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B.A. (Chemistry) University of Oxford

Ph.D. Physical organic chemistry and NMR studies of mono- and bicyclic crown ethers and their complexes.

Post-Doc at Oxford University: Electronic structure of organometallic compounds studies by photoelectron spectroscopy.

1989-1992: Regulatory Toxicologist, UK HSE Toxicology Unit.


Applications of Quantitative Measurements of Reactivity with Model Nucleophiles to the Toxicological Hazard Assessment of Chemicals:


Martin Payne

Lhasa Limited
Introduction → Defra-LINK project
Toxicity vs chemical reactivity – Qualitative or quantitative correlations (or neither)?
Aquatic toxicity prediction in Derek for Windows
Peptide reactivity assays and skin sensitisation
SAR and in vitro assays for skin sensitisation potential
Putting it all together – Integrated testing strategies for skin sensitisation.
Conclusions
Lhasa Limited

In Silico Tools

**Meteor**
An expert system for the prediction of the metabolic fate of xenobiotics

**Derek for Windows**
An expert system for the prediction of toxicity

**Vitic**
A structure-searchable toxicity database
Defra-LINK Project (2008-2010)
http://inchemicotox.org/

Defra-LINK Project Participants
(from http://inchemicotox.org/Collaborating_Partners/collaborating_partners.html)

Liverpool John Moores University
- Prof. Mark Cronin (Project Leader)

University of Tennessee (subcontractor)
- Prof. Terry Schultz

Lhasa Limited
- MPP and Dr. Philip Judson

Fund for the Replacement of Animals in Medical Experimentation (FRAME) (subcontractor)
- Prof. Michael Balls

British Union for the Abolition of Vivisection
- Dr. Katy Taylor

International Foundation for QSAR to Reduce Animal Use
- Prof. Gill Veith

Proctor and Gamble
- E.g. Dr Petra Kern

Shell Chemicals
- Dr. Graham Whale

Unilever Research (SEAC):
- Drs. Nora Aptula and Steve Gutsell

WCA Environmental Ltd:
- Dr. Mark Crane

Marks and Spencer
- Dr. Katy Taylor

Defra-LINK Project Participants
(from http://inchemicotox.org/Collaborating_Partners/collaborating_partners.html)
Quality datasets:
- http://inchemicotox.org/Results/results.html (.xls, .pdf, .txt)

“Tools, for reactive toxicity”:
- “Derek Alerts for Excess Aquatic Toxicity with Semi-Quantitative Predictions”
  Presentation: https://www.lhasalimited.org/icgm/icgm_reports/
  (February 2010)

Integrated testing strategies for skin sensitisation and acute fish toxicity.

Toxicity and Chemical Reactivity – Qualitative or Quantitative Correlations (or neither)?
Quantitative In Vivo Toxicity - Reactivity Correlations? “Get real!”

Toxicity depends on
- The nature, extent and consequences of reactions/interactions with a range of biologically significant and less significant molecules at various points in time and in different tissues, physiological matrices and cellular regions.

Toxicokinetics:
- Absorption, distribution, metabolism and elimination
  - Metabolism to reactive intermediate(s)
    E.g.: Epoxides, nitrenium ions, quinones etc
- Reactions with cellular nucleophiles can be reversible
- Reactions with cellular nucleophiles may be detoxifying
  - E.g. Glutathione conjugation products, excretable as mercapturates
  - Increases with reaction rates (alkyl halides I>Br>Cl>>F)

Toxicodynamics – Mechanism of Toxicity

Definition:
- Interactions with biologically significant molecules (proteins, DNA, etc) causing adverse change or loss of function

Relevant interactions can be bond forming, non-covalent or both
- Non-covalent interactions:
  E.g. with receptors, enzymes, ion-channels, DNA etc
  Production of gene transcription factors etc

Note:
- Non-metabolic transformation products may be important
  - Terpenoids giving auto-oxidation products (hydroperoxides).
  - Catechols, hydroquinones and amine analogues give quione (or similar) products
- Hydrolysis and volatility may be significant issues in vivo and experimentally
Correlations of In Vitro Toxicity with Reactivity:
Genotoxicity

4-(4-Nitrobenzyl)pyridine (NBP) frequently used to model reactivity of guanine nitrogen on DNA bases.

“In vitro induction of micronuclei by monofunctional methanesulphonic acid esters: possible role of alkylation mechanisms.”
- Good correlations of Ames mutagenicity with reaction rates
- Compounds with high $S_n1$ hydrolysis rates had lower mutagenicity due to hydrolysis.

In vitro micronucleus test – activity inversely correlated with the bacterial mutagenicity:
- Reaction with spindle protein?
- More bulky methane sulphonates more potent - greater disturbance during mitosis?

Correlations of In Vitro Toxicity with Reactivity:
Hepatotoxicity and Neurotoxicity

**Hepatotoxicity:**

“Structure-activity relationships for thiol reactivity and rat or human hepatocyte toxicity induced by substituted p-benzoquinone compounds.”

- Rat hepatotoxicity correlated with:
  - Non-enzymatic reaction rates with glutathione, or QM reactivity descriptors
- Correlations for human hepatocytes were poorer

**Neurotoxicity:**

Correlations of In Vivo Toxicity with Reactivity:

**Skin Sensitisation:**
- Many examples of experimental reactivity based QSARs for closely related reactive chemicals (i.e. same mechanistic domain), but LogK\textsubscript{ow} may also be required.
- “Relative alkylation index” concept – David Roberts et al from c. 1980 onward
  - Sensitisation for a given class dependent on lipophilicity (e.g. LogK\textsubscript{ow}) and reactivity (e.g. with butylamine).

Correlations of In Vivo Toxicity with Reactivity:

**Aquatic toxicity:** Many examples
  - Hermens et al. used reaction rates with 4-nitrobenzyl pyridine.
  - Freidig P and Hermens JLM, QSAR 19, 547-553 (2002)
- Numerous papers by Terry Schultz, David Roberts, Nora Aptula and others using glutathione reactivity (RC\textsubscript{50}) assay results
Correlations of *In Vivo* Toxicity with Reactivity

**Favoured by:**
- a directly reactive toxicant
- easily accessible affected tissues (skin or respiratory sensitisation, acute aquatic toxicity of relatively polar chemicals) and
- a homologous series of chemicals operating by a single reactivity mediated mechanism:

*Other properties (e.g. lipophilicity) may also need to be considered*
Log (1/IGC50) vs CLogP
(Tetrahymena pyriformis)


Chemicals with the Highest Calculated Excess Toxicities
(Log[IGC50(expt.)/IGC50(NPN)]), log(1/IGC50(mM)), CLogP
Aquatic Toxicity Prediction
Example: 2,4-Dinitrophenol

- Predicted potency, log (1/IGC50) = 0.7 to 2.1
- Experimental potency, log(1/IGC50) = 1.06

Peptide (and Related) Reactivity Assays
Peptide Reactivity Assays

**Procter and Gamble: Gerberick F et al 2004, 2007**
- Peptide % depletion assays (GSH, lysine and cysteine peptides).
  - Recent variant includes a metabolic activation model based on incubation with HRP/P.
- Lysine peptide Ac-RFAAKAA-COOH, cysteine: Ac-RFAACAA-COOH
- Glutathione: γ-L-Glutamyl-L-cysteinylglycine

**Givaudan Schweiz: Natsch A et al 2007, 2008**
- Peptide reactivity (LC-MS assay) (lower concentrations), peptide oxidation assayed. Peptide with both cysteine thiol and lysine amino groups in a Ac-NKKCDLF unit

**Unilever SEAC: Aleksic M et al 2008, Optimised assays**
- [Peptides with: lysine pH 10, arginine pH 10, tyrosine pH 10, cf. histidine, cysteine pH 7.4],

Glutathione Reactivity Assays

**University of Tennessee: Schultz assay**
- 50% GSH depletion, RC50 value
- Quantified spectrophotometrically at 412 nm
- Reaction of GSH with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) – can be limited by coloured products
- Cheap and easy to conduct, gives rate constants

**UFZ, Leipzig: Schuurmann et al 2009 assay**
- Kinetic GSH chemoassay, similar to the Schultz assay + GSSG formation from oxidation

Glutathione: ECG
Andreas Natsch, Givaudon Schweiz:

- Cysteine reactivity dependent, antioxidant response element, gene induction assay (Keap1-Nrf2-ARE pathway)
  - 2008: AREc32 from human MCF7 breast cancer cell line
  - 2010: Keratinocyte-based reporter cell line

Correlations with LLNA EC3 Values

- Medium and Strong skin sensitizers:
  > approx. 10% depletion in Gerberick Assay (cysteine plus lysine)
    - Weak or non-skin sensitisers
      < 10% peptide depletion

- Use of cysteine and lysine peptide nucleophiles gives good coverage of sensitisers/non-sensitisers
Correlations of skin sensitisation potential with peptide reactivity

Procter and Gamble: Gerberick F et al 2009

- Classification tree model – average of cysteine (1:10) (pH 7.5) and lysine peptide (1:50) % depletion (pH10.2) :
- Minimal (0-6.4%), low 6.4-22.6, moderate 22.6-42.5, high 42.5-100%

Respectively:
NS/W/M/S: 26/5/1/0 3/6/2/0 0/1/6/3 0/3/11/14

Exceptional:

<table>
<thead>
<tr>
<th>Nonanoyl chloride</th>
<th>Medium (EC3 1.8%)</th>
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Exceptional includes:
- Nonanoyl chloride: Medium (EC3 1.8%) Low (9.1)
- Tetrachlorosalicylanilide: Extreme (0.04%) Moderate (22.9±20?)
- 2,3-Butane-dione: Weak (11%) High (53) (arginine?)
- Ethyl acrylate: Weak (28%) High (95)

Correlations of Skin Sensitisation Potential with Peptide Reactivity

Schultz assay (50% GSH depletion)
- Reactive halobenzenes – at least qualitative correlations (2,4- vs 3,5-dinitro halobenzenes)
- Catechols/hydroquinones etc – aerial oxidation/metabolism influences correlations

Roberts and Natsch:
- \( pEC3 = a \cdot \log K_{cy} + 2.11 \) (Micheal acceptors)

Also Roberts et al on haloaliphatic compounds Chem. Res. Tox. 2010

- \( pEC3 = 0.85 \log k_{NB} + 0.92 \) Micheal acceptors
- \( pEC3 = 0.83 \log k_{NB} + 2.13 \frac{S_1}{S_2} \)
In Vitro Assays for Skin Sensitisation Potential

Three in vitro methods, namely,

- The Direct Peptide Reactivity Assay (Gerberick et al)
- The Myeloid U937 Skin Sensitisation Test (MUSST) (CD86, IL-1β (for DC mobilization), IL-8), and the
- Human Cell Line Activation Test (h-CLAT), optimised through COLIPA ring trials, CD54, CD86

Have entered a formal pre-validation studies (ECVAM/NTP-ICVAM) in which they will be assessed independently (as partial replacement methods) with a set of coded compounds.
Dendritic Cell Activation Assays

- Progressing but not yet validated
- Issues (a personal selection):
  - Includes the reactivity component
  - Excludes role of keratinocytes vs Langerhans and/or other dendritic cells
  - Other cytokine/chemokine signals etc are neglected.
  - No quantitative correlation with sensitisation potency
  - Dendritic cell precursors recruited from circulation > resident ones
    - Inflammation (skin irritation/cytotoxicity) promotes recruitment
  - Inter-individual variation – tolerance and suppression mechanisms

Mechanistic complexity in vivo: e.g. Vocanson M et al. Effector and regulator mechanisms in allergic contact dermatitis. Allergy 64, 1699-1714 (2009).

Lymphocyte Activation/Proliferation Assays

- Little progress on assay
  - T-lymphocyte priming – dependent on large number of additional costimulatory molecules interactions
  - Need to consider MHC-peptide -TCR interaction etc and self vs non-self immunogenic regulation.

Challenge Phase:
  - Inflammatory events in liver and spleen are also significant in giving an optimum immunization: e.g. IL-4 from NK T cells
  - Ability to regulate CD8+ T cell response is subject to genetic variability.
In Vitro Skin Irritancy Assays

EpiDerm or EPIDERm: Three dimension reconstructed epidermis with functional stratum corneum – using 50% cytotoxicity as end-point (MTT assay dehydrogenase activity).

  Tox. Letts 180, 2008 9-20

Models “danger signal” response from keratinocytes in skin sensitisation

Sens-it-iv project: Potentially use to assess skin inflammatory response in skin sensitisation induction. Combine with IL-8 release assay using MUTZ-LC cells for qualitative assessment.

Putting it all together – Integrated testing strategies for skin sensitisation.
Purpose of Integrated Testing Strategy

**Function:** To provide a “strategy” or plan for toxicological hazard assessment that may be applied by industry and adopted by regulators.

The strategy will only use *in vivo* animal test methods as the last resort, and maximise the potential use of *in vitro*, *in chemico* and SAR methodology.

From Grindon et al. ATLA 35, 683-697 (2007)

ITS for Skin Sensitisation

1. Are there existing data to suggest that the substance is, or is not, sensitising to the skin?
   - No

2. Define mechanistic domain (if applicable) and collect any available data on the reactive chemistry of the test substance (or its chemical class; non-validated).
3. Use *in silico* methods (such as DEREK, TIMES, OECD QSAR Application Toolbox) to make predictions on skin sensitisation (non-validated).
4. Perform *in vitro* skin penetration study (OECD TG 428).
6. Perform *in vitro* cell based assays such as those involving dendriticLangerhans cells and/or T Lymphocytes (non-validated).
From Grindon et al. ATLA 35, 683-697 (2007)
ITS for Skin Sensitisation

7. Perform weight of evidence evaluation on all data so far. Does this show whether the substance is a skin sensitiser or not?
   - Yes: C&L and/or RA
   - No:

8. Is a full quantitative risk assessment required?
   - No:
   - Yes:

9a. Perform reduced LLNA assay (validated). (Use results as discussed in the text.)

9b. Perform standard Local Lymph Node Assay (LLNA; OECD TG 429). Are results suitable for RA?
   - Yes: RA
   - No:

Integrating in vitro/in chemico testing and “weight of evidence” evaluation for skin sensitisation

Data on chemical or mechanistic analogue

- Skin Penetration
- SAR Prediction
- Reactivity
- Dendritic Cell Activn.
- Skin Irritation
- Lymphocyte assay Self vs non-self
Integrating in vitro/ in chemico data


- 2,4,6-Trinitrobenzene sulfonic acid
- Hydroxyethyl-p-phenylene diamine LLNA –ve (in aq. acetone, o.oil)
- p-Toluylenediamine (PTD) LLNA –ve (in aq. acetone, o.oil)

CD 86, IL-1β and aquaporin P3 gene expression measured

"Prediction of skin sensitization potential is possible [using in vitro analysis of DC activation] provided that skin penetration data indicate sufficient bioavailability of the test compound"

Integrating in vitro/ in chemico data

Multiplicative functions


- (a) A structural alert (2 if yes, 1 if no)
- (b) Bioavailability (low vs high): Score 2 if high, otherwise 1 range,
- (c) Protein reactivity: Score 0-4
- (d) Dendritic cell maturation: Score 0-4
- (e) T-cell proliferation: Score 0-4

Index of Sensitisation Potency = 0 (none) – 256 (extreme)
Integrating the *in vitro/in chemico* data
Multiplicative functions

**Basketter DA and Kimber I. (J. Appl. Toxicol. 2009), 26, 341-350).**
Updating the skin sensitization in vitro assessment paradigm in 2009.

“This version is somewhat simpler and reflects better the current in vitro methods under active development and evaluation”

- (a) Bioavailability assay: Scale 0, 1, 2
- (b) Protein reactivity assay: Scale 0, 1, 2, 3, 4
- (c) Irritancy assay: Scale 1, 2
- (d) “Immunogenicity” assay: Scale 0, 1

(Actually suggested DC markers, e.g. CD54 or CD86 assays on THP1 cells)

**Sensitisation potency Index (SPI) =**

0 (none) – 1-4 weak > 5 (stronger) -16

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**Natsch A, Ernter R and Ellis G**
*Toxicological Sciences* 107, 106-121 (2009).

“Filling the Concept with Data. Integrating data with different in vitro and in silico assays on skin sensitizers to explore the battery approach for animal-free skin sensitization testing”

- (a) Cysteine depletion (EC50): Score 0-4
- (b) ARE induction (EC 1.5, I_{max}) (Scale 0-4)
- (c) A structural alert from TIMES-SS (2 if yes, 1 if no)
- (d) “Significant” bioavailability (low vs high): Score 2 (if CLogP between -2 and 5) or score 1 out of this range,

**Also used QSAR:**
- E.g. LLNA Class = 0.317 Cys Score + 0.376 EC1.5 (ARE) Score + 0.084 CLogP
- CLogP negligible: 0.357 Cys Score + 0.391 EC 1.5 (ARE) Score
Proposed Integrated Testing Strategy
For Skin Sensitisation (To be further developed)

**Reliable SS Data**

- **Yes**
- **No**

**Reduced sensitisation potential**

- **Yes**
- **No**

**Skin Penetration**

- **Yes**
- **No**

-2< LogKow < 5 (except inorganics)

**Sufficient peptide reactivity**

Cys, Lys, Arg

or ARE assay.

**Dendritic cell activn. assay**

Two or more markers

**Skin Irritation Assay or R38**

**Plus if one +ve**

**Robust SAR Prediction**

**Need clear estimate of reliability**

**If two positive assays, then skin sensitiser**

**Similar to self molecules**

**Reduced sensitisation potential**

**CONCLUSION AND ACKNOWLEDGEMENTS**
Conclusions

Reactivity rate measurements provide a useful tool for indicating potential for several forms of toxicity but may be limited by the additional complexities of *in vivo* cf. *in vitro* responses.

In particular, reactivity measurements using peptides have considerable quantitative predictive utility for skin sensitisation and acute aquatic toxicity.

Dendritic cell activation (DCA) assay results reflect a combination of protein reactivity (with or without metabolic activation), and only some elements of DCA.

An ITS for skin sensitisation can be potentially enhanced by using skin irritation information.

Qualitative and quantitative SARs for skin sensitization (e.g. from Derek) have a key role in any integrated test strategy.

Funding

This project was sponsored by Defra (UK) through the Sustainable Arable Link Programme.
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- Marks and Spencer

Thank you – Questions or Comments Please!