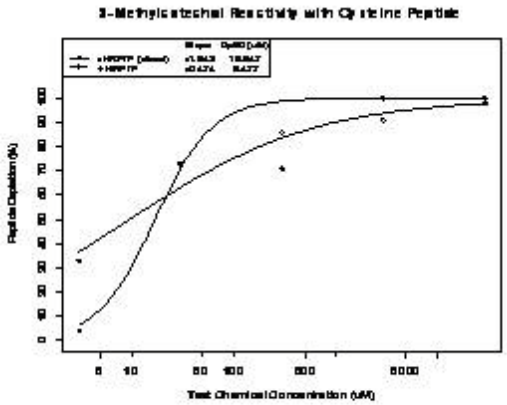
Current Process being considered for RA

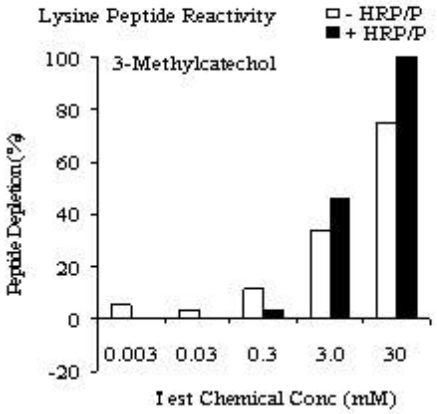
Characterize peptide depletion as a function of test chemical concentration with cysteine and lysine ± HRP/P

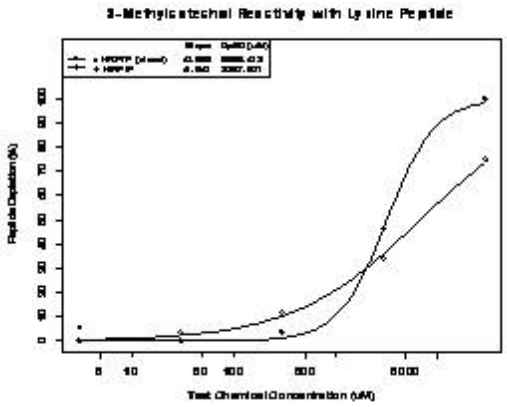
Quantify reactivity by estimating the concentration of test chemical that depletes the peptide by 50% (RC50) within 24 hrs

Correlate with additional endpoints using an integrated testing strategy for predicting skin sensitization potential









Peptide	RC50 (mM) - HRP/P (Direct)	RC50 (mM) + HRP/P
Cysteine	0.0166	0.0101
Lysine	4.68	2.94

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Preliminary Results with Cysteine and Lysine + HRP/P with Dose-Response

		RC50 (mM)*						
		Cysteine Peptide		Lysine Peptide				
Test Chemical	Conc range examined (mM)	–HRP/P (Direct)	+ HRP/P	–HRP/P (Direct)	+ HRP/P	Lowest RC50 Observed (mM)	Corresponding Nucleophile	LLNA Potency Category
Glycerol	0.003-30	NC	NC	NC	NC	NC	minimal reactivity (<10%)	non-sensitizer
Hexane	0.003-30	NC	NC	NC	NC	NC	minimal reactivity (<10%)	non-sensitizer
1-Butanol	0.003-30	NC	NC	NC	NC	NC	minimal reactivity (<10%)	non-sensitizer
(+/-) Lactic acid	0.003-30	NC	NC	NC	NC	NC	minimal reactivity (<10%)	non-sensitizer
Methyl salicylate	0.003-30	NC	NC	NC	NC	NC	minimal reactivity (<10%)	non-sensitizer
Geraniol	0.003-30	NC	NC	NC	NC	NC	minimal reactivity (<10%)	weak
3,4-Dihydrocoumarin	0.003-30	NC	NC	4.2	ND	NC	Lysine (Direct)	moderate
2-Phenylpropionaldehyde	0.003-30	NC	NR	NC	85.1	85.1	Lysine +HRP/P	moderate
Cinnamic alcohol	0.003-30	NC	37	NC	NC	37	Cysteine (+ HRP/P)	weak
Isopropanol	0.003-30	33.2	NC	NC	NC	33.2	Cysteine (Direct)	non-sensitizer
Phenylacetaldehyde	0.003-30	NC	28.4	NC	NC	28.4	Cysteine (+ HRP/P)	moderate
Hydroxycitronellal	0.003-30	NC	NC	NC	17.2	17.2	Lysine (+ HRP/P)	weak
2,3-Butanedione	0.003-30	11.7	NC	NR	NC	11.7	Cysteine (Direct)	weak
1,2-Dibromo-2,4-dicyanobutane	0.003-30	11.0	13.7	NC	NC	11.0	Cysteine (Direct)	strong
Aniline	0.003-30	NC	10.6	NC	NC	10.6	Cysteine (+ HRP/P)	weak
Cinnamaldehyde	0.008-10	5.1	17	NC	ND	5.1	Cysteine (Direct)	moderate
Cinnamaldehyde	0.003-30	4.3	16	NC	ND	4.3	Cysteine (Direct)	moderate
Glyoxal	0.003-30	0.887	6.80	14.4	17.9	0.887	Cysteine (Direct)	moderate
Glutaraldehyde	0.003-30	0.753	9.26	3.74	3.15	0.753	Cysteine (Direct)	strong
1-Chloro-2,4-dinitrobenzene	0.003-30	0.41	31.2	NC	NC	0.41	Cysteine (Direct)	strong
Diethyl maleate	0.003-30	0.409	NC	NC	NC	0.409	Cysteine (Direct)	moderate
Hydroquinone	0.003-30	3.45	0.307	2.64	0.613	0.307	Cysteine (+ HRP/P)	strong
p-Benzoquinone	0.00032-1.0	0.282	0.578	2.5	ND	0.282	Cysteine (Direct)	extreme
1-Naphthol	0.003-30	NR	0.187	22.5	NR	0.187	Cysteine (+ HRP/P)	moderate
4-Amino-m-cresol	0.0001-1.0	0.137	0.420	NC	NR	0.137	Cysteine (Direct)	moderate
Eugenol	0.0001-1.0	NC	0.0813	NC	NC	0.0813	Cysteine (+ HRP/P)	weak
Isoeugenol	0.0001-1.0	0.0676	0.0475	NC	NC	0.0475	Cysteine (+ HRP/P)	moderate
1,4-Phenylenediamine	0.00032-1.0	0.0394	0.0185	NC	ND	0.0185	Cysteine (+ HRP/P)	strong
3-Methylcatechol	0.003-30	0.0166	0.0101	4.68	2.94	0.0101	Cysteine (+ HRP/P)	strong/mod
3-Methylcatechol	0.003-30	0.0168	0.00948	6.89	3.24	0.00948	Cysteine (+ HRP/P)	strong/mod

*RC50 values were estimated using REXcel which fits a two-parameter log-logistic model to peptide reactivity data, and graphs the raw data and fitted curves.
NC = not calculated (peptide depletion did not exceed 10% across concentration range)
NR = not reported (peptide depletion did not exceed 10% for the two highest concentrations tested, or depletion was low (< 20%) and did not increase with an increase in test chemical concentration)
ND = not determined (not tested to date)

Rank order from low to high for the most reactive nucleophile

Trends in peptide reactivity appear to coincide well with general trends in LLNA-based potency classifications

Summary

- Gerberick et al. have made significant progress on the development of a non-animal test for the assessment of skin sensitization potential
- Results with the DPRA have shown great promise and have led to wider validation efforts
- Initial results evaluating the addition of HRP/P to the assay system show promise for the identification of pro-haptens
- Initial RD50 potency assessment approach also looks promising

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