THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

Skin sensitisation prediction using *in vitro* screening methods

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B. Biotechnology (Hons)

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2015

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Abstract

My PhD research project was designed to bring innovation into *in vitro* methods for identification of industrial chemicals, particularly epoxy resin systems (ERS), with skin sensitising potential as a means to minimise the use of animals for this purpose. In my research, the generalisability of two *in vitro* methods originally developed and validated for identification of small molecules to assess the skin sensitising potential of ERS was assessed. Specifically, my research focussed on (i) the human cell line activation test (h-CLAT) which mimics the characteristics of Langerhans cells during the maturation process following their activation by chemical sensitisers, and (ii) the direct peptide reactivity assay (DPRA), that assesses the initial interaction between potential chemical sensitisers with human skin proteins. The ERS data generated using these *in vitro* methods were compared with the skin sensitisation data for the same compounds generated using the widely accepted murine local lymph node assay (LLNA) in order to gain insight into the accuracy and reliability of the *in vitro* methods.

For h-CLAT, I optimised the assay conditions for a 96-well format using $1.6x10^5$ cells/well as well as anti-CD54-FITC and anti-CD86-PE. The relative fluorescent intensity (RFI) of CD54 and CD86 on THP-1 cells was determined using three-coloured flow cytometry. A chemical was regarded as being a positive sensitiser if the RFI of CD54 was >200% and/or that for CD86 was >150%. Five ERS tested in the h-CLAT assay, *viz* bisphenol A diglycidyl ether (DGEBA), trimethylolpropane triglycidyl ether (TMPTGE), poly(ethyleneglycol) diglycidylether (PEGGE) tetraphenylolethae glycidyl ether (THETGE), and poly[(phenyl glycidyl ether)-co-formaldehyde] (PPGE) gave negative results. These findings imply that the h-CLAT assay undertaken in standard format may be unsuitable for assessing skin sensitisation potential of ERS as the CD54 and CD86 were not induced.

To address this issue, I investigated the possibility that ERS induced cytokine release in the h-CLAT assay may be a more sensitive marker of skin sensitisation than changes in expression levels of CD54 and CD86 on THP-1 cells. Encouragingly, concentrations of the cytokines, IL-6 and IL-8, in the cultured THP-1 cell supernatant quantified using a Meso Scale™ Discovery human pro-inflammatory multiplex immunoassay, were markedly increased in DGEBA, TMPTGE, THETGE and PPGE.

For the DPRA, chemicals with known sensitising capacity were incubated with three synthetic heptapeptides, Cor1-C420 (Ac-NKKCDLF), heptapeptides containing cysteine

(Ac-RFAACAA) and lysine (Ac-RFAAKAA) in order to determine the optimal experimental conditions. The sensitising potential of the chemicals were correlated with depletion of each heptapeptide individually in a reaction mixture. The applicability of the DPRA to assess the skin sensitising potential ERS was investigated together with known positive and negative control compounds. The aforementioned heptapeptides were selected as they had been previously shown to have a high correlation with LLNA data when used to assess small molecules.

My DPRA findings show that the optimal incubation temperature for incubation of all heptapeptides was 25°C. Importantly, my data also show that the apparent heptapeptides depletion level is affected by the tube materials used for the DPRA. Specifically, Cor1-C420 was stable in polypropylene tubes but failed to meet the assay acceptance criteria for days 1-3 when borosilicate glass tubes were used. As for cysteine, it was not stable on day 3 post-incubation when glass was used for the assay. Although lysine was stable in both polypropylene and glass tubes during the course of the DPRA, the apparent extent of lysine depleted by the chemical, ethyl acrylate, differed between polypropylene (24.7 ± 5.8%) and glass (47.3 ± 7.7%) vials. Another novel finding was instability of the peptide-chemical complex (i.e. Cor1-C420-cinnamaldehyde and cysteine-2,4-dinitrochlorobenzene) suggesting that the complex formation may be partially reversible. This information suggests that data generated by the DPRA in high-throughput format involving the screening of hundreds of chemicals simultaneously, may not be accurate.

Poor aqueous solubility of ERS in *in vitro* assays is a considerable challenge. To address this issue, the solubility of five ERS using a range of solvent:reaction buffer combinations was compared. In brief, a solvent comprising a 1:1 methanol:acetonitrile containing 1% *tert*-butanol was effective in solubilising these five ERS in reaction buffer. Using this optimised solvent system for dissolution of DGEBA, TMPTGE, THETGE and PPGE, the DPRA data generated were significantly correlated with the LLNA data on skin sensitisation with the exception that PEGGE was positive in the DPRA but classified as a non-sensitiser in the LLNA.

In summary, my findings show that there are many challenges to be overcome in future work beyond the scope of my PhD research project in terms of adapting the DPRA and h-CLAT assays to high-throughput format in order to provide accurate information on the skin sensitisation potential of novel industrial chemicals.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-reviewed journal articles

1. **Wong C.L.**, Ghassabian S, Smith M.T. and Lam A (2015). *In vitro* methods for hazard assessment of industrial chemicals – opportunities and challenges. *Frontiers in Pharmacology* 6:94. doi: 10.3389/fphar.2015.00094.

2. **Wong C.L.**, Lam A, Smith M.T. and Ghassabian S (2015). Optimization of the performance of the direct peptide reactivity assay (DPRA) for assessment of the skin sensitization potential of chemicals. *Frontiers in Pharmacology* (under review).

Conference Abstracts

1. **Wong, C.L.**, Lam, A.L., Wyse, B.D., Smith, M.T. *In vitro* assessment of chemical sensitisation potential using the human cell line activation test (h-CLAT). In: Abstracts of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT) 46th Annual Scientific Meeting; 2012 Dec 2-5, Sydney, Australia. Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists. *(Poster)*
    - ***Awarded a travel grant by ASCEPT to present in the 46th Annual Scientific Meeting***
    - ***Neville-Percy prize finalist***

2. **Wong, C.L.**, Lam, A.L., Ghassabian, S., Smith, M.T. Evaluation of *in vitro* assays as alternative strategies in predicting skin sensitisers. In: Abstracts of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT) 47th Annual Scientific Meeting; 2013 Dec 1-4, Melbourne, Australia. Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists. *(Poster)*
    - ***Awarded a travel grant by ASCEPT to present in the 47th Annual Scientific Meeting***

3. **Wong, C.L.**, Lam, A.L., Ghassabian, S., Smith, M.T. Evaluation of the direct peptide reactivity assay (DPRA) in identifying epoxy resins with skin sensitizing potential. In: Abstract of the Alternatives to Animal Experimentation (ALTEX) 9th World Congress on Alternatives and Animal Use in the Life Sciences (WC9); 2014 Aug 24-28; Prague, Czech Republic. *(Poster)*
    - ***Awarded travel grants by Alternatives Congress Trust (ACT) Germany, Stiftung zur Förderung von Ersatz- und Ergänzungsmethoden zur Einschränkung von Tierversuchen (SET) Foundation (Germany) and Info Kinetics Sdn. Bhd. (Malaysia)***

4. **Wong, C.L.**, Lam, A.L., Ghassabian, S., Smith, M.T. Evaluation of the direct peptide reactivity assay (DPRA) in identifying epoxy resins with skin sensitizing potential. In: Abstract of the Translation Research Excellence (TRX14); 2014 Oct 24; Brisbane, Australia. *(Poster)*

5. **Wong, C.L.**, Lam, A.L., Ghassabian, S., Smith, M.T. Evaluation of the direct peptide reactivity assay (DPRA) in identifying epoxy resins with skin sensitizing potential. In: School of Pharmacy Research Higher Degree Day; 2015 Feb 18; Brisbane, Australia. *(Poster)*

Publications included in this thesis

1. **Wong C.L.**, Ghassabian S, Smith M.T. and Lam A (2015). *In vitro* methods for hazard assessment of industrial chemicals – opportunities and challenges. *Frontiers in Pharmacology* 6:94. doi: 10.3389/fphar.2015.00094 - incorporated as Chapter 1 (refer to Appendix for published paper)

Contributor	**Statement of contribution**
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Author Sussan Ghassabian	Edited paper (5%)
Author Maree T. Smith	Edited paper (15%)
Author Ai-Leen Lam	Wrote (30%) and edited the paper (20%)

2. **Wong C.L.**, Lam A, Smith M.T. and Ghassabian S (2015). Optimization of the performance of the direct peptide reactivity assay (DPRA) for assessment of the skin sensitization potential of chemicals. *Frontiers in Pharmacology* – incorporated as Chapter 3 (under review).

Contributor	**Statement of contribution**
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Author Maree T. Smith	Designed experiments (30%) Analysis and interpretation of data (10%) Edited paper (20%)
Author Sussan Ghassabian	Designed experiments (30%) Analysis and interpretation of data (10%) Edited paper (20%)

Contributions by others to the thesis

The publications submitted as part of this thesis have had contributions from other authors. The nature and extent of these contributions are detailed above.

Statement of parts of the thesis submitted to qualify for the award of another degree

None

Acknowledgements

First of all I would like to express my deepest gratitude to my supervisors, Dr Ai Leen Lam, Dr Sussan Ghassabian and Professor Maree T. Smith, who have supported me throughout my doctoral tenure along with encouragement and patience. I am grateful for the freedom to work on the research independently as they see fit. They have taught me the research methodologies as well as other soft skills which will definitely prepare me in my future career. Working in an accredited laboratory equipped me with management skill and interpersonal skills which help me in my future undertakings.

A special thank goes to all the members of Centre for Integrated Preclinical Drug Development (CIPDD)/TetraQ based in both St Lucia and Herston who offered their valuable guidance during my candidature. I would like to thank Dr Bruce Wyse for the planning and guidance on my research during my first year of candidature. A special thanks to Ms Suzanne O'Hagan for assisting me with animal ethics application and assistance in animal model, the murine local lymph node assay (LLNA). I would like to acknowledge with much appreciation to Dr Hui Jiang and Dr Hao Wang who advise and assist me with the technical issues involving high performance liquid chromatography tandem mass spectrometry (LC-MS/MS).

Furthermore, I would like to acknowledge Mr Geoffrey Osborne and Ms Virginia Nink from Queensland Brain Institute (QBI) for assisting me in operating flow cytometry and data analysing as well as working with me in developing high-throughput sample analysis methods using flow cytometry.

I would like to thank the Graduate School and School of Pharmacy, The University of Queensland for providing The University of Queensland International (UQI) scholarship to cover my tuition fees and stipend throughout my candidature. I would also like to thank various organisations, such as the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT), Alternatives Congress Trust (ACT) Germany, the Stiftung zur Förderung von Ersatz- und Ergänzungsmethoden zur Einschränkung von Tierversuchen (SET) Foundation (Germany) and Info Kinetics Sdn. Bhd. (Malaysia) for the travel grant awards to attend and present my research findings at various national and international conferences.

Lastly, I would like to express a special gratitude and thanks to my family and friends in both Malaysia and Australia who gave me encouragement throughout my candidature.

Keywords

allergic contact dermatitis, DPRA, epoxy resins, h-CLAT, *in vitro* methods, LLNA, skin sensitisation

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 111506 Toxicology (incl. Clinical Toxicology), 100%

Fields of Research (FoR) Classification

FoR code: 1115, Pharmacology and Pharmaceutical Sciences, 100%

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List of Abbreviations

7-AAD	7-amino actinomycin D
ACD	Allergic contact dermatitis
ANOVA	Analysis of variance
AOO	Acetone:olive oil
AOP	Adverse outcome pathways
APCs	Antigen presenting cells
ARE	Antioxidant response element
Arg	Arginine
ATCC	American Type Culture Collection
BD	Becton Dickinson
BG	Birbeck granule
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
BT	Buehler test
CABA	Contact Allergen Bank Australia
CAS	Chemical abstract service
CD	Cluster of differentiation
CU	Contact urticaria
CV	Coefficient of variation
CV50	50% of cell viability
CV75	75% of cell viability
Cys	Cysteine
DCs	Dendritic cells
DEREK	Deductive estimation of risk from existing knowledge
DETA	Diethylenetriamine
DGEBA	Diglycidyl ether of bisphenol A
DGEBF	Diglycidyl ether of bisphenol F
DMSO	Dimethylsulfoxide
DNBS	2,4-dinitrobenzenesulfonic acid
DNCB	2,4-dinitrochlorobenzene
DNFB	2,4-dinitrofluorobenzene
dpm	Disintegrations per minute
DPRA	Direct peptide reactivity assay

DTT	DL-dithiothreitol
EC	Estimated concentration
ECL	Electrochemiluminescence
ECVAM	European Centre for the Validation of Alternative Methods
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
ERS	Epoxy resin systems
EU	European Union
EURL-ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
FACS	Fluorescent activated cell sorter
FBS	Fetal bovine serum
FCA	Freund's complete adjuvant
FITC	Fluorescein isothiocyanate
FSC-A	Forward scatter area
GARD	Genomic allergen rapid detection
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GPMT	Guinea pig maximisation test
GSH	Glutathione
h-CLAT	Human cell line activation test
His	Histidine
HMT	Human maximisation test
HPLC	High performance liquid chromatography
HRIPT	Human repeated insult patch test
HRP/P	Horseradish peroxide and hydrogen peroxide
hTCPA	Human T-cell priming assay
HTD	Highest technical dose
ICAM	Intercellular adhesion molecule
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICD	Irritant contact dermatitis
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukins
ISP	Index of sensitising potency

LCs	Langerhans cells
LCSA	Loose-fit coculture-based sensitisation assay
LLNA	Local lymph node assay
LLOQ	Lower limit of quantitation
log $K_{o/w}$	log octanol/water partition coefficient
Lys	Lysine
MALDI-TOF/MS	Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry
MCASE	Multi computer automated structure evaluation
MEST	Mouse ear swelling test
Met	Methionine
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MOA	Mode of action
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSD	Meso Scale Discovery®
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUSST	Myeloid U937 skin sensitisation test
NESIL	No expected sensitisation induction level
NOEL	No observed effect level
OCD	Occupational contact dermatitis
OECD	Organisation for Economic Co-operation and Development
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PEGGE	Poly(ethylene glycol) diglycidyl ether
PGE	Phenyl glycidyl ether
PPGE	Poly[(phenyl glycidyl ether)-co-formaldehyde]
Pro	Proline
QBI	Queensland Brain Institute
QC	Quality control
QSAR	Quantitative structural-activity relationship
R^2	Regression coefficient

RASH	Resources About Skin Health
REACH	Registration, evaluation, authorization and restriction of chemicals
RFI	Relative fluorescent intensity
RHE	Reconstructed human epidermal
RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
SD	Standard deviation
SI	Stimulation index
SMARTS	Smiles arbitary target specification
SMILES	Simplified molecular-input line-entry system
SPT	Schwartz-Peck test
SSC-A	Side scatter area
TETA	Triethylenetetramine
TGF	Transforming growth factor
THETGE	Tetraphenylolethae glycidyl ether
TIMES-SS	Tissue metabolism simulator for skin sensitisation
TMB	Tetramethylbenzidine
TMPTGE	Trimethylolpropane triglycidyl ether
TNF	Tumour necrosis factor
TOPKAT	Toxicity prediction by komputer assisted technology
TOPS-MODE	Topological substructural molecular descriptors
TRUE	Thin-layer Rapid Use Epicutaneous Tests
ULOQ	Upper limit of quantitation
US	United States
UV	Ultraviolet
VEGA	Virtual models for property evaluation of chemicals within a global architecture

Chapter 1: Literature Review

1.1. Human skin

Human skin is the largest organ in the human body. It plays crucial roles against physical injury, primary defence against infection, prevents excessive water loss as well as detoxification and metabolism of xenobiotic compounds from our surroundings, such as pollutants, drugs and cosmetics (Kao and Carver, 1990, Brodell and Rosenthal, 2008). In general, human skin consists of several layers, the epidermis, dermis and the subcutaneous layer. The epidermis functions as a primary physical defence barrier against large molecules that are not permeable to the lipid bilayer of the skin (Lee et al., 2006). The epidermis is divided into various strata, with the stratum corneum comprising the outermost layer, followed by the stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale before the dermis layer (Figure 1-1) (Venus et al., 2011). Within the stratum corneum, there are numerous cells and signalling mediators involved in mediating the cutaneous immune responses.

Dermatological disorders or skin diseases refer to the physical impairment of the skin. Skin disease is a very common physical disorder as almost everyone in the community has been diagnosed with at least one skin condition once in their lifetime (Marks et al., 1999). The most common skin diseases reported in Australia are acne, atopic dermatitis, psoriasis and warts. Skin diseases vary immensely from mild conditions such as simple visible skin alterations, like a rash and redness to severe ailments which cause scarring, disfigurement or even fatality (Marks et al., 1999).

Dermatitis is a skin condition clinically characterised by inflammation of the skin in response to external stress or irritants (Patel and Nijhawan, 2008). Exposure to exogenous causative allergens, including both naturally occurring and synthetic substances may lead to the elicitation of visible clinical symptoms such as eczema, erythema and pruritus (Bourke et al., 2009, Cashman et al., 2012). Dermatitis can be categorised into several types that include contact dermatitis, atopic dermatitis, stasis dermatitis, nummular dermatitis and seborrheic dermatitis. One of the most commonly reported types of dermatitis in the workplace is contact dermatitis (Lushniak, 2000).

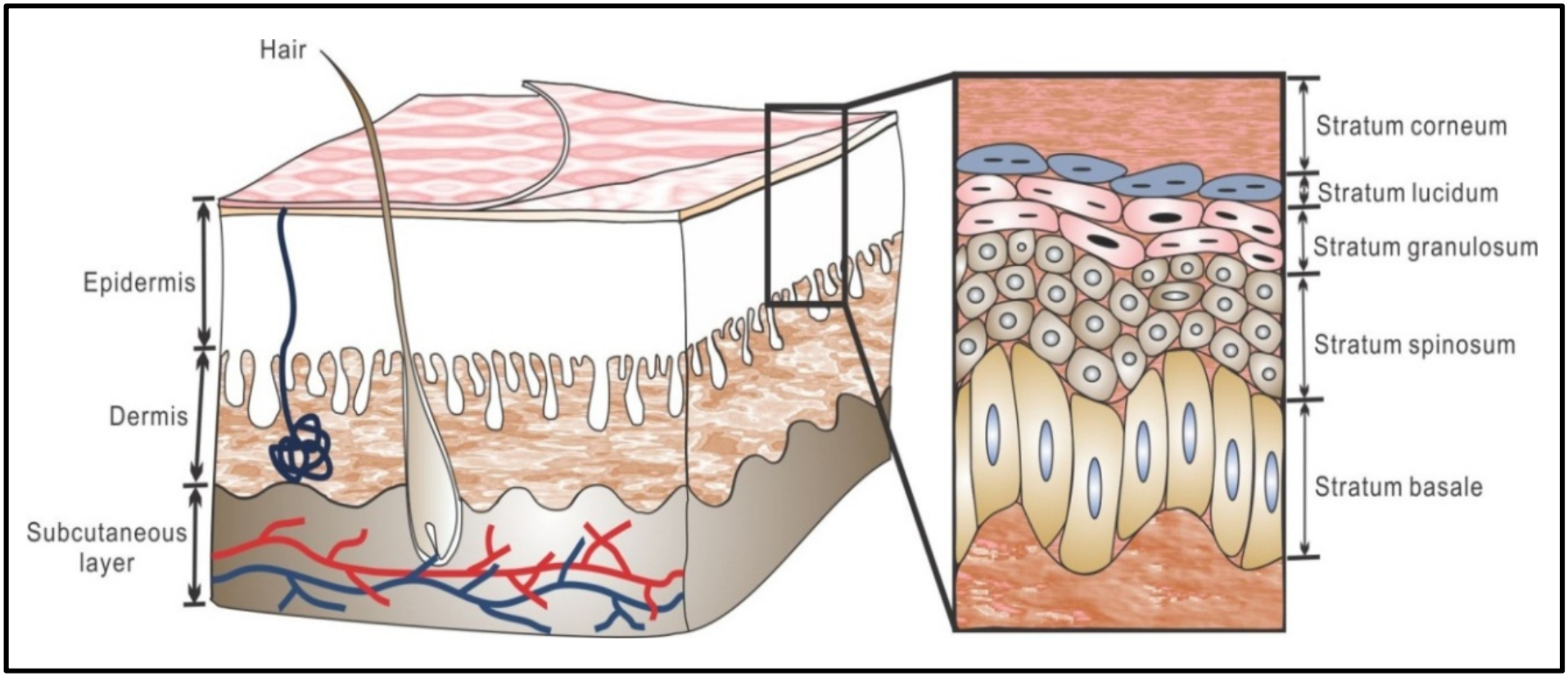

Figure 1-1: Human skin structure. Human skin is divided into three main layers which are the epidermis, dermis and subcutaneous layers. The epidermis is further divided into several layers with the stratum corneum comprising the outermost layer. The stratum corneum is cornified with a layer of keratinocyte cells which act as an initial barrier to the penetration of exogenous compounds.

1.2. Contact dermatitis: The skin disease[1]

Contact dermatitis is defined as an inflammatory reaction in response to foreign substances that come into contact with the skin (Patel and Nijhawan, 2008). It mainly affects exposed skin areas, such as the hands, legs, arms and face, although it may occur on other parts of the body (Belsito, 2005). Typically, it can be classified into three phases, acute, subacute and chronic inflammatory reactions (Ghosh, 2009). An acute inflammatory reaction is characterised by pruritus, erythema and vesiculation owing to vasodilation and migration of leukocytes to the site of invasion (Krasteva et al., 1999). This may be within a short period of time, which can be either minutes or delayed up to 24 hours after skin contact with exogenous compounds (Krasteva et al., 1999). A chronic inflammatory reaction involves pruritus, xerosis, lichenification, hyperkeratosis and fissuring as a result of prolonged and persistent inflammation caused by external stimuli (Bourke et al., 2009, Cashman et al., 2012). A state between acute and chronic reactions is known as subacute dermatitis. Similar to acute and chronic reactions, subacute dermatitis has noticeable erythema and scaling on the skin surface (Ghosh, 2009).

Contact dermatitis is influenced by both intrinsic and extrinsic factors. Intrinsic factors include age, gender, ethnicity, epidermal barrier texture and genetic polymorphism whilst extrinsic factors comprise geographical and environmental factors, as well as biochemical properties of the exogenous compounds, all of which may have an impact on skin reactivity (Belsito, 2005, Cashman et al., 2012, Zhai et al., 2012, Landeck et al., 2012).

Contact dermatitis accounts for up to 95% of reported work-related skin diseases (Lushniak, 2000). Epidemiological studies of contact dermatitis across different regions, such as in the United States (US), Europe, Africa and Asia illustrate that occupational contact dermatitis (OCD) is the most common occupational skin disease (Keegel et al., 2009). OCD is frequently reported among medical practitioners, hairdressers, beauticians, chefs, cleaners, farmers, construction and specialised epoxy workers as well as those within manufacturing industries. This apparent higher incidence rate of OCD in the aforementioned professions is due to the use of distinctive chemical components in latex, hair dyes, cosmetics, pesticides, metals, cement and epoxy resins (Abbott et al., 2009,

[1] This section of the literature review of my PhD thesis has been published in the journal, *Frontiers in Pharmacology*, as a review article.

Wong C.L., Ghassabian S, Smith MT and Lam A (2015). *In vitro* methods for hazard assessment of industrial chemicals – opportunities and challenges. *Front. Pharmacol.* 6:94. doi: 10.3389/fphar.2015.00094.

Cahill and Andersen, 2010, Gimenez-Arnau, 2011, Idriss et al., 2012, Liippo and Lammintausta, 2011, Lowney and Bourke, 2011, Mahler, 2011, Mikov et al., 2011, Sosted, 2011, Yasky et al., 2011). OCD has become both a significant public health concern in terms of employee pain and suffering as well as a considerable socioeconomic burden due to reduced productivity comprising lost work days and higher manufacturing costs (Cashman et al., 2012, Frosch et al., 2011, Lyons et al., 2013). In the US alone, the estimated annual direct and indirect costs of occupational skin diseases for 2012 exceeded USD1 billion; a value that was likely underestimated due to many unreported cases that remain unaccounted for (Cashman et al., 2012, Lushniak, 2004). Additionally, the cost of dermatological treatments in the US is forecast to reach USD18.5 billion per annum by 2018 (Evers, 2013).

1.3. Types of contact dermatitis

Contact dermatitis can be classified into contact urticaria (CU), an immediate immunological or non-immunological reaction after external cutaneous contact with an exogenous stimulus (Davari and Maibach, 2011); irritant contact dermatitis (ICD), a non-allergic inflammatory reaction by a combination of endogenous and exogenous factors (Slodownik et al., 2008); or allergic contact dermatitis (ACD) which involves sensitisation of the immune system in response to specific causative agents (Rustemeyer et al., 2011). In most cases, the various types of contact dermatitis are indistinguishable clinically as they exhibit similar signs and symptoms involving similar basic underlying inflammatory pathways (Rustemeyer et al., 2011).

1.3.1. Contact urticaria

In general, contact urticaria (CU) is a less common type of contact dermatitis. According to Jacob and Steele (2006b), CU represented only approximately 0.5% of all reported contact dermatitis cases. CU is provoked by a type I hypersensitivity immune response where it evokes a rapid but transient vascular response of the skin upon direct contact with an exogenous stimulus (Grabbe, 2010, Davari and Maibach, 2011). The clinical appearance of CU is usually characterised by papules and plaques with erythematous patches (Kossard et al., 2006). Symptoms presented by CU normally resolve without scarring in a short period of time and rarely last for more than several days (Zuberbier et al., 2009). The underlying mechanism of CU is similar to that underpinning most types of hypersensitivity responses that involve the association of antigen with immunoglobulin E (IgE) molecules on the surface of basophils and mast cells (Harvell et al., 1994). The stimulation of IgE

leads to the local release of mediators, such as histamine resulting in the visible wheal-and-flare clinical manifestation as well as itching and a localised burning sensation (Harvell et al., 1994).

1.3.2. Irritant contact dermatitis

Irritant contact dermatitis (ICD) is the most common type of OCD which comprises 80% of all occupational skin diseases (Sasseville, 2008). ICD is well recognised as non-immunological local skin inflammation triggered by injury as a direct result of skin contact with caustic chemicals (Levin and Maibach, 2002). However, it has been suggested that multiple immunologic-like pathways may also trigger ICD (Levin and Maibach, 2002, Slodownik et al., 2008). ICD is commonly accompanied by erythema, oedema and corrosion following single or repeated exposure of an irritant to the same cutaneous site (Kartono and Maibach, 2006, Mathias and Maibach, 1978).

ICD can be either an immediate or a cumulative reaction (Levin and Maibach, 2002). The immediate causative agents are often related to strong acid and alkaline agents with corrosive properties or highly concentrated chemicals that are capable of generating chemical burns rapidly after skin contact (Kartono and Maibach, 2006, Sasseville, 2008). In contrast, cumulative ICD occurs when an individual is continuously exposed to weak irritants such as detergents or wearing gloves over long periods of time (Sasseville, 2008). ICD is a consequence of cell disruption in the epidermis and dermis layers by direct cytotoxic action of the irritants (Ale and Maibach, 2010). The noticeable physical changes in the skin are due to alterations in the skin's biological system, such as transepidermal water loss, epidermal barrier disruption as well as the release of vasoactive peptides and pro-inflammatory signalling molecules (Sasseville, 2008).

The threshold of susceptibility to irritants is varied among individuals owing to multiple factors such as the thickness of the physical barrier, the volume and concentration of the irritant, as well as the frequency, duration and area of exposure (Landeck et al., 2012, Slodownik et al., 2008). The fundamental mechanisms involved in the inter-individual variation of susceptibility to skin irritants are yet to be defined (Watkins and Maibach, 2009). Studies on the genetic susceptibility to ICD have mainly focused on genes involved in skin inflammation such as the pro-inflammatory cytokines, interleukin (IL)-1α, IL-1β, IL-8 and tumour necrosis factor (TNF)-α and the anti-inflammatory cytokine, IL-10 (Kezic et al., 2009). As TNF-α plays a role in inducing inflammation, its expression level were thought to affect an individual's response towards irritants, which later led to the discovery of

polymorphic variants of TNF-α in normal and ICD individuals (Allen et al., 2000). Recently, Landeck and colleagues (2012) found that individuals with the TNF-α-308 polymorphism were more likely to develop ICD whereas the TNF-α-238 variant had a shielding effect against the development of ICD in those individuals. Hence, susceptibility to development of ICD appears to be associated with genetic heritability, at least in part.

1.3.3. Allergic contact dermatitis[2]

Allergic contact dermatitis (ACD) is a type IV delayed hypersensitivity cutaneous immune reaction which is mediated by T-lymphocytes occurring upon repeated skin exposure to contact allergens (Kimber et al., 2002a). Similar to ICD, the chances of an individual becoming sensitised to a particular chemical are dependent upon both the specific properties of the chemical as well as the particular individual's susceptibility (Basketter and Maxwell, 2007). ACD develops in two stages, the sensitisation phase and the elicitation phase (Figure 1-2) (Kimber et al., 2011).

1.3.3.1. Sensitisation phase

During the sensitisation phase, skin sensitisers or haptens initially come into contact with the stratum corneum and subsequently gain access to the body system through the viable epidermis. The invasion of haptens triggers the local release of pro-inflammatory molecules which subsequently induce the binding of the haptens with skin proteins (Kimber et al., 2002a). The release of pro-inflammatory molecules also stimulate the disentanglement and subsequent migration of Langerhans cells (LCs) from the surrounding keratinocytes towards the hapten-protein complex (Schwarzenberger and Udey, 1996). The hapten-protein complex binds to the major histocompatibility complex (MHC) on LCs and is then transported into lymph nodes via the afferent lymphatics (Toebak et al., 2009). During the transitory migration to the lymph nodes, the activated LCs differentiate into mature antigen presenting cells (APCs) resulting in morphological changes such as the loss of endocytic/phagocytic receptors and the upregulation of co-stimulatory molecules and MHC molecules (Toebak et al., 2009). The hapten-protein complex is presented by the APCs to the naïve hapten-responsive T-lymphocytes, followed by selective clonal expansion of effector and memory T-cells. The proliferated

[2] This section of the literature review of my PhD thesis has been published in the journal, *Frontiers in Pharmacology*, as a review article.

Wong C.L., Ghassabian S, Smith MT and Lam A (2015). *In vitro* methods for hazard assessment of industrial chemicals – opportunities and challenges. *Front. Pharmacol.* 6:94. doi: 10.3389/fphar.2015.00094.

population of primed antigen-specific T-lymphocytes are then disseminated into the blood circulation resulting in the sensitisation of an individual (Kimber et al., 2011).

1.3.3.2. Elicitation phase

Elicitation arises when a sensitised individual is re-exposed to the same or structurally similar haptens (Basketter and Maxwell, 2007). Elicitation is triggered when the haptens interact with the skin, either at the same or different skin site (Kimber et al., 2011). Upon re-exposure, epidermal cells release a cocktail of pro-inflammatory cytokines and chemokines which draw the previously primed hapten-specific T-lymphocytes from the peripheral circulation into the epidermal layer (Kimber et al., 2011). The infiltrating T-cells produce inflammatory cytokines which in turn trigger the secretion of chemokines by keratinocytes, resulting in increased lymphocyte infiltration from blood vessels into the epidermal compartment leading to the development of ACD (Basketter and Maxwell, 2007, Toebak et al., 2009).

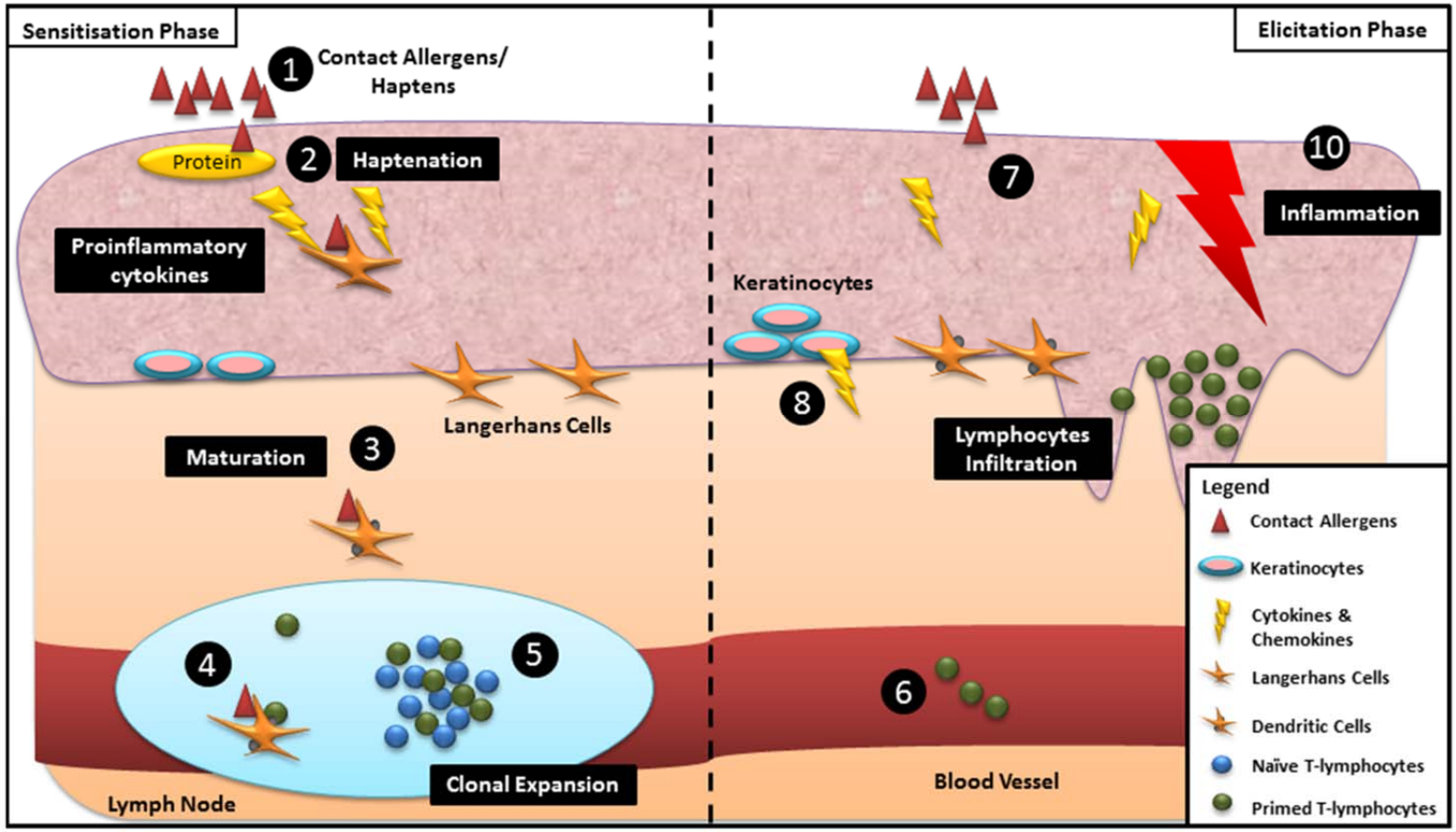

Figure 1-2: Schematic overview of the mechanisms underpinning skin sensitisation during the sensitisation and elicitation phases. 1. Haptens gain access through the viable epidermis. 2. Binding of haptens and skin proteins. 3. LCs bind to the hapten-protein complex and differentiate into matured DC's during migration to the lymph node. 4. LCs present haptenated protein to the naïve T-lymphocytes. 5. Clonal expansion of specific effector and memory T-cells. 6. Proliferated T-lymphocytes disseminate into the blood circulation resulting in sensitisation of an individual. 7. Re-exposure of similar haptens to the same individual. 8. Release of pro-inflammatory cytokines and chemokines by epidermal cells. 9. Infiltration of T-cells from blood vessel into site of contact. 10. Development of ACD.

1.4. Mechanisms of ACD

1.4.1. Haptens and skin proteins

A hapten is generally a small compound with low molecular weight, generally less than 500 Daltons, that is chemically reactive (Kaplan et al., 2012, Mowad, 2006). In addition to low molecular weight, a hapten is presumed to have a log $K_{o/w}$ (octanol/water partition coefficient) in the range -1.4 to 4, which facilitates penetration of the stratum corneum (Kaplan et al., 2012, Martin, 2004, Chipinda et al., 2011a). In the epidermal compartment, the hapten modifies and binds with self-proteins in the skin which then further develop into immunogenic antigens, a process known as haptenation (Chipinda et al., 2011a). Haptens bind to skin proteins through an irreversible covalent modification or by establishing a chelation complex (Kaplan et al., 2012), with high bond energies varying between 200 and 420 kJ/mol (Chipinda et al., 2011a). Specifically, the covalent binding between a hapten and a specific protein carrier is a result of a nucleophilic-electrophilic interaction between the nucleophilic residue of the skin protein and the electrophilic domain of the hapten (Basketter et al., 1995).

Reactive skin proteins and their nucleophilic residues such as cysteine (-SH), histidine (=N-), lysine ($-NH_2$), methionine (-S-), and tyrosine (-OH) are hapten-specific (Lepoittevin and Leblond, 1997, Vocanson et al., 2009). Haptens contain distinct mechanistic domains that are accessible by specific skin proteins (Chipinda et al., 2011a). The most common mechanistic domains found on haptens are Michael acceptors, acylating agents, Schiff base formers, S_NAr electrophiles and S_N1/S_N2 electrophiles (Divkovic et al., 2005, Chipinda et al., 2011a). These different types of nucleophilic-electrophilic binding mechanisms can influence the type and strength of adducts formed for downstream processing which subsequently affects the allergenic potency of chemicals (Chipinda et al., 2011a).

Currently, 40% of skin sensitisers have been demonstrated to have electrophilic centres that are vulnerable toward nucleophilic attack (Chipinda et al., 2011a). The remaining skin sensitisers trigger the immunogenic system through different mechanisms that are independent of the nucleophilic-electrophilic interaction system. For instance, sensitising metal ions such as nickel and cobalt react with skin protein by forming non-covalent binding leading to the formation of protein-metal chelate complexes (Gamerdinger et al., 2003). Regardless of the type of interactions, both processes are able to generate stable

bonding between hapten and skin protein to facilitate the activation of acquired immunity (Chipinda et al., 2011a).

One of the most commonly identified haptens, 2,4-dinitrochlorobenzene (DNCB) is classified as a strong sensitiser based on human patch test and animal testing; it is widely used as a positive skin sensitiser control compound, for development of skin sensitisation assays (Gerberick et al., 2001). Extensive investigation into the sensitising chemical structure-activity relationships of haptens has led to the development of computer-based prediction models and peptide reactivity tests (Gerberick et al., 2004, Lepoittevin, 2006). However, some of the known sensitisers identified by the human patch test and in animal studies did not show any response when analysed using these non-animal testing methods. These false negative results suggested that not all chemical sensitisers were chemically reactive in nature and therefore could not be assigned into a single classification as haptens (Lepoittevin, 2006).

Two distinct terms, prohapten and prehapten have since been designated to illustrate the different types of chemical sensitisers (Lepoittevin, 2006). These unique haptens are not reactive in their native forms and require additional modification in order to exhibit their sensitising potential (Lepoittevin, 2006). A prohapten describes a sensitising chemical that requires metabolic alteration by either an enzyme-induced reaction or oxidation to form a protein-conjugated hapten in order to activate the immune system (Aptula et al., 2007). Conversely, a prehapten is used to define a chemical sensitiser that requires abiotic transformation, not via biochemical processes to attain a protein-reactive derivative (Lepoittevin, 2006). Examples of pro- and prehaptens are listed in Table 1-1 (Troutman et al., 2011). On occasion, chemical sensitisers can act as both pro- and prehaptens given the optimal conditions (Aeby et al., 2008). For example, *p*-phenylenediamine undergoes enzyme activation at pH 9-10 with approximately 30 minutes exposure time, but it undergoes auto-oxidation when exposed to air or oxygen for more than 48 hours at pH 5-6 (Aeby et al., 2008).

The understanding of different chemical sensitisers and their derivatives/adducts is crucial for development of *in vitro* assays that can accurately identify potential sensitisers. To date, more than 4,350 chemicals have been identified as contact allergens through human patch tests with the number increasing over the years (De Groot and Frosch, 2011). It has been reported that an additional 675 new allergens were found between 1994 and 2008; an 18% increase over the last 14 years (De Groot and Frosch, 2011). With the increasing

number of potential allergens coming to light, there is an urgent need to develop high-throughput screening assays to prospectively identify allergens. The currently available *in vitro* testing approaches for identification of chemicals with skin sensitisation potential, is discussed in Sections 1.8.

1.4.2. Signalling mediators

Pro-inflammatory cytokines, inflammatory cytokines as well as chemokines facilitate signal transduction pathways of immunological activation in response to a skin sensitiser (Kimber et al., 2002a). When the skin is first exposed to allergens or haptens, keratinocytes in the epidermal layer release numerous pro-inflammatory mediators (IL-1α, IL-1β , IL-18, TNF-α, prostaglandin E2), immunosuppressive mediators (transforming growth factor (TGF)-β, IL-10 and IL-1 receptor antagonist) and growth factors (IL-6, IL-7, IL-15, and granulocyte/macrophage colony-stimulating factor (GM-CSF)) (Basketter and Maxwell, 2007) to assist the subsequent activation of the immunogenic pathways. Activated keratinocytes will further induce the release of signalling mediators by innate immune resident-skin cells, such as LCs in the epidermis as well as mast cells, DCs and fibroblasts in the dermis (Basketter and Maxwell, 2007, Griffiths et al., 2005).

During subsequent hapten exposure, Type I inflammatory cytokines such as gamma interferon (IFN-γ) and IL-4 are produced in addition to the abovementioned pro-inflammatory cytokines (Toebak et al., 2009, Vocanson et al., 2009). These inflammatory cytokines stimulate the resident-skin cells, predominantly mast cells to produce TNF-α and IL-8 (also called CXCL8) which in turn generate the release of chemokines from keratinocytes such as CXCL9, CXCL10, and CXCL11 (Toebak et al., 2009). These chemokines attract diffusion of $CXCR3^+$ T-cells to the site of allergen contact. CXCR3 is a receptor expressed on the surface of effector memory $CD4^+$ and $CD8^+$ T-cells (Toebak et al., 2009, Vocanson et al., 2009). The recruitment of the effector memory T-cells along with leukocytes, DCs and non-hapten-specific cells into the epidermal layer of the skin leads to the secretion of chemokines CCL5, CCL17, CCL18, CCL21, and CCL22 which in turn amplify T-cell influx into the epidermal layer (Goebeler et al., 2001, Serra et al., 2004). The rapid infiltration of these signalling mediator cocktails leads to development of ACD (Kimber et al., 2002a).

Table 1-1: Examples of pro- and prehaptens

Prohaptens	Prehaptens
a) Eugenol	a) Geraniol
b) Polyaromatic hydrocarbons	b) Hydroquinone
c) Cinnamic alcohol	c) Isoeugenol
d) Carvone oxime	d) Methylcatechol
e) Diphenylthiourea	e) *p*-phenylenediamine
f) *p*-phenylenediamine	f) D-limonene
	g) β-carophyllene

1.4.3. Langerhans and dendritic cells

LCs are immature DCs that are largely found in the epidermis, particularly the stratum spinosum layer as well as in the lymph nodes. LCs are derived from $CD34^+$ progenitor cells in the bone marrow which are thought to be equipped with both immuno-stimulatory and -regulatory features (Strunk et al., 1997, Streilein et al., 1990). LCs comprise the minority of the total cell population within the epidermal layer and are consistently spread throughout the epidermis. LCs are characterised by the presence of adhesins (CD11a, CD11b, CD54, and E-cadherin), co-stimulators (CD86 and CD40), major histocompatibility complex (MHC class I and class II), receptors (FcεRI, CD32, CD205 and CD207) and lineage restricted molecules (CD1a and CD1c) (Kimber et al., 2011). Furthermore, LCs contain a unique intracytoplasmic organelle known as Birbeck granule (BG) which is thought to play a role in receptor-mediated endocytosis and antigen-presentation (Kimber et al., 2000, Mc Dermott et al., 2002, Valladeau et al., 2000).

As previously detailed in section 1.4.2, the invasion of haptens induces the local release of a number of signalling mediators by keratinocytes which lead to the engagement of LCs, specifically the pro-inflammatory mediators, IL-1β and TNF-α (Basketter and Maxwell, 2007). The release of IL-1β and TNF-α down-regulates membrane-bound E-cadherin expression in LCs, thereby unravelling the LCs from the surrounding keratinocytes (Schwarzenberger and Udey, 1996). Along with the release of LCs and pro-inflammatory mediators, LCs are drawn toward the hapten-protein complex (Schwarzenberger and Udey, 1996). During the migration of the hapten-protein complex into draining lymph nodes, the LCs differentiate into mature DCs which in turn, function as APCs to present the hapten to specific T-cells in the lymph nodes (Ainscough et al., 2013).

1.4.4. Proliferation and recruitment of hapten-specific T-cells

T-cells are subdivisions of lymphocytes which have fundamental roles in the immune system. ACD development is highly dependent on the priming and expansion of T-cells (Vocanson et al., 2009, Kimber et al., 2011). There are two essential signals involved in regulating T-cell activation (Basketter and Maxwell, 2007). The initial signal encompasses the interaction of the T-cell receptor and an APC which is initiated by the presentation of the hapten-protein complex to naïve hapten-specific T-lymphocytes, $CD4^+$ and $CD8^+$ T-lymphocytes within the groove of its MHC Class I and II surface molecules (Kaplan et al., 2012). Allergen-specific effector $CD8^+$ T-cells are activated by the APCs and further differentiated into cytotoxic T-cells (Tc1/Tc17) while $CD4^+$ T-cells develop into T-helper

cells (Th1/Th17) (Vocanson et al., 2009). $CD8^+$ T-cells primarily produce cytokines, typically IFN-γ and IL-2 which initiate the inflammatory reaction in ACD (Kimber et al., 2011). $CD4^+$ T-cells are speculated to have an immunoregulatory role in ACD where they regulate both priming and expansion of specific $CD8^+$ T-cells in the lymphatics, along with activating $CD8^+$ T-cells in the skin (Kimber et al., 2011, Vocanson et al., 2009). The second signal involves interaction of co-stimulatory molecules, CD28 on T-cells with the cell surface molecules, CD80 or CD86 on DCs, which promotes cell cycle progression, T-cell activation and amplification (Basketter and Maxwell, 2007).

1.5. Etiology of ACD[3]

To date, more than 4000 chemicals are linked to induction of ACD in humans (Cahill et al., 2012). The 18-year retrospective analysis of ACD patients identified a number of frequently defined contact allergens, some of which have been summarised in Table 1-2 (Cahill et al., 2012). Human patch tests series including the North American Series, the European Baseline Series, the International Standard Series and the Thin-layer Rapid Use Epicutaneous Tests (TRUE) were developed to identify chemical sources of ACD. Their development involved compilation of chemical substances commonly implicated in the population of a given geographical area to cause ACD (Spiewak, 2008).

1.5.1. Epoxy resin system (ERS)

Epoxy resin-induced ACD was first reported in the 1950s, a time when extensive development of epoxy resin systems (ERS) was taking place in industry (Broughton, 1965). The ERS is comprised of an epoxy resin, reactive diluent, hardener or other additives such as solvents, modifiers and fillers which together control the chemical and physical properties of the ERS (Geraut et al., 2009, Nixon et al., 2012). Both epoxy resins and reactive diluents contain epoxide groups in their molecules (Figure 1-3) (Muskopf and McCollister, 1987). In general, epoxy resins are defined by the coupling reaction of epichlorohydrin with compounds that have at least two reactive hydrogen atoms in their chemical structure (Gooch, 2007).

ERS are regarded the third most common allergen type for occupational ACD after chromates and rubber allergens, with ERS reported as the primary source of ACD in the

[3] This section of the literature review of my PhD thesis has been published in the journal, *Frontiers in Pharmacology*, as a review article.

Wong C.L., Ghassabian S, Smith MT and Lam A (2015). *In vitro* methods for hazard assessment of industrial chemicals – opportunities and challenges. *Front. Pharmacol.* 6:94. doi: 10.3389/fphar.2015.00094.

plastics manufacturing industry (Geraut et al., 2009). The prevalence of ERS-induced ACD by country is summarised in Table 1-3.

It was estimated that for individuals with ERS-associated ACD, ~60-80% were sensitised to diglycidyl ether bisphenol A (DGEBA), an ERS that is widely used in industry (Björkner et al., 2011). This high prevalence resulted in the inclusion of DGEBA in the human patch test series since the 1960s (Geraut et al., 2009). Other epoxy resins including diglycidyl ether bisphenol F (DGEBF) and tetraglycidylmethylenedianiline are also associated with the induction of ACD (Nixon et al., 2012, Geraut et al., 2009).

Apart from epoxy resins, epoxy hardeners, predominantly polyamine compounds such as triethylenetetramine (TETA) and diethylenetriamine (DETA), as well as reactive epoxy diluents (e.g. phenyl glycidyl ether and *p*-tert-butylphenyl glycidyl) (Geier et al., 2004), also cause ACD. A retrospective analysis of the records of 182 patients with ACD induced by epoxy resins over a 22-year period showed that 23.6% had developed an allergic response to epoxy hardeners (Jolanki et al., 2001). In a prospective study involving 92 individuals with suspected and/or prior exposure to ERS, patch tests showed that they were responsive to the epoxy diluents, 1,6-hexanediol diglycidyl ether (19.5%) and 1,4-butanediol diglycidyl ether (18.5%) (Geier et al., 2004), highlighting cross-reactivity between epoxy compounds for induction of ACD in humans.

Although the high propensity of ERS to induce ACD is known, they are nevertheless used widely in commercial thermosetting products due to their strong adhesive bonding properties between different surfaces while exhibiting excellent resistance in harsh chemical and environmental conditions (Cahill et al., 2012). Worldwide demand for epoxy resins is forecast to reach ~3 million tons by the end of 2017, with an estimated value of USD8.4 billion per annum (GIA forcasts the global market, 2012, Markets and Markets, 2014). The high global demand for epoxy resins is due to their ever increasing utility in a wide range of industrial applications including automotive coatings, electronic coatings, construction and adhesive products (Dietrich and Mirasol, 2012, GIA forcasts the global market, 2012). At present, research on assessment of the generalisability of *in vitro* tests developed for identifying the skin sensitising potential of small molecules used in the toiletries and cosmetics industries, to that of industrial chemicals, is limited. Hence, this knowledge gap needs to be addressed.

Table 1-2: Common allergens and sources of exposure.

Allergens	Source
Epoxy resin system (ERS)	Adhesives, paints
Formaldehyde	Pesticides, home cleansers
Fragrance mix	Toiletries, cosmetics
Neomycin sulphate	Creams, deodorants
Nickel sulphate	Costume jewellery, tools

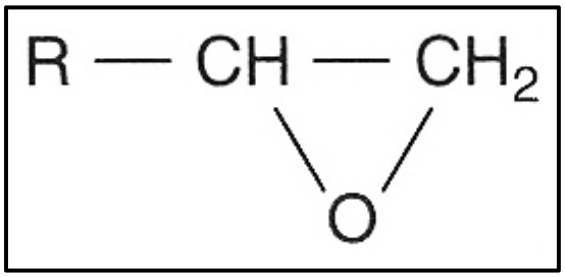

Figure 1-3: Chemical structure of the epoxide group.

Table 1-3: Reported prevalence of occupational ACD due to epoxy resin systems (ERS).

Study Period	Country	Study Population (number of individuals)	Prevalence of ERS-induced ACD (%)	References
1993-2002	Australia	1354	3.0	Cahill et al., (2005)
1996-2006	North America	2540	0.9	Amado and Taylor, (2008)
1997-2001	Norway	2336	1.0	Romyhr et al., (2006)
1999-2008	Portugal	2440	0.6	Canelas et al., (2010)
2001-2010	Denmark	219	8.2	Mose et al., (2012)
2001-2006	China	1354	8.5	Cheng et al., (2011)
2005-2009	Denmark	20 808	1.3	Bangsgaard et al., (2012)
2006-2008	Lithuania	816	1.5	Beliauskiene et al., (2011)

1.5.2. Diagnosis and prevention of ACD

Currently, ACD is assessed via the human patch test or through physical examination and history taking by an experienced dermatologist (Geraut et al., 2009, Jacob and Steele, 2006a). At present, ACD can be diagnosed by two different methods, the skin tests and immunological tests (Nosbaum et al., 2009). The skin tests include the human patch test which is detailed in Section 1.6.1 (Kanerva et al., 1999). The immunological tests involve assessment of allergen-specific T-cells in skin and/or blood by using methods such as immunohistochemistry, analysis of cytokines, culturing leucocytes from biopsy samples, the lymphocyte transformation test and an ELISPOT assay (enzyme-linked immunospot) (Nosbaum et al., 2009). Early diagnosis of ACD is crucial for appropriate prevention and treatment (Jacob and Steele, 2006a). Treatment is provided to severe cases by applying skin creams containing corticosteroids to reduce local inflammation and relieve itching (Cohen and Heidary, 2004).

In order to reduce the incidence of ACD in Australia, an Australian Government statutory body, Safe Work Australia has made an effort to collate all cases of occupational diseases, including ACD, reported in Australia (Safe Work Australia, 2012). This governmental body aims to improve occupational health and safety of workers in Australia by providing the platform for risk management in the workplace (Safe Work Australia, 2012). In addition, the occupational skin disease database and Contact Allergen Bank Australia (CABA) have been established to collect national data on the incidence rates and allergens associated with occupational skin diseases to improve diagnosis as well as implement effective risk management policies (Safe Work Australia, 2012). Furthermore, awareness programs such as Resources about Skin Health (RASH) have been introduced to create awareness and educate workers on appropriate preventative measures towards OCD (Safe Work Australia, 2012). Since the establishment of education programs and risk assessments, the incidence rate of contact dermatitis in Australia based on workers' compensation claims has declined significantly from 155 claims per million employees in 2005 to 98 claims per million employees in 2009 (Safe Work Australia, 2012).

Additionally, the prevention of ACD can be further improved by accurate identification of potential allergens that might lead to ACD through various skin sensitisation tests, which have commonly included the human patch test or animal testing (Geier et al., 2004, Kanerva et al., 1999, Gamer et al., 2008). The currently available skin sensitisation tests/assays are outlined in Section 1.6.

1.6. Contact allergens' screening approaches

Allergic contact dermatitis (ACD) is an indication of immunotoxicity in human skin (Kimber et al., 2002a). While it is a non-reversible skin disorder, it is a preventable disease. Therefore, it is important to understand the relevant biological and immunological mechanisms of skin sensitisation in developing new and enhanced screening approaches for hazard identification and characterisation. Several skin sensitisation assays have been developed since the early 20th century (Kligman, 1966a, Schwartz and Peck, 1944) and these will be discussed in the next section.

1.6.1. Human test

In the early 1940s, Schwartz and Peck developed the Schwartz-Peck Test (SPT) for estimating the total incidence rate of skin sensitisation in a population (Schwartz and Peck, 1944). SPT was carried out by applying a test chemical on a healthy individual's skin for 24 to 48 hours. An interim resting period of two weeks was given to the individual before a new piece of test chemical patch was reapplied for the next 24 to 48 hours (Schwartz and Peck, 1944). The patching site was monitored throughout the study for the development of clinical symptoms such as erythema, oedema, papules and vesicles. The clinical manifestation of contact dermatitis at the re-exposure stage signified the test chemical as a prospective sensitiser (Kligman, 1966a, Schwartz and Peck, 1944).

Likewise, the Human Maximisation Test (HMT) entailed a patch with test chemical applied on either the forearm or the lower leg in the calf region of a healthy volunteer (Kligman, 1966c). Initially, the patch was placed for 48 hours, followed by five repeat treatments applied over alternate days. The individual was subsequently challenged with the same test article for an hour and the test site monitored closely to determine any physical abnormality (Kligman, 1966c). The advantage of HMT over SPT is the inclusion of the detergent sodium lauryl sulphate to provoke and utilise the resulting initial skin irritancy as a provocative agent for the subsequent application of the test chemical (Kligman, 1966d). Use of detergent in the HMT was to facilitate detection of a compound's potential variability in its sensitisation capacity after being formulated into a different product, for example, drugs, cosmetics and industrial chemicals (Kligman, 1966c). The aim of the HMT was to give an estimate of potential hazard and the degree of impairment caused by chemicals, which were then classified accordingly by means of physical examination (Kligman, 1966c).

However, a major concern of conducting human tests is the high possibility of a healthy individual being sensitised to a chemical eventually leading to ACD. Therefore, ethical issues with respect to human patch tests specifically human welfare, dignity, rights and safety have to be taken into consideration while conducting human research (Carlson et al., 2004). Further details on human research ethics with regard to sensitising chemical testing, are discussed in Section 1.7.

Aside from ethical concerns, both SPT and HMT were only carried out within small populations and therefore could not provide an accurate reflection of the sensitisation rate of contact dermatitis within the wider and ethnically diverse population (Kligman, 1966b). For example, an individual with pigmented skin was found to be less responsive to inflammation caused by irritants and allergens (Kligman, 1966b). Furthermore, no clear consensus was made regarding exposure time, frequency, area and intensity that would ideally portray chemical exposure in real-life situations (Kligman, 1966c, Kligman, 1966b). Currently, the Human Repeated Insult Patch Test (HRIPT) (Kligman, 1966a) is an accepted human test (McNamee et al., 2008). HRIPT involves nine repeated exposures to a test chemical patch applied for 24 hours over a three week period, followed by a two week incubation period and subsequent application of a challenge patch to a naïve site of the skin for 24 hours (McNamee et al., 2008, Politano and Api, 2008). The challenge site is then scored for skin responses, such as erythema, oedema, papules and vesicles for the next three days (Politano and Api, 2008). Patients exhibiting equivocal results undergo a rechallenge phase where both occlusive and semi-occlusive patches of the test article and vehicle control are placed upon the naïve sites. The post-application effect is then monitored over three consecutive days (McNamee et al., 2008, Politano and Api, 2008).

HRIPT is primarily used to confirm the safety of finished products based on the threshold level derived from an animal model, quantitative structure-activity relationships (QSAR) or available human clinical data (Basketter et al., 2005). HRIPT does not assess dose-response relationships of a test chemical as ACD largely depends on the frequency of chemical exposure rather than the initial induction dose (Basketter et al., 2005). HRIPT assists risk assessment processes by confirming a No Observed Effect Level (NOEL) of a chemical which is the maximum safety level that does not induce skin sensitisation in a healthy human population (Api et al., 2008, Gerberick and Robinson, 2000). NOEL in turn has been used as a data source for fostering the No Expected Sensitisation Induction Level (NESIL), which indicates the absence of an unfavourable response in humans to a particular formulation (Api et al., 2008).

However, as human tests are considered unethical with their results affected by numerous unclarified variable factors, extensive efforts have been made to provide alternative assessments that suitably represent a human's immunological response to chemical sensitisation (Basketter, 2009).

1.6.2. Animal tests

The use of various animal models in predicting skin sensitisation, developed since the mid-1930s, was the first step to minimise and avoid human testing (Landsteiner and Jacobs, 1935).

1.6.2.1. Guinea pig model

The guinea pig test on contact hypersensitivity was first developed in the mid-1930s (Voss, 1958, Landsteiner and Jacobs, 1935, Draize et al., 1944). Guinea pig tests were later revised and named as the Buehler Test (BT) (Buehler, 1965) and the Guinea Pig Maximisation Test (GPMT) (Magnusson and Kligman, 1969). Both BT and GPMT mimic the induction and challenge phases in human skin tests, in that the test substance was initially applied to the back of a shaved guinea pig, followed by a subsequent application (challenge phase) two weeks later (Buehler, 1965, Magnusson and Kligman, 1969). The test site was observed and graded according to the severity of erythema manifested (Magnusson and Kligman, 1969, Robinson et al., 1990). GPMT which incorporated the use of Freund's Complete Adjuvant (FCA) to enhance the immunological response of the animal was considered to a more sensitive test than BT in detecting chemical allergens (Maurer et al., 1994). While BT and GPMT have successfully identified numerous chemicals, false negative and inconsistent results were obtained in part due to the difficulty in determining the ideal exposure frequency and duration as well as concentration of test substance (Robinson et al., 1990, Maurer et al., 1994). Furthermore, inconsistencies in classification of the sensitisers were largely due to the subjective nature of determining the severity of the animal response, and therefore not quantifiable (Vial and Descotes, 1994). Overall, sensitivity and specificity of the guinea pig tests were reported to be 85% and 83%, respectively when compared to known human clinical cases that involved 70 selected chemicals (Vial and Descotes, 1994).

1.6.2.2. Mouse model

The mouse Ear Swelling Test (MEST) was first described in the 1980s as an alternative assay to the guinea pig model in assessing contact allergens (Moller, 1984, Gad et al., 1986). The mouse test was preferred over the guinea pig tests as the former was cost effective and had a shorter experimental period which entailed use of fewer animals and required less space (Gad et al., 1986). More importantly, Asherson and Ptak (1968) had successfully shown that the passive transfer of delayed-type contact hypersensitivity could be generated in a mouse.

During the induction phase of MEST, intradermal injections of FCA emulsion followed by test substance are applied to the mouse (Gad et al., 1986). Application of the test substance was repeated on the abdomen for four consecutive days, and the mouse was challenged with a final application on the ears one week later with test substance and vehicle control substance, each on a separate ear of the mouse (Gad et al., 1986). MEST uses the swelling of mouse ears as a quantitative end point, by measuring the thickness of both ears. An increment of thickening of the ears as result of the applied test substance would indicate the sensitisation potential of the test substance (Gad et al., 1986).

While MEST had been reported to successfully identify sensitisers (Gad et al., 1986), a later report contradicted the earlier findings whereby more than half of the same sensitisers tested using MEST at two separate laboratories resulted in inconsistent results (Dunn et al., 1990). Moreover, MEST was not thought to be a suitable assay for classifying weak or moderate sensitisers as these sensitisers would be unable to produce significant ear swelling and lead to false negative results (Gad, 1994). However, use of vitamin A as a supplement in MEST was suggested to boost the immune response in mice which could potentially help in detecting weak sensitisers (Maisey and Miller, 1986).

Unlike MEST, the Local Lymph Node Assay (LLNA) uses T-cell proliferation in the lymph nodes as a quantitative endpoint (Basketter et al., 2002, Kimber et al., 1986, Kimber et al., 1989, Kimber et al., 2002b). LLNA provides a better platform for discriminating the potency of allergens as LLNA has the advantage of producing a dose response to sensitisers, in that the extent of T-cell proliferation in the lymph nodes is proportional to the dose or potency of the sensitiser (Basketter et al., 2002). This basic mechanism underlying the LLNA is therefore similar to the sensitisation phase in humans (Figure 1-2) where naïve mice exposed to a test substance on the ears resulted in a corresponding degree of sensitisation by T-cell expansion that is measured by the amount of radiolabelled

thymidine (^{3}H-thymidine) incorporation into the cellular DNA of draining lymph nodes (Kimber and Basketter, 1992). Moreover, the challenge phase is not required in the LLNA which reduces the duration and magnitude of animal pain and suffering (Kimber et al., 1986).

Results of each test treatment in the LLNA are expressed as a stimulation index (SI), which is the ratio of the T-cell proliferation in the treated group of mice to that of the concurrent vehicle/solvent control group (Omori and Sozu, 2007, Basketter et al., 1999b). A chemical with SI≥3 is regarded as a sensitiser (Basketter et al., 1999b). The potency of skin sensitisers are expressed as an EC3 value, which represents the estimated chemical concentration (as a percentage value) that is able to induce a three-fold increase in T-cell proliferation, i.e. SI=3 (Basketter et al., 1999b). The level of radiolabelled thymidine incorporated into the DNA of auricular draining lymph nodes is proportional to the potency of the hapten (Basketter et al., 1999a, Basketter et al., 1999b, Gerberick et al., 2001). The EC3 values derived from the LLNA correlates well with sensitising potencies of compounds obtained from human clinical data, with non-sensitisers having an EC3 value of more than 100% while strong sensitisers with low EC3 values, generally less than 1% (Basketter et al., 1999a, Gerberick et al., 2001, Kimber et al., 2003). This classification allowed haptens to be classified as non-sensitisers, weak, moderate, strong or extreme sensitising agents (Table 1-4) (Kimber et al., 2003).

At present, there are two additional modified versions of the LLNA that employ different techniques in quantitating lymph node T-cell proliferation (OECD, 2010b, OECD, 2010c). The conventional LLNA employs the use of ^{3}H-thymidine to determine the total radiolabelled thymidine incorporated into the DNA of newly divided T-cells in the lymph nodes (OECD, 2010a). However, while the conventional LLNA is well established and provides consistent data, there is a huge concern with using radioactive material in the test system (Takeyoshi et al., 2001). The current modifications made on the typical LLNA protocol incorporating the use of non-radioactive materials such as LLNA:BrdU-ELISA (5-bromo-2-deoxyuridine-enzyme-linked immunosorbent assay) and LLNA:DA (developed by Daicel Chemical Industries, Ltd.) for quantifying T-cell proliferation (OECD, 2010b, OECD, 2010c).

To date, the LLNA is the only *in vivo* method that has been subjected to a formal validation process by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 1999). The LLNA has been accepted as a stand-alone testing method

for hazard identification and risk assessment of chemicals (ICCVAM, 1999). In addition, the LLNA was incorporated as a Test Guideline (No. 429) by the Organisation of Economic Cooperation and Development (OECD) in 2002 (OECD, 2002, OECD, 2010a).

1.6.2.3. Rat model

Apart from the guinea pig and mouse models, contact hypersensitivity has been tested in other animal models. Arts and colleagues (1996) performed contact hypersensitivity tests using five different rat strains. The experiment was performed similarly to the murine LLNA and the results were found to be comparable with those from the murine LLNA (Arts et al., 1996). However, due to the larger ear surface and slower immune response in rats, the LLNA in rat models required a higher treatment volume of test chemicals and an additional day for radiolabelling proliferating T-cells (Arts et al., 1996). Additionally, both facial and auricular draining lymph nodes were required for analysing the proliferation of T-cells, as the facial lymph nodes in rats act as the draining lymph nodes from the ears (Tilney, 1971).

Table 1-4: Potency classification of LLNA (Kimber et al., 2003)

EC3 Value (%)	Potency Classification
≥10 - ≤100	Weak
≥1 - <10	Moderate
≥0.1 - < 1	Strong
<0.1	Extreme

1.7. Animal and human welfare

The use of animals and humans as testing models for identifying potential skin sensitisers has long provoked much ethical debate (Basketter, 2009, Carlson et al., 2004). To that end, the Helsinki declaration produced by the World Medical Association since 1964 with six revisions (most recent revision in 2008) provides guidance on the involvement of humans as test subjects in research studies (WMA, 2008). It is also compulsory that approval from the Independent Ethics Committee has to be obtained prior to research commencement (WMA, 2008). A major concern of using human subjects in chemical sensitisation trials is the high risk potential for development of sensitisation after the first exposure to the test compound (WMA, 2008). Hence, strict documentation and care must be taken to protect the health and welfare of the individual participants in the trial (WMA, 2008).

In 2003, the European Union (EU) Member States adopted the 7th amendment to the Cosmetic Directive (76/768/EEC) which not only imposed a ban on testing finished cosmetic products and ingredients on animals (testing ban), but further prohibited the marketing of cosmetic products or any of their raw ingredients which have been tested on animals (marketing ban) (EU, 1976). Since July 2013, the Cosmetic Directive (76/768/EEC) was replaced by a new regulatory act, the EC1223/2009 which similarly requires animal testing to be replaced by alternative methods and bans the marketing of any products that have been subjected to animal testing (EU, 2009). Without a validated alternative method of assessing potential sensitisers, the enforcement of the EC1223/2009 regulation may severely affect the cosmetics and toiletries industries, in that the safety of the products for the end users remain undetermined due to the lack of animal data with respect to their toxicity, carcinogenicity and sensitisation potential (EU, 2009).

Furthermore, the EU REACH regulation (registration, evaluation, authorisation and restriction of chemicals) EC1907/2006 that came into force on 1 June 2007, put pressure on the testing of thousands of chemicals that had not been previously assessed for skin sensitisation potential, further driving the necessity to seek alternative testing approaches that involve fast and cost effective screening (EU, 2006).

At present, both the ICCVAM and European Centre for the Validation of Alternative Methods (ECVAM) have formed cooperative measures in implementing and validating alternative testing approaches (Stokes et al., 2002), which will be further discussed in Section 1.8.

1.8. Non-animal screening assays

1.8.1. *In chemico* assays: Peptide-chemical interactions[4]

Epoxy resins and/or epoxy resin composite materials, in common with other classes of haptens, react with skin proteins. The hapten-protein complex is then internalised and processed by LCs (Aleksic et al., 2007). Protein modification, in a process known as haptenation, is a key step in the initiation of skin sensitisation (Chipinda et al., 2011b). The majority of contact allergens are electrophilic in nature, comprising Michael acceptors, S_NAr and S_N2 electrophiles, Schiff base formers or acylating agents, which underpin their ability to react with the nucleophilic amino acid residues of skin proteins (Chipinda et al., 2011b, Lalko et al., 2012). For epoxy resins, the electrophilic epoxide groups react with the nucleophilic moieties of skin proteins via S_N1 or S_N2 type nucleophilic reactions (Obach and Kalgutkar, 2010).

This haptenation process is mimicked *in vitro* by the direct peptide reactivity assay (DPRA) (Figure 1-4(A)) that assesses depletion of small proteins (peptides) secondary to their interaction with potential haptens (Gerberick et al., 2007). Briefly, in this model, synthetic peptides containing nucleophilic residues including cysteine or lysine are incubated with test chemicals at a pre-determined ratio for 24 hours to allow the binding of the active side chain of the peptide to the hapten. Based upon the irreversible covalent bond formation that occurs between haptens and amino acid residues in proteins, the DPRA quantifies the amount of unbound (remaining) peptide in the reaction mixture using high performance liquid chromatography (HPLC). Subsequently, quantification of the bound (depleted) peptide is determined as a measure of reactivity of the test chemical (Gerberick et al., 2004). At present, several detection methods and peptides have been used in the DPRA and these methods are summarised in Table 1-5.

At present, the DPRA has been validated by the ECVAM for the assessment of contact allergens as a replacement for the *in vivo* LLNA (Troutman et al., 2011). A test guideline has been promulgated by the Organization for Economic Co-operation and Development (OECD) highlighting the generalisability of peptide reactivity for small molecules (OECD,

[4] This section of the literature review of my PhD thesis has been published in the journal, *Frontiers in Pharmacology*, as a review article.

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2015a). However, the suitability of the DPRA test system for other chemical classes such as epoxy resins that contain an epoxide group remains to be assessed.

1.8.1.1. DPRA: Chemicals tested to date

Use of the DPRA to assess the ability of 82 compounds that are mainly used as ingredients in cosmetic and toiletry products, to deplete cysteine-, lysine- and glutathione-based peptides, indicated a significant correlation between peptide depletion and their sensitiser potency as previously established from the *in vivo* LLNA data (Gerberick et al., 2007).

Steps undertaken to improve the accuracy of the DPRA for identification of potential skin sensitising chemicals have included incorporation of oxidising agents such as horseradish peroxidase and hydrogen peroxide (HRP/P) as well as cytochrome P450 enzymes to metabolically activate unreactive haptens into their more reactive hapten form, a process that may take place in human skin *in vivo* (Bergström et al., 2007, Troutman et al., 2011). By incorporating HRP/P into the DPRA, 83% of 70 chemicals with known sensitising potential were identified accurately as compared with the standard DPRA reported previously (89%) (Troutman et al., 2011). The apparently reduced accuracy of the HRP/P-added DPRA analysis is misleading however, as the initial chemical set used to evaluate the previous DPRA prediction model did not include pre-/pro-haptens (Gerberick et al., 2007).

More recent refinements aimed at increasing the robustness of the DPRA to identify skin sensitising chemicals include using pH conditions that more closely mimic human skin pH and measurement of concomitant chemical-specific mass changes indicative of peptide adduct formation (Dietz et al., 2013). In other work, the rate constant for reactivity of various test chemicals with the DPRA peptide was determined to assess the extent to which quantitative kinetic reactivity data generated by measuring cysteine depletion at multiple test chemical concentrations and at various incubation times, were correlated with their potency as sensitisers (Roberts and Natsch, 2009, Natsch et al., 2015). However, drawbacks of this approach are that chemical reactivity varies markedly between various functional groups and the reaction rate of test chemicals with the DPRA peptide may not be linearly related to their *in vivo* sensitisation potency (Roberts and Natsch, 2009).

1.8.1.2. DPRA: Application to ERS

While heptapeptides containing cysteine and lysine are the most widely used for the *in vitro* DPRA, other modified peptides have been investigated. More recently, the utility of the DPRA for classifying the sensitising capacity of several epoxies including novel analogues of DGEBF and phenyl glycidyl ether (PGE), has been examined using a synthetic peptide, *viz* PHCKRM (Pro-His-Cys-Lys-Arg-Met). The extent of peptide (PHCKRM) depletion by six novel epoxy analogues and the parent epoxide, PGE, was correlated with the sensitising potency of these epoxies determined using *in vivo* LLNA assessment (Niklasson et al., 2009). The strong sensitiser, PGE produced 88% peptide depletion whereas the weak epoxide sensitisers, butyl glycidyl ether and butenyl glycidyl ether produced 46% and 54% peptide depletion, respectively (Niklasson et al., 2009). In a DPRA evaluation of DGEBF (containing 2 epoxide groups) and two variants (Variant A and Variant B) using the same synthetic peptide (PHCKRM), the thiol (cysteine) binding of DGEBF and its variants appeared to be affected by the terminal epoxide groups (O'Boyle et al., 2012). Variant A (DGEBF without terminal epoxide groups) did not react with free thiols whereas variant B (DGEBF with 1 terminal epoxide group) did react with thiol groups albeit to a slightly lesser extent than the diepoxide DGEBF. Interestingly, the reaction rate for DGEBF that contains 2 terminal epoxide groups was slightly faster than that of variant B. These findings are aligned with the sensitising capacity of DGEBF and its variants determined using the LLNA and the KeratinoSens™ assay (O'Boyle et al., 2012).

To date, reports on the applicability of the incorporation of enzymes into the DPRA, as a means of bioactivation for assessing the skin sensitisation potential of epoxy resins, are lacking. It is known that the enzyme, epoxide hydrolase, catalyses the hydrolysis of epoxides to their respective dihydrodiol metabolites which react readily with skin proteins. Conversely, the enzyme, glutathione-*S*-transferase catalyzes the detoxification of epoxides by formation of glutathione conjugates (Obach and Kalgutkar, 2010). Hence, future investigation involving incorporation of epoxide hydrolase and/or glutathione-*S*-transferase into the DPRA for analysis of epoxy resin compounds is warranted, to more closely mimic possible bioactivation and deactivation processes within human skin that produce reactive electrophilic intermediates and detoxified species, respectively.

Issues relating to the poor aqueous solubility of industrial compounds that have high log $K_{o/w}$, present another obstacle for use of the DPRA to assess skin sensitising potential of compounds such as epoxy resins. Although various solvents including dimethylsulfoxide

(DMSO), methanol and acetonitrile have been used to dissolve lipophilic compounds, only small volumes of these solutions can be used due to their limited miscibility with an aqueous solution of the peptide to be depleted. To that extent, microemulsion systems have potential to improve miscibility between an organic solution of a lipophilic test compound and that of an aqueous peptide solution; preliminary data suggest that this approach is worthy of further investigation (Merckel et al., 2010). Additionally, the modulation of *in vitro* test systems such as the DPRA by organic solvents can limit the range of solvents that can be used for dissolution of epoxy resin compounds. This limitation will be addressed in more detail in Section 1.9.

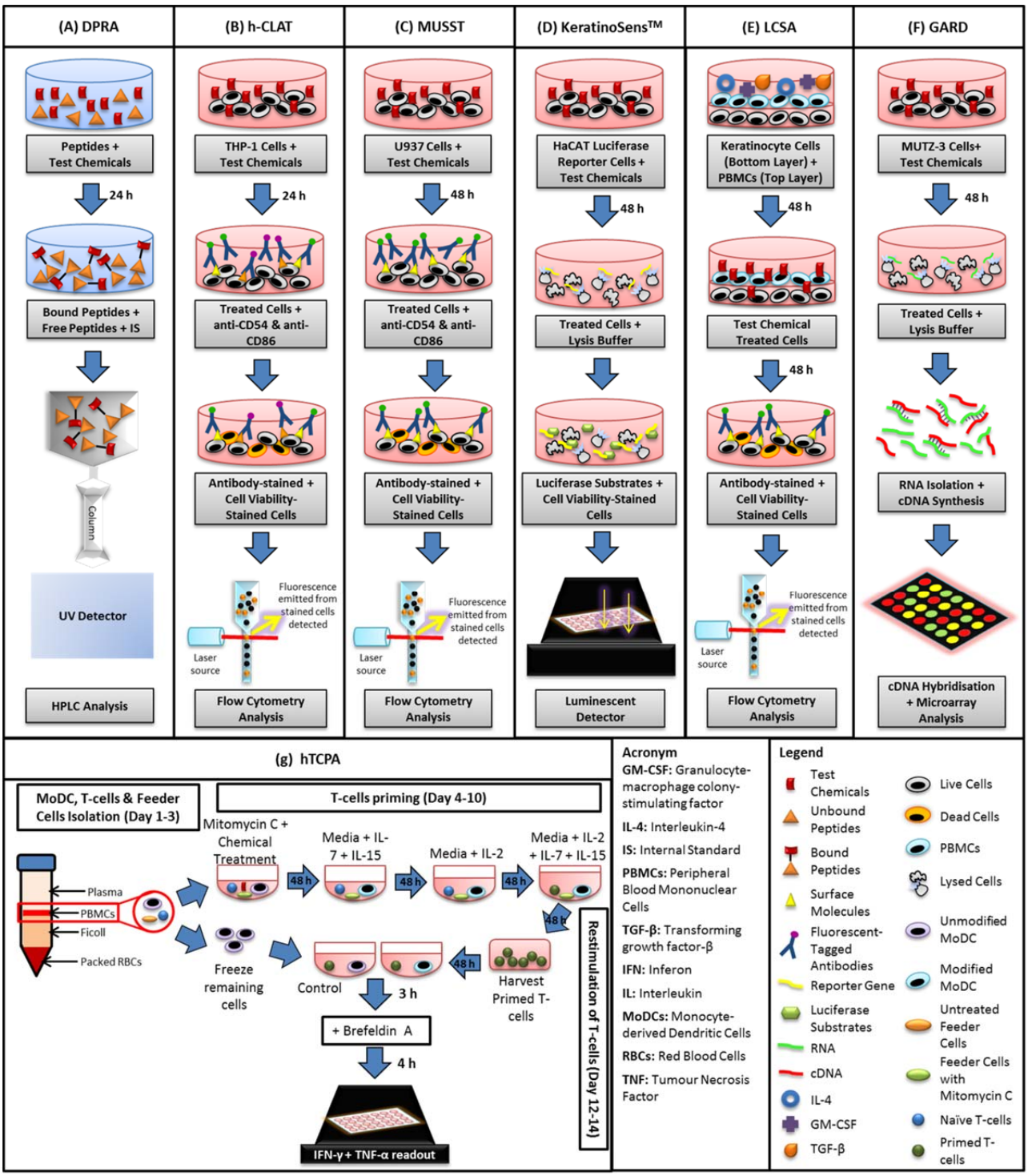

Figure 1-4: Schematic diagram summarising the steps involved in the conduct of *in vitro* assays currently available for assessment of skin sensitisation potential. (A) Direct peptide reactivity assay (DPRA), (B) human cell line activation test (h-CLAT), (C) myeloid U937 skin sensitisation test (MUSST), (D) KeratinoSens™, (E) loose-fit coculture-based sensitisation assay (LCSA), (F) genomic allergen rapid detection (GARD) and (G) human T-cell priming assay (hTCPA).

Table 1-5: Summary of currently available detection methods and peptides used for haptenation.

Detection Method	Peptide/Nucleophile	Measurement Endpoint	Reference
Spectrophotometric	Tripeptide: GSH Cys: RWAACAA Lys: RWAAKAA 4-nitrobenzenethiol (NBT) Pyridoxylamine (PDA)	Measure peptide depletion	Schultz et al. (2005) Jeong et al. (2013) Chipinda et al. (2010), Chipinda et al. (2014)
HPLC-DAD	Cys: RFAACAA Lys: RFAAKAA His: RFAAHAA CysReact: RFAACAA Keap-257: KYDCEQR Keap-297: DSRCKDY RP17-34: KRVCEEF Cor1-C420: NKKCDLF Cofill-138: QANVYEE	Measure peptide depletion Measure peptide depletion and adduct formation	Gerberick et al. (2004), Gerberick et al. (2007) Natsch et al. (2007)
HPLC-UV	Tripeptide: GSH Cys: RFAACAA Lys: RFAAKAA N-(2-(1-naphthyl)acetyl)-L-cysteine (NAC) α-N-(2-(1-naphthyl)acetyl)-L-lysine (NAL)	Measure peptide depletion Measure peptide depletion	Gerberick et al. (2007) Fujita et al. (2014)

Table 1-5: *cont.*

Detection Method	Peptide/Nucleophile	Measurement Endpoint	Reference
LC-MS	Corl-C420: NKKCDLF	Measure peptide depletion, peptide oxidation, adduct formation and thiol reactivity	Natsch and Gfeller (2008)
LC-MS/MS	Cys: RFAACAA Lys: RFAAKAA	Measure peptide depletion with the presence and absence of horseradish peroxidase and hydrogen peroxide	Gerberick et al. (2009), Troutman et al. (2011)
HPLC-ESI-MS	Hexapeptide: PHCKRM	Measure peptide depletion	Niklasson et al. (2009)
EC-ESI-TOF/MS	Tripeptide: GSH Cys: RFAACAA-COOH Lys: RFAAKAA-COOH KCN	Determine autoxidation, oxidative biotranformation	Jahn et al. (2012)
MALDI-TOF/MS	Dual Peptide: Peptide-20: LHKSMGRTWQFDYNPEAAVIK (minus Cys) Peptide-21: LHKSMGRTWQFDYNPEACVIK (plus Cys)	Determine the chemical-specific peptide mass shifts and peptide depletion	Dietz et al. (2013)
GeLC-MS/MS	Human Serum Albumin	Determine the total modified amino acid residues on HSA	Aleksic et al. (2007), Parkinson et al. (2014)

GSH: Glutathione, Cys: Cysteine, Lys: Lysine, His: Histidine, KCN: Potassium cyanide

1.8.2. *In vitro* assays: cell-based models

Human LCs and DCs play key roles in skin sensitisation (Coutant et al., 1999). Hence, there has been considerable research attention on development of *in vitro* systems that mimic the roles of LCs and DCs in skin sensitisation. Initial *in vitro* assays using LCs/DCs were limited due to the scarcity of available LCs and inter-donor variability of DCs (Yoshida et al., 2003). These factors were compounded by between-laboratory variability in cell isolation and cell culture techniques, which led to assay reproducibility problems (Yoshida et al., 2003). The inter-donor variability was circumvented by the use of human myeloid cell lines, such as KG-1, THP-1, MUTZ-3, and U937 that have the ability to differentiate into cells with DC-like characteristics (Yoshida et al., 2003, Hu et al., 1996, Koss et al., 1996). Several *in vitro* model systems using human cell lines to assess the skin sensitising potential of contact allergens have been developed and are discussed in the following sections.

1.8.2.1. Human Cell Line Activation Test (h-CLAT)

The human Cell Line Activation Test (h-CLAT) (Figure 1-4(B)) was developed by Ashikaga et al. (2006) using THP-1 cells as a model for the mechanism underpinning human skin sensitisation. THP-1 cells are human monocytic leukemia cells which were derived from the peripheral blood of a male with acute monocytic leukemia (Tsuchiya et al., 1980). THP-1 was chosen for h-CLAT as THP-1 is easily cultured and re-capitulates several desired *in vivo* mechanisms, such as esterase activity, lysozyme production and phagocytosis (Tsuchiya et al., 1980, Ashikaga et al., 2006). In addition, naïve undifferentiated THP-1 cells have low endogenous expression levels of typical monocytic surface markers such as CD54 and CD86 (Santegoets et al., 2008) that were upregulated by skin sensitisers (Sakaguchi et al., 2006). Previous research was mainly focused on expression levels of CD86 as it is a hallmark of DCs activation and maturation, and therefore a useful marker to differentiate potential skin allergens/sensitisers and irritants (Ashikaga et al., 2002). However, the singular assessment of CD86 expression level alone was insufficient as sensitisers such as metal allergens failed to augment CD86 expression but alternately induced CD54 expression (Yoshida et al., 2003). Inter-laboratory studies showed that inclusion of the assessment of the upregulation of CD54 together with CD86 in the h-CLAT improved both the sensitivity and reproducibility of the assay (Sakaguchi et al., 2006, Ashikaga et al., 2006, Sakaguchi et al., 2010).

Further improvements to the h-CLAT were established with respect to the optimal cell culture and fluorescent-labelled antibody conditions to facilitate optimum binding to and detection of the CD54 and CD86 surface molecules by flow cytometry analysis (Ashikaga et al., 2006). Inter-laboratory data further revealed that threshold limits for relative fluorescence intensity (RFI) of 200% and 150% for CD54 and CD86 respectively allowed for better predictive identification of potential skin sensitisers by h-CLAT (Sakaguchi et al., 2007). However, as some of the sensitisers appeared to augment only CD54 or CD86 (Sakaguchi et al., 2009), the use of a weight-of-evidence approach was proposed where a chemical would be considered a positive sensitiser, if two out of any three independent data values for any one concentration fell above the threshold limits for CD54 or CD86 (Sakaguchi et al., 2006, Ashikaga et al., 2006, Sakaguchi et al., 2010).

A comparative study of h-CLAT, LLNA and HRIPT found that the h-CLAT gave an accuracy of 93.1% for the 29 chemicals tested (Sakaguchi et al., 2009). In a separate study, accuracy was 83% for the 51 sensitisers tested (Nukada et al., 2011). The false negatives from h-CLAT were attributed to weak sensitisers and the inability of THP-1 cells to metabolise and oxidise prohaptens and prehaptens respectively (Ashikaga et al., 2010).

1.8.2.2. Myeloid U937 Skin Sensitisation Test (MUSST)

The Myeloid U937 Skin Sensitisation Test (MUSST) (Figure 1-4(C)) utilises the human myeloid cell line U937 to determine chemical sensitisers (Ade et al., 2006, Python et al., 2006). U937 which originated from the histiocytic lymphoma of a male patient is able to differentiate in response to external stimuli to form macrophage-like morphology and features (Sundstrom and Nilsson, 1976, Ade et al., 2006, Python et al., 2007). MUSST was initially developed by L'Oréal (Ade et al., 2006) and Procter & Gamble (Python et al., 2006) whereby the upregulation of U987 surface molecular marker CD86 expression and cell viability are determined after 48 h exposure to a range of test chemicals. A chemical is regarded as a sensitiser if it results in a concentration-dependent upregulation of CD86 at non-cytotoxic concentrations in two concordant experiments (Ade et al., 2006). MUSST was further modified to include the concurrent assessment of additional markers, IL-1β and IL-8, where a chemical showing significant upregulation of at least two markers was considered a sensitiser (Python et al., 2007). Further method improvement to increase the detection capacity of MUSST by optimising cell density, concentration and marker thresholds were undertaken during the Colipa (the European Cosmetics Association)

interlaboratory ring trials, with the supporting results currently submitted to ECVAM for pre-validation (Aeby et al., 2010).

1.8.2.3. KeratinoSens™ Assay

The KeratinoSens™ assay (Figure 1-4(D)) is a cell-based reporter gene assay for screening chemical compounds that provides a full concentration-response assessment (Emter et al., 2010). The assay is based upon a stable transgenic immortal human keratinocyte (HaCaT) cell line that expresses luciferase under the control of an SV40 promoter and an antioxidant response element (ARE) derived from the human *AKR1C2* gene (Emter et al., 2010). The ARE-driven luciferase reporting cell line was developed based on previous findings on the stimulation of the nuclear factor erythroid-derived 2-related factor 2 (Nrf2)-Keap1-ARE regulatory pathways by skin sensitisers (Natsch and Emter, 2008, Vandebriel et al., 2010). Furthermore, gene expressions studies using microarray and reverse transcriptase-polymerase chain reaction (RT-PCR) found ARE as a reliable marker for screening skin sensitisers (Emter et al., 2010, Gildea et al., 2006, Ryan et al., 2004). Briefly, in the KeratinoSens™ assay, the transgenic cell line is treated with a range of test chemical concentrations and the resulting luciferase activity used to determine the EC1.5. The EC1.5 value is denoted as the chemical concentration that induces a 50% increase in luciferase expression above background (Emter et al., 2010). The KeratinoSens™ assay yielded high accuracy (85%) in predicting skin sensitisers with high reproducibility found within and between different laboratories (Natsch et al., 2011a). However, as expected, the KeratinoSens™ assay failed to predict sensitisers which do not activate the Nrf2-Keap1-ARE regulatory pathways, such as anhydrides which are reactive toward amine-groups (Emter et al., 2010). Interestingly, the KeratinoSens™ is able to predict some prohaptens as HaCaT cells are capable of metabolising prohaptens, such as the putative prohaptens cinnamic alcohol, ethylenediamine and diethylentriamine that were initially identified as non-sensitisers in the DPRA (Emter et al., 2010). Recently, KeratinoSens™ has been formally validated and the OECD test guideline has been published (OECD, 2015b).

1.8.2.4. Loose-fit coculture-based sensitisation assay (LCSA)[5]

An allergen-sensitive *in vitro* method that combines two layers of cells, termed the loose-fit coculture-based sensitisation assay (LCSA), was developed using human primary keratinocytes from healthy donors, and mobile DC-like cells *viz* peripheral blood mononuclear cells (PBMCs) (Figure 1-4(E)) (Schreiner et al., 2008). As keratinocytes are proposed to have a role in haptenation via maturation of DCs, this assay has the advantage of being able to detect prohaptens such as isoeugenol (Schreiner et al., 2008), that are not detected by many *in vitro* model systems. In short, inclusion of keratinocytes in this two-tiered cell-based system facilitated metabolic activation of prohaptens into sensitising agents akin to that which occurs in the skin *in vivo* (Wanner et al., 2010).

Similarly to h-CLAT and MUSST (as depicted in Figure 1-4(B) and (C)), LCSA quantifies the increase in expression of the cell surface marker, CD86 (Schreiner et al., 2007). Additionally, LCSA accuracy and sensitivity for assessing metal allergens such as nickel and cobalt, were improved by measuring accumulation of the pro-inflammatory cytokine, IL-6 and the chemokine macrophage inflammatory protein 1-beta (MIP-1β) (Schreiner et al., 2008). In a comparative evaluation of the *in vitro* LCSA relative to the *in vivo* LLNA for assessing the skin sensitising potential of a group of textile disperse dyes, both methods identified 87.5% of these dyes as having skin sensitising potential. Hence, the LCSA is a promising *in vitro* method for identifying agents with skin sensitising potential for use in combination with other non-animal testing methods (Sonnenburg et al., 2012). However, the current challenges in using the LCSA include the necessity to obtain keratinocytes and PBMCs from healthy human donors which makes the method susceptible to inter-donor variability. Additionally, the complexity and time required for seeding keratinocytes and PBMCs in this co-culture assay makes it low throughput and so future innovation is required to adapt the LCSA to high-throughput format.

[5] This section of the literature review of my PhD thesis has been published in the journal, *Frontiers in Pharmacology*, as a review article.

Wong C.L., Ghassabian S, Smith MT and Lam A (2015). *In vitro* methods for hazard assessment of industrial chemicals – opportunities and challenges. *Front. Pharmacol.* 6:94. doi: 10.3389/fphar.2015.00094.

1.8.2.5. Genomic allergen rapid detection (GARD)[6]

Apart from quantification of changes in cell surface expression of molecules of interest, genomic methods may offer an alternative or complementary *in vitro* testing paradigm. For example, genomic allergen rapid detection (GARD) employs the myeloid cell line, MUTZ-3 that resembles skin DCs with respect to transcriptional profiles and the ability to activate specific T-cell populations (Figure 1-4(F)) (Johansson et al., 2013). GARD uses a complete genome expression array approach to measure expression levels of 200 transcripts involved in the activation of various signalling pathways involved in skin sensitisation.

Unlike the KeratinoSens^{TM}, MUSST and h-CLAT *in vitro* methods that use specific markers for classifying sensitisers, GARD utilises 'biomarker signatures' for identifying skin sensitisers, thereby potentially increasing the predictive ability of the method. An added advantage of GARD is that it can distinguish respiratory and skin allergens by their unique biomarker signatures (Johansson et al., 2013). Encouragingly, use of GARD to assess 38 chemicals with known skin sensitisation potential in a preliminary study, showed that the accuracy, sensitivity and specificity of the method was high at 99% (Johansson et al., 2011).

Recently, Albrekt et al. (2014) stressed that chemical reactivity properties were key factors for consideration when developing *in vitro* screening models of chemical sensitisers. Sensitising chemicals were divided into groups based upon their mechanistic reactivity and assessed against various cell-signalling pathways using the GARD assay. Interestingly, different chemical reactivity groups induced differential changes in various cell signalling pathways, particularly those involved in cell cycling and metabolism. Potency in modulating these pathways appeared to be correlated with skin sensitisation potential (Albrekt et al., 2014). However, care is required to avoid over-interpretation of these associations with respect to potential sensitiser classification. More work is clearly required using larger numbers of chemicals with a broad range of functional groups of varying reactivity, as well as a range of concentrations and reaction times. Nevertheless, the GARD assay can provide invaluable information on the various cell signalling pathways underpinning the sensitisation process which is invaluable in informing further

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development of *in vitro* skin sensitisation test methods. Future research is warranted to assess the extent to which the epoxide group in ERS will modulate cell-signalling responses based upon their reactivity domain and/or their sensitising potency.

1.8.2.6. Human T-cell priming assay (hTCPA)[7]

During skin sensitisation, specific effector and memory T-cells are activated by DCs triggered by sensitising agents. While activation and proliferation of T-cells reflect the ultimate step in inducing sensitisation, there are very few assays that address this aspect of the sensitisation process. At present, only the *in vivo* LLNA is used widely to evaluate the activation and expansion of T-cells. More recently, an *in vitro* assay known as the human T-cell priming assay (hTCPA) was developed to assess T-cell responses initiated by contact allergens (Figure 1-4(G)) (Dietz et al., 2010, Richter et al., 2013). The hTCPA uses naïve T-cells isolated from PBMCs of healthy donors that are depleted in $CD25^+$ and $CD45RO^+$, a T-cell population responsible for regulating hapten-specific IFN-γ-producing T-cells in lymph nodes (Vocanson et al., 2013). The modified T-cells are co-cultured with hapten-treated monocyte-derived DCs at two stages, priming and re-stimulation. After re-stimulation, the increase in T-cell production and the cytokines, IFN-γ and TNF-α, are quantified using an enzyme-linked immunosorbent assay (ELISA) and an intracellular cytokine assay (Richter et al., 2013, Vocanson et al., 2013).

The hTCPA has been used successfully to assess the skin sensitising potential of the strong sensitisers, 2,4-dinitrochlorobenzene (DNCB), 2,4-dinitrobenzenesulfonic acid (DNBS) 2,4,6-trinitrobezene sulfonic acid (TNBS), and moderate/weak sensitisers, fluorescein isothiocyanate (FITC) and α-hexylcinnamaldehyde as well as the non-sensitisers, methyl salicylate, DMSO and sodium lauryl sulfate (Vocanson et al., 2014). Hence, the hTCPA has considerable potential as an *in vitro* method for assessing the skin sensitising propensity of contact allergens. However, similar to the LSCA, this method is time-consuming and fraught with difficulty in assay reproducibility due to the scarcity of T-cell donors and inter-donor variability. More work is warranted to assess the applicability and generalisability of the hTCPA system using a larger number and a wider range of chemical classes. For example the hydrophobicity of DNCB led to its reduced uptake by DCs which in turn did not stimulate T cell proliferation (Dietz et al., 2010). While the use of

[7] This section of the literature review of my PhD thesis has been published in the journal, *Frontiers in Pharmacology*, as a review article.

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nanoparticle encapsulation of lipophilic compounds significantly increased the ability of DNCB to stimulate T-cell proliferation and thus increase the assay sensitivity (Vocanson et al., 2013), inclusion of this additional step adds another level of complexity and increases assay costs.

1.8.3. *In silico* assays: QSAR and expert system

The QSAR and expert system were developed to predict the sensitisation potential of a novel compound using computer modelling (Cronin, 2010). While neither of these assays is suitable for stand-alone use for replacement of animal testing to identify potential skin sensitisers, they provide a widely acceptable tool for data mining and screening of large numbers of compounds (Gombar et al., 1997, Cronin, 2010). The primary function of QSAR studies is to predict the biological activity of a query compound based on the presence of its structural characteristics (Cronin, 2010). By contrast, the expert system is a tool that applies a database collection of computational rules, principles and facts that are derived from existing knowledge of known chemicals to provide a rational prediction on the activity of a query compound (Gombar et al., 1997).

In general, QSAR can be divided into two models, the global and local models (Chaudhry et al., 2010, Vandebriel and van Loveren, 2010). The global model aims to read-across compounds in all chemical classes that have been identified previously from human and animal data as sensitisers (Chaudhry et al., 2010). The global model is suitable for identifying structurally dissimilar compounds with a range of different mechanistic actions (Chaudhry et al., 2010). Nevertheless, the global model has comparatively low predictive power as any target compound that fell outside the predicted range gave rise to an inaccurate prediction (Chaudhry et al., 2010). By contrast, the local model investigated compounds that belong to a similar class either structurally or mechanistically but may overlook the compounds in distinct classes during sensitisation estimation (Vandebriel and van Loveren, 2010). In addition, QSAR also categorise compounds based on mechanistic platforms (mechanistic models) and empirically derived statistical approaches (empirical models) (Vandebriel and van Loveren, 2010). Multiples QSAR and expert systems have been developed. Table 1-6 summarises the current *in silico* models used for classifying and identifying skin sensitisers. These *in silico* models have been validated according to the OECD validation principles for QSAR models (OECD, 2004).

Table 1-6: List of currently available *in silico* models for assessment of skin sensitisation potential of chemicals

***In silico* approaches**	**Description**	**References**
TOPS-Mode (Topological Substructural Molecular Descriptors)	• Developed based on 93 compounds • Predicts skin sensitisers based on various mechanisms involved in skin sensitisation • Prediction based on structural understanding of compounds in the database (Structural Alert) • Successfully identified prohaptens	Estrada et al. (2003)
TOPKAT (Toxicity Prediction by Komputer Assisted Technology)	• Developed based on available guinea pig test data. • Provides prediction on toxicity endpoint • Provides an algorithm for reactivity and domain prediction information • Uses univariate analysis[8] and optimum prediction space (OPS)[9] system	Gombar et al. (1996)
DEREK Nexus (Deductive Estimation of Risk from Existing Knowledge)	• Expert system • Predicts sensitisers using structural alert and rule-based approach • Associated with metabolism prediction program (METEOR) for prohapten prediction	Ridings et al. (1996)
MCASE (Multi Computer Automated Structure Evaluation)	• Expert system • Developed based on human and animal studies • Rule-based approach • Prediction based on structural fragments (subunit) of compounds • Estimates potency of a target compound • Associated with metabolism tool (META)	Saiakhov and Klopman (2008)

[8] Univariate analysis: Statistical approach performed based on a single variable.
[9] Optimum prediction space (OPS): Prediction models are fine-tuned based on the multivariate space associated with target chemical. The model is applicable when the target compound is laid within or near the boundary of the training set.

Table 1-6: *Cont.*

In silico approaches	Description	References
SMARTS Patterns (Smiles Arbitary Target Specification)	• Developed based on LLNA data • Defines target compound in the two dimensional string which allow database screening of SMILES strings • Identifies potential mechanisms of action which enable it to be categorised in an appropriate reactivity domain • Trend analysis carried out followed by assigning reactivity domain of target compound	Enoch et al. (2008)
TIMES-SS (Tissue Metabolism Simulator for Skin Sensitisation)	• Expert system • Based upon the data from a consortium comprising industry and regulatory agencies • Utilises electrophilic mechanistic information • Integrates structure-toxicity and structure-metabolism relationships through a number of transformations simulating skin metabolism and interaction of the generated reactive metabolites with skin proteins • Incorporates Phase I and II skin enzymes that catalyse biotransformation reactions • Predicts the interaction between reactive target compound with skin proteins • Includes 3D QSAR submodel for evaluating compound reactivity (sensitisation rate) covering numerous alerting groups	Patlewicz et al. (2007)
OECD Application Toolbox	• Served as a platform for incorporating various modules and databases • Identifies target compound based upon structural similarity, mechanism of action, similar metabolites • Predicts target compound by using read-across, trend analysis (interpolating or extrapolating from a trend in existing database) and/or QSAR models. • Predicts peptide binding potency	OECD http://www.qsartoolbox.org/
VEGA (Virtual Models for Property Evaluation of Chemicals within a Global Architecture)	• Read-across strategy where target compound assessment is based upon that of structurally related substances. • Provides algorithms that are discrete from other QSAR models where similar compounds will be identified and the applicability domain will be analysed.	CAESAR http://www.vega-qsar.eu/

1.8.4. *Ex vivo* assays: Reconstructed human epidermis (RHE)

Although the aforementioned alternative testing methods focused on the key events within the sensitisation phase, they did not take into account the initial penetration phase of haptens through the viable epidermis as well as enzymatic activities within the skin compartments. Human skin that mainly functions as a protective layer, affects the penetration rate of haptens (Berard et al., 2003). Aeby and colleagues (2004) revealed that haptens with similar sensitising characteristics in *in vitro* models possessed distinct sensitising properties when tested in *in vivo* models. Human skin is a metabolically active organ which contains various enzymes and signalling molecules (Gibbs et al., 2007). Hence, to ideally reflect the human skin response in ACD and accurately predict the sensitising properties of haptens in assays, it is important to consider factors such as the incorporation of metabolically active molecules/enzymes that simulate the actual skin environment as well as the filtering function of human skin to estimate the minimal concentration that lead to sensitisation (Frankart et al., 2012).

Currently, there are four commercially available reconstructed human skin models, also known as 3D-recontructed human epidermal (RHE) models, including EpiDerm™ (MatTek Corporation), EpiSkin™ (Imedex) and SkinEthic™ (Laboratoire Skin Ethic) (Gibbs et al., 2007). RHE is constructed of an acellular dermal matrix without fibroblasts that contains a combination of cytokine and growth factors to mimic the actual skin environment (Gibbs et al., 2007). In addition to RHE models, another model comprised of a keratinocyte and fibroblast populated dermal matrix is called the skin equivalent model (Bell et al., 1983). It is commercially available as TestSkin™ (Organogenesis Inc). These commercial skin models have been validated and accepted for use in skin corrosion and skin irritation tests (Gibbs et al., 2007). However, at present, there is no commercially available skin model that contains DCs or LCs for predictive identification of skin sensitising compounds (Uchino et al., 2011). A recent promising study by Uchino et al. (2011) incorporated DCs, keratinocytes and fibroblasts into their newly reconstructed skin model and preliminary results showed that the skin model was responsive to sensitisers.

Even though RHE models are more complete models mimicking human skin compared with other non-animal testing methods that only concentrate on a single key event in the ACD mechanism, the challenge remains for development of a complete human skin model that has high predictive capacity for skin sensitisation identification (McKim et al., 2012, Gibbs et al., 2007). This challenge is largely due to inter-individual differences at both the

cellular and molecular levels, including genotypic variation, different epidermal thickness, and different levels of metabolic activity and rates of skin cell differentiation (Gibbs et al., 2007).

More recently, EpiSensA, an *in vitro* skin sensitisation assay that utilises a commercially available RHE (Saito et al., 2013) has become available. In brief, using this skin model system, skin sensitising potential of test compounds is assessed based upon changes in the expression of genes related to the cellular stress response. Preliminary data obtained from the analysis of 16 test compounds using EpiSensA were promising (Saito et al., 2013). Despite considerable progress, the challenge remains for a more complete human skin model system to become available that has a high degree of accuracy for correctly identifying and classifying the skin sensitisation potential of novel compounds. Nevertheless, EpiSensA has promise for improving *in vitro* assessment of the skin sensitising properties of compounds with poor aqueous solubility such as epoxy resins.

1.9. Challenges in assessing epoxy resin compounds using *in vitro* model systems[10]

Despite significant progress in the development and optimisation of non-animal testing assays, a major limitation in their use for accurately identifying the skin sensitising capacity of test compounds, is poor water solubility, particularly for aqueous-based assays (McKim et al., 2012). Maintaining a suitable balance between the final solvent composition, test compound solubility and deleterious solvent-related effects within the assay, is pivotal for generating meaningful data on skin sensitisation potential. In general, the solvent-related issues associated with *in vitro* assays are related to the toxicity and/or solvent-mediated modulation of the assay, thereby confounding assay readouts resulting in inaccurate assessment of skin sensitisation potential. High solvent concentrations in cell-based assays adversely affect cellular integrity, resulting in cell death (Tapani et al., 1996, Galvao et al., 2014). Concentration-related toxic effects of the solvent are needed to be evaluated to identify the maximum 'no effect' levels for each *in vitro* assay. The balance between acceptable solvent percentage in the aqueous cell-based test system whilst maintaining solubility of high molecular weight and low solubility test compounds, particularly industrial epoxy resin compounds is yet to be adequately addressed. This

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issue is arguably the most significant obstacle to be overcome in adapting current *in vitro* skin sensitisation assays to assessment of epoxy resin hazard risk.

Moreover, unacceptable modulation of the *in vitro* test system by organic solvents limits the range of solvents that can be used for dissolution of epoxy resin compounds. For example, organic solvents routinely used in laboratories inhibit cytochrome P450-mediated metabolic reactions, and may potentially fail to activate the enzyme-dependent sensitising chemicals in the test system (Troutman et al., 2011, Li et al., 2010). DMSO is unsuitable for use in the DPRA as its high reactivity means that it may react with assay peptides resulting in false positive results. The use of DMSO in the DPRA would require an additional costly step of purging the reaction system with an inert gas such as argon, to prevent oxidation of DMSO (Niklasson et al., 2009).

While selection of solvents compatible with *in chemico* assays may improve the ability of the DPRA to identify epoxy resins that have skin sensitising properties, it is more difficult to attain a suitable balance between epoxy resin solubility and cell viability in aqueous culture-based assays. Moreover, future investigation is required regarding the fact that most test compounds are applied in solution to *in vitro* assays which may not necessarily be reflective of the situation in humans where there may be topical application of the compound in the solid state to the skin. To address this issue, the RHE has considerable potential. The RHE comprises an acellular dermal matrix mimicking the human skin epidermis layer. It has been used together with cytokines and growth factors to better represent the human skin microenvironment (Gibbs et al., 2007). Preliminary data using the RHE system showed that it was responsive to known sensitisers (Uchino et al., 2011).

Apart from use of RHE model systems, the accuracy of *in vitro* methods for skin sensitisation assessment of industrial chemicals may be improved by including multiple assay readouts using an ‘assay panel’ approach (Bauch et al., 2012, Jaworska et al., 2011, Natsch et al., 2009). However, questions on the generalisability of these *in vitro* methods to accurately identify chemicals containing very different functional groups, is as yet unclear. In particular, most *in vitro* methods were developed and evaluated using small molecule chemicals that are widely utilised in the manufacture of cosmetic and toiletry products. This is a significant limitation as it has now been shown that different functional groups with varying chemical reactivity produce differential engagement of cell signalling pathways (Albrekt et al., 2014).

For example, from a dataset of 145 chemical compounds assessed using the KeratinoSens™ and MUSST assays, those that were preferentially lysine-reactive resulted in false negatives (Natsch et al., 2013). These findings mirror work by others (Migdal et al., 2013) whereby chemicals with high reactivity towards cysteine, and not lysine, activated the Nrf2-ARE pathway in THP-1 cells, a well-known toxicity pathway activated by skin sensitisers (Natsch, 2010) that underpins the design principles of both the KeratinoSens™ and LuSens tests. ERS compounds such as DGEBA, DGEBF and PGE react selectively with thiol groups (cysteine) (Natsch et al., 2013, O'Boyle et al., 2012). Hence, the KeratinoSens™ and LuSens assays that are based on the aforementioned pathway are worthy of future investigation for their applicability and reliability to assessment of the skin sensitising potential of epoxy resins.

However, it is important to bear in mind that a single stand-alone method based upon a single mechanistic pathway may be an insufficient approach to assess novel derivatives of skin sensitisers. These compounds may evoke a different mechanistic pathway in the skin leading to skin sensitisation. Indeed, ECVAM recently recommended that both the KeratinoSens™ and DPRA can be used as part of an integrated assessment approach to assess skin sensitisers (ECVAM, 2014). Hence, future research is required to assess the applicability of current *in vitro* methods to assess the skin sensitising potential of a broader range of chemical compounds as a means to identify the most appropriate *in vitro* assays and assay readout ranges, for establishing benchmarks to use for classifying the skin sensitisation potency of novel compound classes.

Another consideration to this discussion is the inherent accuracy of the LLNA itself with respect to existing human data. The LLNA is widely utilised as the benchmark for evaluating the predictive accuracy of non-animal methods. However, when compared against the human maximisation and patch test, the accuracy of the LLNA was only 72% (Anderson et al., 2011). More recently, a retrospective comparison of a moderately large dataset (>100) of test compounds revealed an 82% predictive accuracy for LLNA when compared with established human data (Urbisch et al., 2015). In other work, use of an integrated testing strategy-based on data from ‘two out of three *in vitro* prediction models’ resulted in a higher overall accuracy (≥90%) when compared with human data, as opposed to ≤83% using the LLNA dataset (Urbisch et al., 2015, Bauch et al., 2012). Factors potentially contributing to the discordance between human and LLNA data include the difference in skin penetration rates between the mouse and human, as well as the application method of the test compounds on the skin (Anderson et al., 2011; Delaine et

al., 2011). The volatility and cytotoxicity of compounds such as the components of ERS, could affect potency outcomes given the open nature of substance application to the mouse ear in the LLNA in contrast with the occluded dressing used in human patch tests (Delaine et al., 2011). Hence, where possible, it is important to compare data produced by various *in vitro* skin sensitisation tests with human data rather than relying solely on comparisons with LLNA data.

1.10. Rationale for my PhD Research Project

With worldwide economic growth, the global demand for epoxy resins is forecast to reach ~3 million tons by the end of 2017, projected at ~USD9.2 billion annually (GIA forcasts the global market, 2012). However, existing composite resin systems do not meet current and forthcoming manufacturing requirements as they are hazardous to both the environment and public health. Modification and improvement of current ERS are deemed unfeasible at this stage due to the complication of developing a non-sensitising composite resin with the desired chemical and physical characteristics. The prevalence of ACD in industrial workers dealing with ERS has increased considerably over the years and it is one of the most common occupational contact allergies reported (Niklasson et al., 2009). Hence, it is fundamental to develop a new generation of safe composite resins that still retain their high performance properties. At present, research on skin sensitisation has focused on use of the LLNA as it is widely accepted as a stand-alone method for identifying skin sensitisers and between-sensitiser discrimination based upon potency. In spite of the accessibility of the LLNA for skin sensitisation assessment of chemicals, ethical issues related to animal testing, has raised many concerns. The ethical mantra of the 3Rs, reduction, refinement and replacement in animal testing has gained political and economic momentum. As a result, a battery of validated high-throughput non-animal test methods to accurately identify the skin sensitisation potential of new resin composite materials and their constituents, will provide critical information that can be incorporated into the establishment of risk assessments. This in turn will lead to significant improvements in public health, including decreasing the socioeconomic burden due to reduced productivity comprising lost work days and higher manufacturing costs.

1.10.1. Research hypothesis

Optimisation of the current *in vitro* methods, DPRA and h-CLAT is required in order to improve the early detection of potential skin sensitisation caused by epoxy resin compounds.

1.10.2. Research objective and aims

The specific aims to be achieved are to:

- Optimise the DPRA to improve the efficacy of assessing chemicals, predominantly epoxy resins using peptides (with distinct side chains).
- Determine the sensitivity of hapten prediction using various peptide combinations in the DPRA.
- Optimise h-CLAT for assessment of hydrophobic chemicals, predominantly epoxy resin compounds and to explore potential biomarker use for refinement of the assay.
- Assess sensitivity of the h-CLAT in a high-throughput format to accommodate screening of a large number of chemicals.
- Compare the test outcomes of the DPRA and h-CLAT for assessment of the skin sensitisation potential of epoxy resins with that predicted by the available QSAR toolbox and murine LLNA methods.

Chapter 2: Establishment and optimisation of the THP-1 human cell line for assessing the skin sensitisation potential of chemicals

2.1. Introduction

As noted in Chapter 1, allergic contact dermatitis (ACD) is a T-cell mediated, delayed-type hypersensitivity immune response, which involves four key events in the skin, *viz* protein binding between the sensitising chemical and skin proteins (haptenation), keratinocyte activation, dendritic cell (DCs) activation and proliferation of hapten-specific T-cells (OECD, 2012a). Although several non-animal testing methods have been developed to examine these key events, none of these *in vitro* assays can be used as a stand-alone method for assessing skin sensitisation potential due to the complexity of the human response to a chemical allergen (Aeby et al., 2010).

The focus of this chapter of my PhD thesis was to bring innovation into the *in vitro* method known as the human cell line activation test (h-CLAT) and then use this improved method to assess the skin sensitisation potential of representative test chemicals, i.e. epoxy resin systems (ERS). The h-CLAT (reviewed in Chapter 1, Section 1.8.2.1) uses THP-1 cells (a human monocytic cell line) as a model system to mimic the maturation of Langerhans cells (LCs) to DCs, an essential step in the human skin sensitisation pathway (Ashikaga et al., 2006). THP-1 cells were chosen for the h-CLAT as they are readily cultured whilst maintaining several desired *in vivo* activities such as esterase activity, lysozyme production and phagocytosis (Tsuchiya et al., 1980, Ashikaga et al., 2006). More importantly, naïve undifferentiated THP-1 cells have low endogenous expression levels of typical cell surface markers such as CD54 and CD86 (Santegoets et al., 2008) that are specifically up-regulated when treated with skin sensitisers (Sakaguchi et al., 2006).

In the past several decades, there has been exponential growth in the use of ERS in commercial applications due to their strong adhesive bonding properties when applied to a range of surface materials and their excellent resistance to harsh chemical and environmental conditions (Cahill et al., 2012). However, this widespread use has led to many incidences of ACD in humans associated with the handling of epoxy resin compounds as raw materials in the workplace. Hence, it is crucial to have accurate and cost-effective methods that can be used to screen industrial chemicals for their potential as skin sensitisers for hazard assessment in the workplace.

Previous work by others has shown that the h-CLAT has have high predictive capacity for identifying chemical allergens commonly found in preservatives, hair dyes and fragrances (Kosaka et al., 2010, Nukada et al., 2012, Okamoto et al., 2010, Sono et al., 2010). Specifically, the overall test accuracy of the h-CLAT was 84% and 83% compared with the

murine local lymph node assay (LLNA) and human data respectively, for 100 chemicals assessed (Ashikaga et al., 2010, Nukada et al., 2011). However, the suitability of h-CLAT as a screening method for industrial chemicals such as ERS, is unknown and this knowledge gap is addressed in this chapter. The h-CLAT is typically performed in a 24-well plate using anti-CD54 and anti-CD86 that are conjugated to the same fluorochrome (Ashikaga et al., 2006). In this chapter, I investigate the feasibility to adapt h-CLAT into a 96-well assay format using dual flurochrome antibody-staining of CD54 and CD86 in order to improve the efficiency of the h-CLAT.

Apart from measurement of CD54 and CD86 up-regulation in response to sensitisers, several studies have assessed pro-inflammatory cytokine production, including interleukin (IL)-8, IL-12p40, tumour necrosis factor (TNF)-α, and IL-1β by DCs and/or surrogate cell lines (e.g. THP-1 cells) by allergens with sensitising potential (Aiba et al., 2003, De Smedt et al., 2001, Takahashi et al., 2011, Toebak et al., 2006). The augmentation of production of one or more pro-inflammatory cytokines by sensitising chemicals has been further assessed in keratinocytes and DC-type surrogate cells with a view to differentiating between contact allergens (skin sensitisers), respiratory allergens and irritants. For example, IL-18 was induced following exposure of the human keratinocyte cell line, NCTC2455, to contact allergens but not to respiratory allergens or irritants (Corsini et al., 2009). Apart from the cell surface molecular markers, CD86, CD54 and CD40, contact allergens but not non-allergens triggered the up-regulation of TNF-α and IL-8 expression in THP-1 cells (Miyazawa et al., 2007). Hence, in this chapter, the effect of contact allergens on cytokine production in THP-1 cells, specifically IL-1β, IL-12p70, IFN-γ, IL-6, IL-8, IL 10 and TNF-α as potential biomarkers for ERS, was evaluated. Cytokine quantification was performed using a high-throughput multiplexed assay herein.

2.2. Materials and methods

2.2.1. Chemicals and reagents

2.2.1.1. Test chemicals

Bisphenol A diglycidyl ether (DGEBA, CAS 1675-54-3), trimethylolpropane triglycidyl ether (TMPTGE, CAS 3454-29-3, technical grade), poly(ethylene glycol) diglycidyl ether (PEGGE, CAS 72207-80-8), tetraphenylolethane glycidyl ether (THETGE, CAS 7328-97-4), poly[(phenyl glycidyl ether)-co-formaldehyde] (PPGE, CAS 28064-14-4), 2,4-dinitrochlorobenzene (DNCB, CAS 97-00-7) and methyl salicylate (CAS 119-36-8) were supplied by Sigma-Aldrich Corporation (NSW, Australia).

2.2.1.2. Reagents

Dimethylsulfoxide (DMSO), bovine serum albumin (BSA), phosphate buffered saline (PBS), γ-globulin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), isopropanol, tetramethylbenzidine (TMB) liquid substrate system, and Tween-20 were purchased from Sigma-Aldrich Corporation (NSW, Australia). Monoclonal mouse anti-human CD54-fluorescein isothiocyanate (FITC) (clone 6.5B5) was purchased from Dako (Japan). Monoclonal mouse anti-human CD86-phycoerythrin (PE) (clone FUN-1), 7-amino actinomycin D (7-AAD), and IL-6 OptEIA™ Enzyme-linked Immunosorbent Assay (ELISA) kit (catalogue no. 555220), IL-8 OptEIA™ ELISA kit (catalogue no. 555244), IL-10 OptEIA™ ELISA kit (catalogue no. 555157) and IL-1β OptEIA™ ELISA kit (catalogue no. 557953) were obtained from Becton Dickinson (BD) Biosciences (NJ, USA). Meso Scale Discovery® (MSD) human pro-inflammatory 7-plex tissue culture kit (catalogue number N75008B-1) was supplied by Meso Scale Diagnostics, LLC (MD, USA). 0.4% Trypan blue was obtained from Life Technologies Invitrogen (VIC, Australia). Sulphuric acid, formic acid, sodium carbonate anhydrous and sodium hydrogen carbonate were purchased from Ajax Finechem Pty. Ltd. (NSW, Australia) and sodium hydroxide was provided by Chem-Supply (SA, Australia).

2.2.1.3. Cell culture media

Fetal bovine serum (FBS), PBS and RPMI 1640 (ATCC modification) media (catalogue no. A10491) were supplied by Life Technologies Invitrogen (VIC, Australia). 2-mercaptoethanol was purchased from Sigma-Aldrich Corporation (NSW, Australia).

2.2.2. Experimental design

2.2.2.1. Cell culture

Monocytic THP-1 cells (catalogue no. TIB-202^{TM}) were purchased from the American Type Culture Collection (ATCC; VA, USA). These cells were cultured in vented cell culture flasks, passaged and harvested as per the manufacturer's instructions. The cell passages used in all experiments did not exceed 20 passages (after thawing). The cell cultures were routinely maintained at approximately 8x10^5 cells/mL in the cell culture flasks. THP-1 cells were propagated in a humidified incubator at 37°C (5% CO_2: 95% air) in RPMI 1640 medium supplemented with 10% FBS and 2-mercaptoethanol (0.05 mM). The concentration of viable THP-1 cells in suspension was determined by trypan blue exclusion assay prior to seeding of cells into 96-well cell culture plates. Briefly, a small aliquot (~20µL) of THP-1 cells was aseptically transferred to a small vial and diluted at a 1:1 ratio with 0.4% trypan blue. The cell-trypan blue mixture was then loaded onto an Improved Neubauer haemocytometer to determine the number of viable (unstained) cells. The suspension of THP-1 cells was then harvested by centrifugation at 130 xg for 5 min. The resulting cell pellet was re-suspended in fresh growth medium to a final concentration of 2x10^5 viable cells/mL for maintaining the cell culture or to a final concentration of 2x10^6 viable cells/mL for the purpose of seeding THP-1 cells (80 µL per well) into 96-well assay plates.

2.2.2.2. Cytotoxicity test

Prior to the selection of concentrations of each test chemical in subsequent cell-based assays (Sections 2.2.2.4), concentration-response curves for all test chemicals were established for THP-1 cells, to determine the concentration of each chemical that resulted in 75% cell viability (CV75). All test chemicals were freshly prepared on the day the assay was performed. Seven representative test chemicals (DNCB, methyl salicylate, DGEBA, PEGGE, TMPTGE, THETGE and PPGE) were prepared in DMSO at 250 mg/mL. A further 11 working concentrations of each chemical were subsequently prepared by 2-fold serial dilutions of the 250 mg/mL chemical stock in a 96-well plate. Each of these 12 working stock concentrations were then further diluted 250-fold in cell culture medium and subsequently added in triplicate (80 µL per well) to 96-well culture plates containing 80 µL (per well) of 2x10^6 cells/mL. Essentially the assay plates contained a final THP-1 concentration of 1x10^6 cells/mL and a final test chemical concentration of a 1/500 dilution of each of the previously prepared 12 working stock solutions for each test chemical. The

chemical-treated cells were incubated at 37°C (5% CO_2: 95% air) for 24 (±1) h. The final concentration of the solvent, DMSO, used in this cell-based assay did not exceed 0.2%.

After 24 h incubation, the chemical-treated cells were transferred to a 96-well v-bottomed plate and centrifuged at 300 xg at 4°C for 5 min. The cell culture medium was aspirated and discarded. The cells were washed twice with fluorescent activated cell sorter (FACS) buffer (1x PBS supplemented with 0.1% BSA). The cell pellets were then resuspended in 150 µL of 0.5 µg/mL 7-AAD, a cell-viability stain to gate out the dead cells and the cell viability of the chemically-treated cells were determined by flow cytometric analysis.

The cell viability of each well was determined using the formula in Equation 1 below (OECD, 2014):

$$\text{Cell viability } (\%) = \frac{Number\ of\ living\ cells}{Total\ number\ of\ acquired\ cells} \times 100\% \quad \text{----- (1)}$$

The concentration of each test chemical that gave CV75 was determined from a calibration curve using log-linear interpolation using the formula in Equation 2 below:

$$\log CV75 = \frac{(75-C)\times\log(B)-(75-A)\times\log(D)}{A-C} \quad \text{----- (2)}$$

Where

A is the minimum value of cell viability over 75% in testing groups
B is the maximum value of cell viability below 75% in testing groups
C or D is the concentration showing the value of cell viability A or B

2.2.2.3. Optimisation of antibodies for h-CLAT

The optimal concentrations of anti-CD86-PE and anti-CD54-FITC for use in the h-CLAT (Section 2.2.2.4) were determined using an antibody titration curve. Specifically, THP-1 cells were treated with the positive (sensitiser) control, DNCB, at four different concentrations based upon the CV75 determined from the cytotoxicity assay (Section 2.2.2.2), i.e. 1.2 x CV75, 1 x CV75, 1/1.2 x CV75, and $1/1.2^2$ x CV75. As per Section 2.2.2.2, these four working stock concentrations were then further diluted 250-fold in cell culture medium and added in triplicate (80 µL) to 96-well culture plates containing 80 µL aliquots of the $2x10^6$ cells/mL cell suspension. After 24 h incubation, the cells were transferred to a 96-well v-bottom plate and centrifuged at 300 xg at 4°C for 5 min. The resulting cell pellets were washed twice with FACS buffer where 150 µL/well of FACS

buffer was added and the plate was centrifuged at 300 xg at 4°C for 5 min. 1% BSA in PBS was added into each well (100µL/well) and incubated for at least 15 min on ice. After incubation, the plate was centrifuged at 300 xg at 4°C for 5 min and the resulting supernatant was discarded. The cell pellets were then washed twice with FACS buffer. Five working concentrations of each antibody were prepared by 2-fold serial dilution from the neat antibody solution (1/10, 1/20, 1/40, 1/80 and 1/160) using 1x PBS. Each antibody concentration (50 µL aliquots) for anti-CD86-PE and anti-CD54-FITC were added to each set of DNCB-treated cells and incubated for at least 30 min on ice to allow the binding of the antibodies to the cell surface molecules, CD54 and CD86. The plate was then centrifuged at 4°C at 300x g for 5 min and washed twice with FACS buffer. The cells were then resuspended with 150 µL of FACS buffer containing 0.5 µg/mL 7-AAD and analysed by flow cytometry to determine the optimal dilution/concentration of anti-CD86-PE and anti-CD54-FITC that produced the highest signal-to-noise ratio.

2.2.2.4. h-CLAT protocol

Test chemicals with eight concentrations based upon their respective CV75 concentrations (predetermined in Section 2.2.2.2) were prepared as per the Organisation for Economic Co-operation and Development (OECD) draft guideline for the h-CLAT (OECD, 2014). Briefly, 1.2-fold serial dilution of the test chemicals (1.2 x CV75, 1 x CV75, 1/1.2 x CV75, $1/1.2^2$ x CV75, $1/1.2^3$ x CV75, $1/1.2^4$ x CV75, $1/1.2^5$ x CV75 and $1/1.2^6$ x CV75) were performed in 96-well plates using DMSO as the solvent. The THP-1 cells were treated and incubated in triplicates and three independent experiments were performed with the above range of chemicals as per section 2.2.2.2. After 24 h incubation, the cells were transferred to 96-well v-bottom plates and centrifuged at 300 xg at 4°C for 10 min. The cell culture supernatant was collected and stored at -80°C to be used for cytokine quantification as a separate experiment (Section 2.2.2.6). The cell pellets were washed twice with FACS buffer. The cell pellets were subsequently resuspended in 1% BSA in PBS and incubated for at least 15 min on ice and washed with FACS buffer. 50 µL aliquots of anti-CD54 and anti-CD86 antibodies (in FACS buffer) at their predetermined optimal concentrations (Section 2.2.2.3) were added to each well and incubated for at least 30 min on ice. The plate was then centrifuged at 4°C at 300x g for 5 min and the resulting cell pellets washed twice with FACS buffer. The cells were then resuspended with 150 µL of FACS buffer containing 0.5 µg/mL of 7-AAD. The plate was incubated on ice for at least 10 min prior to being analysed with flow cytometry.

2.2.2.5. Quantification of 7-AAD, CD54 and CD86

3-coloured flow cytometry acquisition was performed using 96-well plate format, a BD LSRII analyser (BD Biosciences, USA) and an in-house designed autosampler unit integrated with a Gilson 232XL sampling injector at the Queensland Brain Institute (QBI), with the system parameters as summarised in Table 2-1 and Table 2-2, respectively. The autosampler was controlled by in-house designed software. The band-pass filters used for 7-AAD were 660/20, 530/30 for FITC and 575/26 for PE. Spectral overlap was compensated for in channels using anti-CD54-FITC and anti-CD86-PE. The data were analysed using the BD FACSDiva Version 6.1.3 analysis software and the geometric mean fluorescence intensity (MFI) was obtained.

The relative fluorescence intensities (RFIs) of CD54 and CD86 were calculated based upon Equation 3 below:

$$Relative\ Fluorescence\ Intensity\ (RFI)(\%) = \frac{\left(\begin{matrix}Geometric\ MFI\ of\\ treated\ cells\ with\ antibody\end{matrix}\right) - \left(\begin{matrix}Geometric\ MFI\ of\\ treated\ cells\ without\ antibody\end{matrix}\right)}{Geometric\ MFI\ of\ control\ with\ antibody - Geometric\ MFI\ of\ control\ without\ antibody} X100\% \quad \text{----- (3)}$$

The RFI value was not calculated when the cell viability was less than 50% (CV50). The thresholds were set at RFI≥150% for CD86 and RFI≥200% for CD54. Finally, a chemical was classified as a sensitiser if a chemical at any concentration exceeded either of the CD86 or CD54 thresholds in two out of three independently conducted experiments.

Table 2-1: Instrument parameters used for analysing samples. The parameters were optimised by using the BD FACSDiva™ Software Version 6.1.3.

Parameters	Value
FSC-A	305 volts
SSC-A	348 volts
Cy5-PE	667 volts
Threshold	20,000
Event	5,000 event
Stopping time	20 seconds

Table 2-2: The parameters used for analysing samples using a 96-well plate format.

Parameters	Value
Flow rate	200 µL/min
Injection volume	80 µL
Rinsing volume	500 µL

2.2.2.6. Cytokine quantification

Cytokine concentrations in the THP-1 cell culture supernatant from the h-CLAT experiments were quantified using the MSD human pro-inflammatory 7-plex tissue culture kit as per the manufacturer's protocols. Three concentrations of each test chemical (i.e. CV75, CV75/1.2 and CV75/1.2^2) were used in the assay. All proprietary kit reagents and wash buffers used were supplied by the manufacturer. Briefly, 150 µL of the 1% Blocker B in PBS was added to each well of the 96-well human pro-inflammatory 7-plex electrochemiluminescence (ECL) plate and allowed to incubate on a plate shaker at 700 rpm at room temperature (RT) for 1 h. After incubation, the plate was washed three times with wash buffer. A range of eight calibrator concentrations (0.610 – 10 000 pg/mL) for each of IL-1β, IL-12p70, IL-6, IL-8, IL-10, TNF-α and IFN-γ were prepared. The calibrators and supernatant samples were aliquoted in duplicate wells (25 µL/well) and incubated on a plate shaker at 700 rpm at RT for 2 h. The plate was washed three times with wash buffer before the addition of 25 µL of the respective 1x detection antibody solution. The plate was incubated on a plate shaker at 700 rpm at RT for an additional 2 h. Lastly, the plate was washed three times with wash buffer and 150 µL of 2x Read Buffer T was added to all wells. The plate was read using the MSD Sector Imager 2400A (Meso Scale Diagnostics, LLC, MD, USA). The MSD Discovery Workbench Version 4.0.12 software was used to analyse the data.

2.2.2.7. Cytokine stability test

Cytokine stability was assessed using BD OptEIA^{TM} ELISA kits as per the manufacturer's protocols. The cytokine (IL-1β, IL-6, IL-8 and IL-10) quality control (QC) samples were prepared at the following concentrations, IL-1β: 11.7, 125 and 200 pg/mL; IL-6: 14.1, 150 and 240 pg/mL; IL-8: 9.4, 100 and 160 pg/mL; IL-10: 23.4, 250 and 400 pg/mL and stored at -80°C. Each of the prepared cytokine samples was treated according to the two following experimental conditions (1) incubation at 37°C for 24 h to determine the stability of the cytokines at the given temperature and (2) 1x, 2x, 3x and 4x freeze-thaw cycles prior to assay. For each freeze-thaw cycle, frozen samples were left at RT until they were fully thawed. Samples were then frozen with dry ice to ensure the samples were fully frozen prior to the second cycle of freeze-thaw.

All cytokine test samples (37°C incubated and freeze-thawed) were stored at -80°C until analysis. Briefly, a 96-well ELISA plate was coated with 100 µL of anti-human IL-8 monoclonal antibody (capture antibody) diluted 1 in 250 in 0.1 M sodium carbonate, pH 9.5

(coating buffer) overnight at 4°C (~18 h). The wells of the ELISA plate were washed three times with 0.05% Tween-20 in PBS (wash buffer). The plate was then blocked with 200 µL of 10% FBS in PBS (assay diluent) and incubated for 1 h at RT. Next, the standard calibration curve for IL-8 (3.13 - 200 pg/mL) was prepared in assay diluent. After 1 h incubation, the plate was washed three times with wash buffer and 100 µL aliquots of each calibration standard and cytokine test samples were pipetted in duplicate into appropriate wells. The plate was sealed with a plate sealer and incubated for 2 h at RT. The plate was washed five times with wash buffer and 100 µL aliquots of working detector (biotinylated anti-human IL-8 and streptavidin-horseradish peroxidase conjugate diluted 1/250 in assay diluent) were added to all wells and incubated for 1 h at RT. After incubation, the plate was washed with wash buffer seven times. 100 µL aliquots of TMB substrate solution were added to all wells and incubated for an additional 30 min at RT in the dark. 50 µL aliquots of 2 N sulphuric acid (stop solution) were then added to each well and absorbance of 450 nm corrected to the background absorbance at 570 nm was measured using the TECAN Sunrise™ reader (TECAN, Männedorf, Switzerland) coupled with Magellan Tracker Version 7.1. The stability of IL-6, IL-10 and IL-1β were similarly assessed using the above method. The concentrations range of the calibration curves for the three cytokines were prepared as followed, IL-1β: 3.91 – 250 pg/mL; IL-6: 4.69 – 300 pg/mL; IL-10: 7.81 – 500 pg/mL.

2.2.2.8. Data analysis

The acceptance criterion for the cytokine concentrations measured using the MSD and ELISA assays was ±25% for both the LLOQ and ULOQ of the calibration curve. The accuracy of the low, medium and high QC samples was set at ±20%. The cytokine concentrations quantified using the MSD are presented as fold changes relative to those measured in the cell culture supernatant of vehicle-treated THP-1 cells (Equation 4).

$$Fold\ change = \frac{Concentration\ of\ cytokine\ in\ stimulated\ cell\ culture\ supernatant}{Concentration\ of\ cytokine\ in\ vehicle{-}control\ cell\ culture\ supernatant} \quad \text{----- (4)}$$

2.3. Results

2.3.1. Cytotoxicity test

Cell viability of the THP-1 cells incubated with the various test chemicals was assessed using the viability stain, 7-AAD, to distinguish between dead (stained) and live (unstained) cells (Figure 2-1). A total of 5,000 cells were used in all experiments. The chemical concentration that produced CV75 was calculated as described in Section 2.2.2.2 and these concentrations are listed in Table 2-3.

2.3.2. Optimisation of anitibodes for h-CLAT

For THP-1 cells treated with 4.6 µg/mL DNCB, the MFI for anti-CD86-PE and anti-CD54-FITC determined by flow cytometry was found to plateau after the 1/40 dilution of the manufacturer-provided antibody solution (Figure 2-2). The area of plateau for the MFI of both antibodies were also similarly observed in THP-1 cells treated with the remaining three DNCB concentrations (2.7, 3.2 and 3.8 µg/mL) (data not shown) albeit to a lesser extent that of the 4.6 µg/mL DNCB-treated cells. Hence, the optimal antibody dilution chosen for both anti-CD54 and anti-CD86 for all subsequent h-CLAT assays was 1/40 of the manufacturer supplied antibody solution.

2.3.3. h-CLAT

Expression levels of CD54 and CD86 in THP-1 cells treated with the various chemicals were determined by three-coloured flow cytometry analysis (Figure 2-3). Consistent with expectations, DNCB was classified as a sensitiser by h-CLAT as the expression levels of both CD54 and CD86 at various concentrations exceeded the RFI thresholds of ≥200% and ≥150% respectively, in all three independent experiments (Figure 2-4). By contrast, the non-sensitiser, methyl salicylate, did not induce CD54 and CD86 (Figure 2-5). However, all five epoxy resins tested, *viz* DGEBA, PEGGE, TMPTGE, THETGE and PPGE failed to meet these criteria for classification as sensitisers, as the epoxy resin compounds did not induce expression of CD54 and CD86 in two out of three independent experiments at their respective tested concentrations. Hence, these chemicals were classified as negative sensitisers (Figure 2-6 to Figure 2-10).

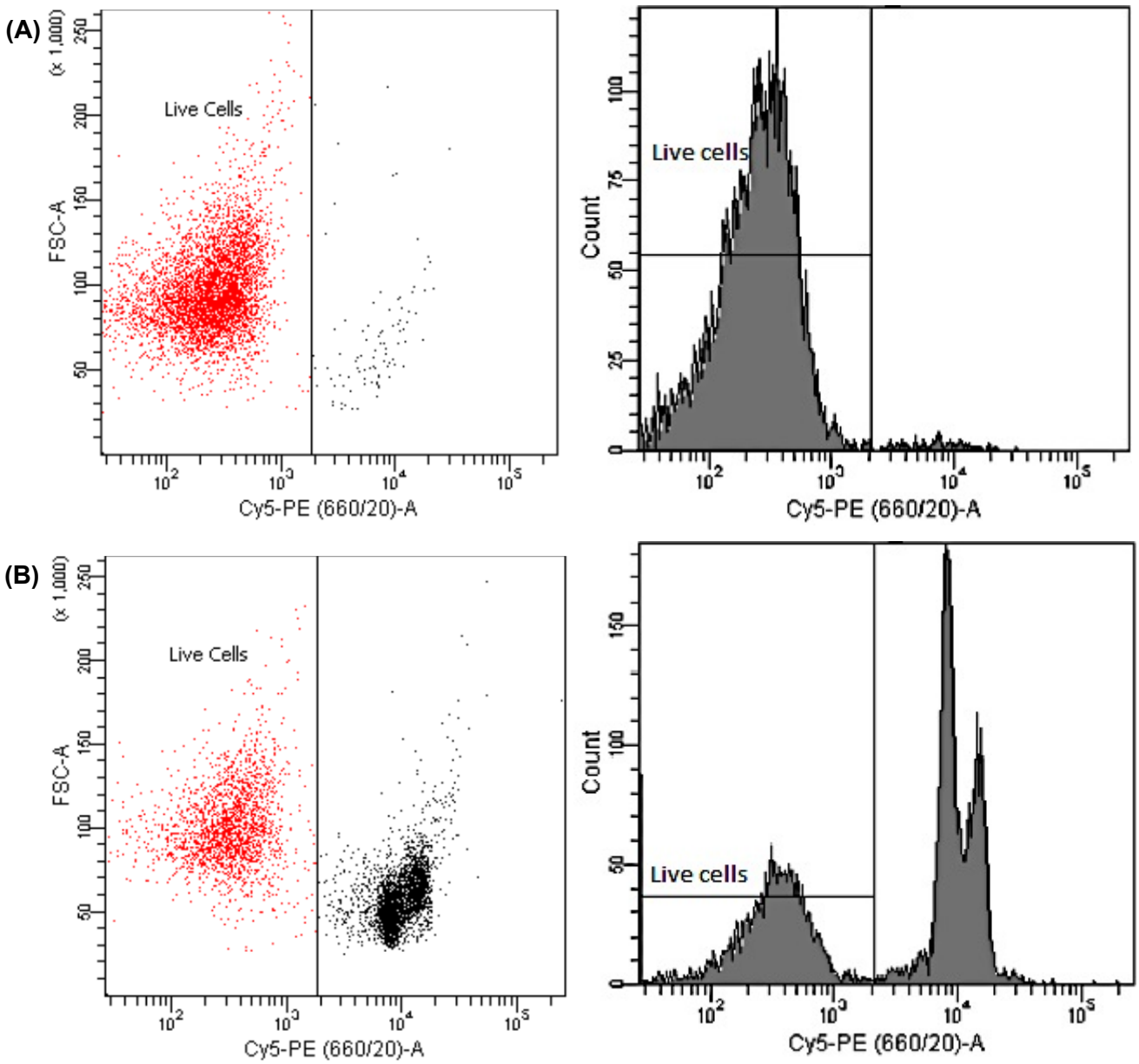


Figure 2-1: Graphical representation of cell viability gating in flow cytometry using the BD FACSDiva Software Version 6.1.3. Forward scatter area (FSC-A) data estimate the relative size of the cells while side scatter area (SSC-A) data provide information on the cell granularity. Cy5-PE represents the cells stained with 7-AAD. (A) vehicle-treated cells with 98.1% live cells (B) 7.8 µg/mL DNCB-treated cells with 40.3% live cells.

Table 2-3: Chemical concentrations that result in cell viability of 75% (CV75).

Chemicals	CV75 (µg/mL)
DNCB	4.63
Methyl salicylate	500
DGEBA	32.6
PEGGE	98.5
TMPTGE	27.8
THETGE	27.4
PPGE	79.5

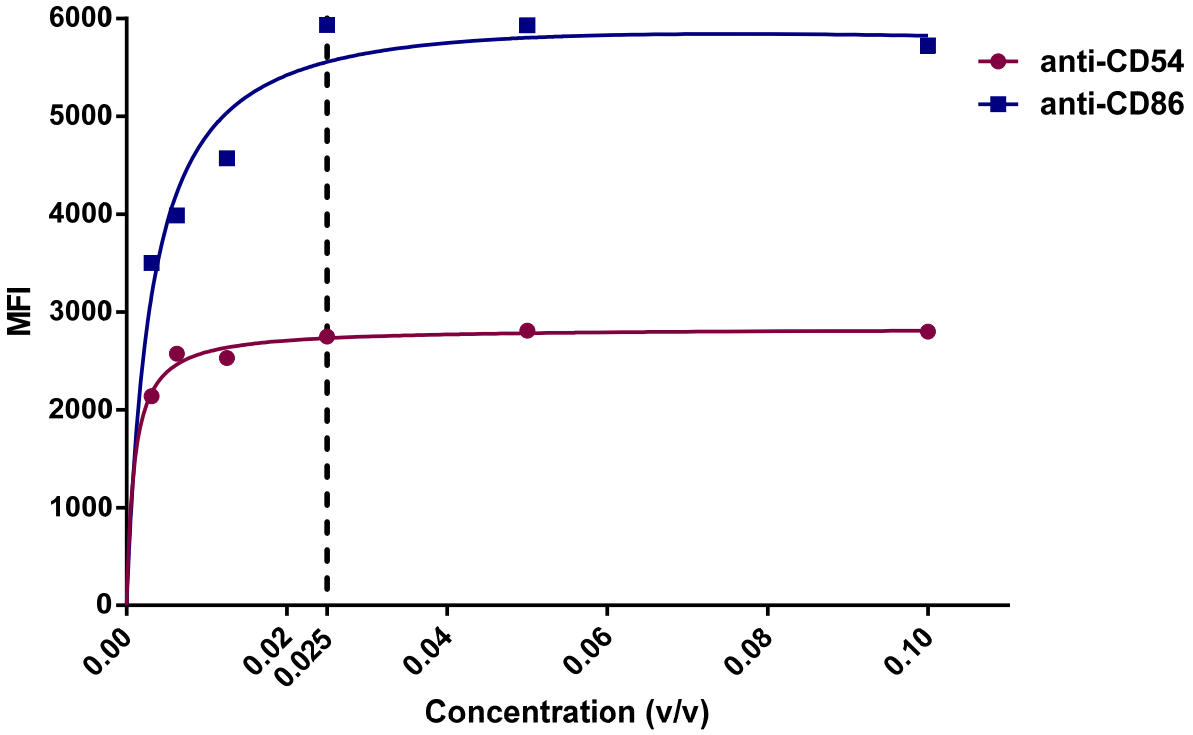


Figure 2-2: Determination of the optimal concentration of anti-CD54 and anti-CD86 antibodies for use in the h-CLAT. THP-1 cells were incubated with DNCB at a concentration of 4.6 µg/mL for 24 h and stained with 6 different concentrations of antibodies (neat solution and five 2-fold serial dilution working concentrations).

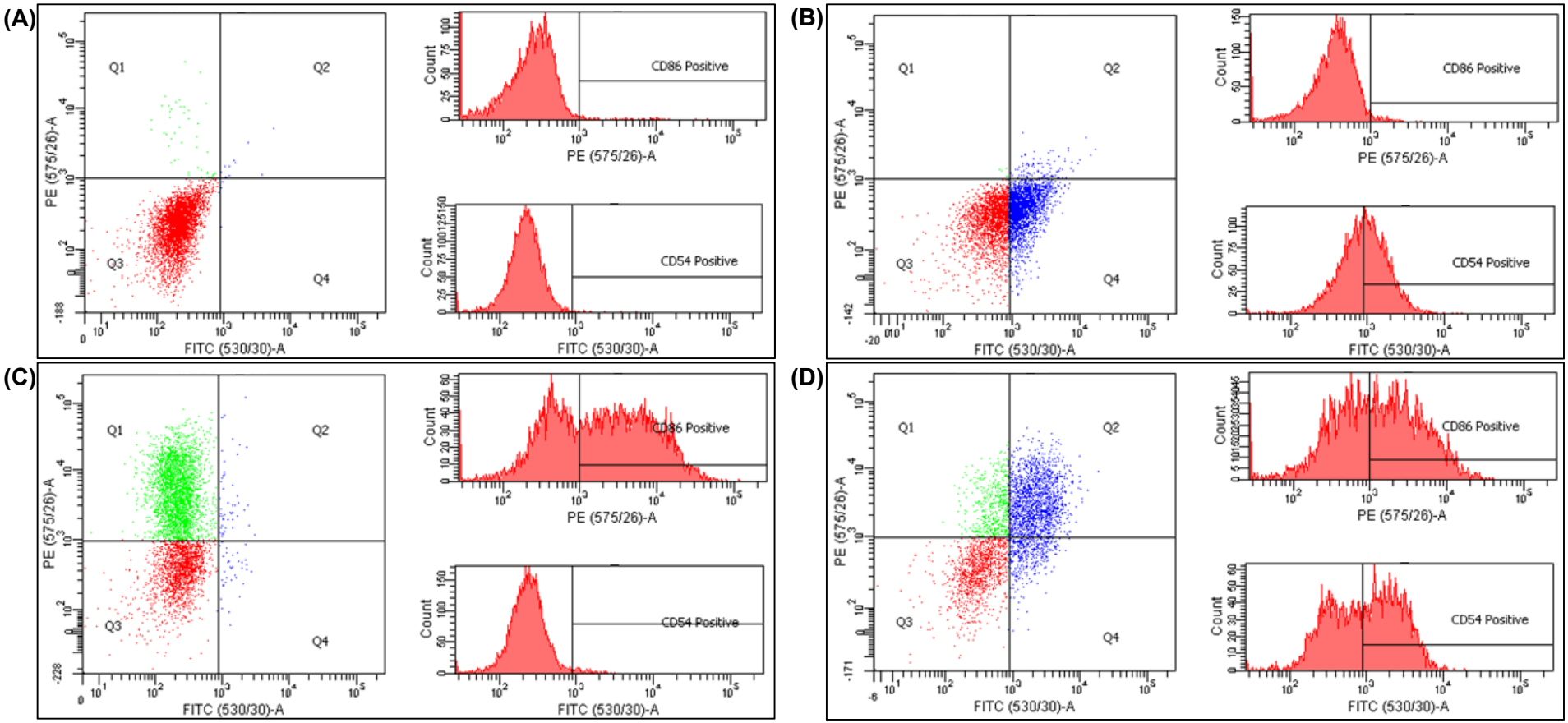


Figure 2-3: Graphical representation of cell gating with 3-coloured fluorochromes used in the flow cytometry data analysis. The phycoerythrin area (PE-A) represents CD86 positive cells while the fluorescein isothiocyanate area (FITC-A) represents CD54 positive cells. The total live cells after incubation with DNCB at 4.6 μg/mL were gated as per Figure 2-1 followed by subsequent gating for PE or FITC stained cells. (A) DNCB (4.6 μg/mL)-treated cells without staining (B) DNCB (4.6 μg/mL)-treated cells with CD54-FITC staining only (C) DNCB (4.6 μg/mL)-treated cells with PE-CD86 staining only (D) DNCB (4.6 μg/mL)-treated cells with both CD54-FITC and CD86-PE staining.

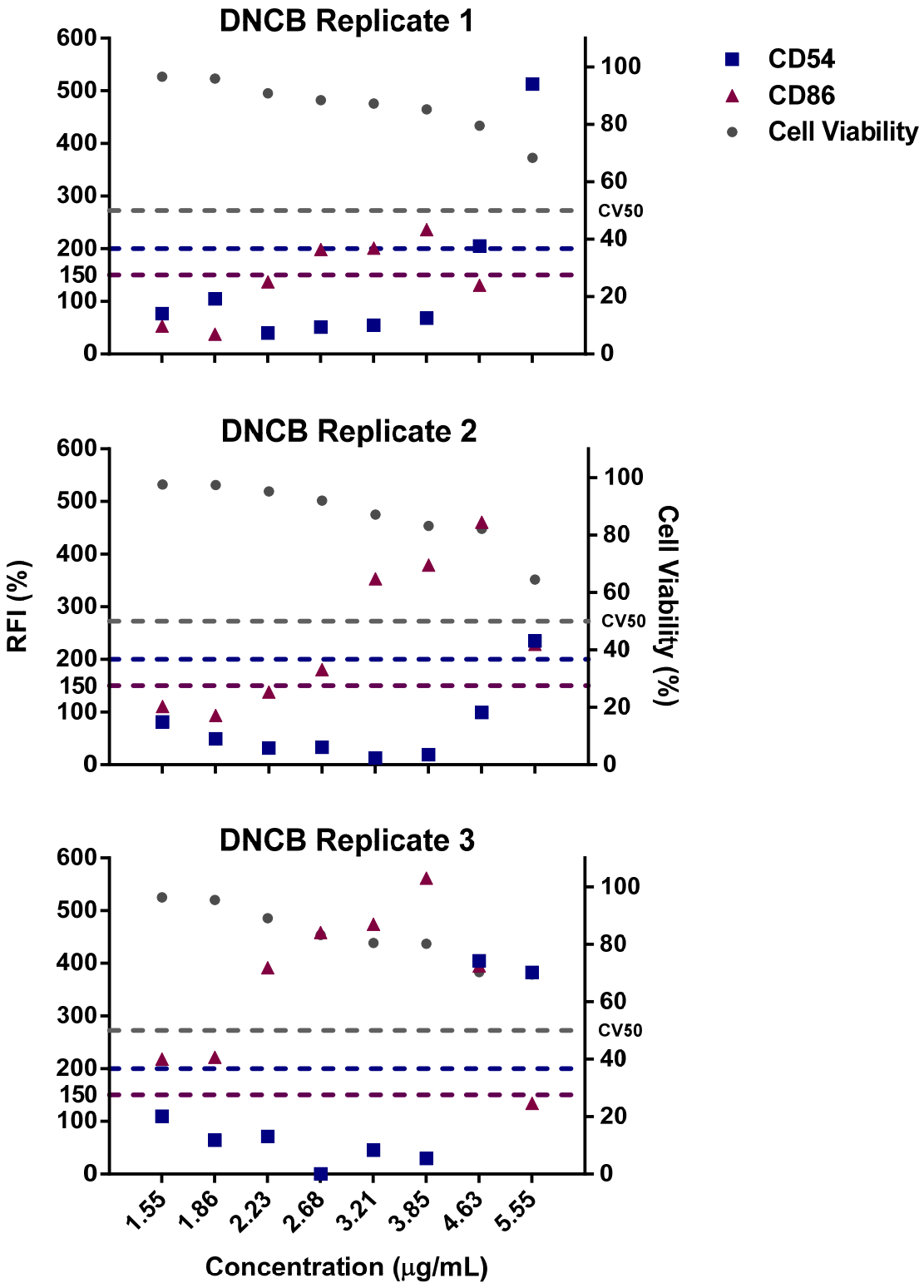


Figure 2-4: Expression levels of the cell surface molecules CD54 (■) and CD86 (▲) shown as RFI%, as well as percentage cell viability (●) of THP-1 cells incubated with 8 concentrations of 1.2-fold serially diluted DNCB. The blue dotted line (---) represents the threshold value of CD54, RFI=200 while the red dotted line (---) represents the threshold value of CD86, RFI=150. DNCB was classified as a positive sensitiser.

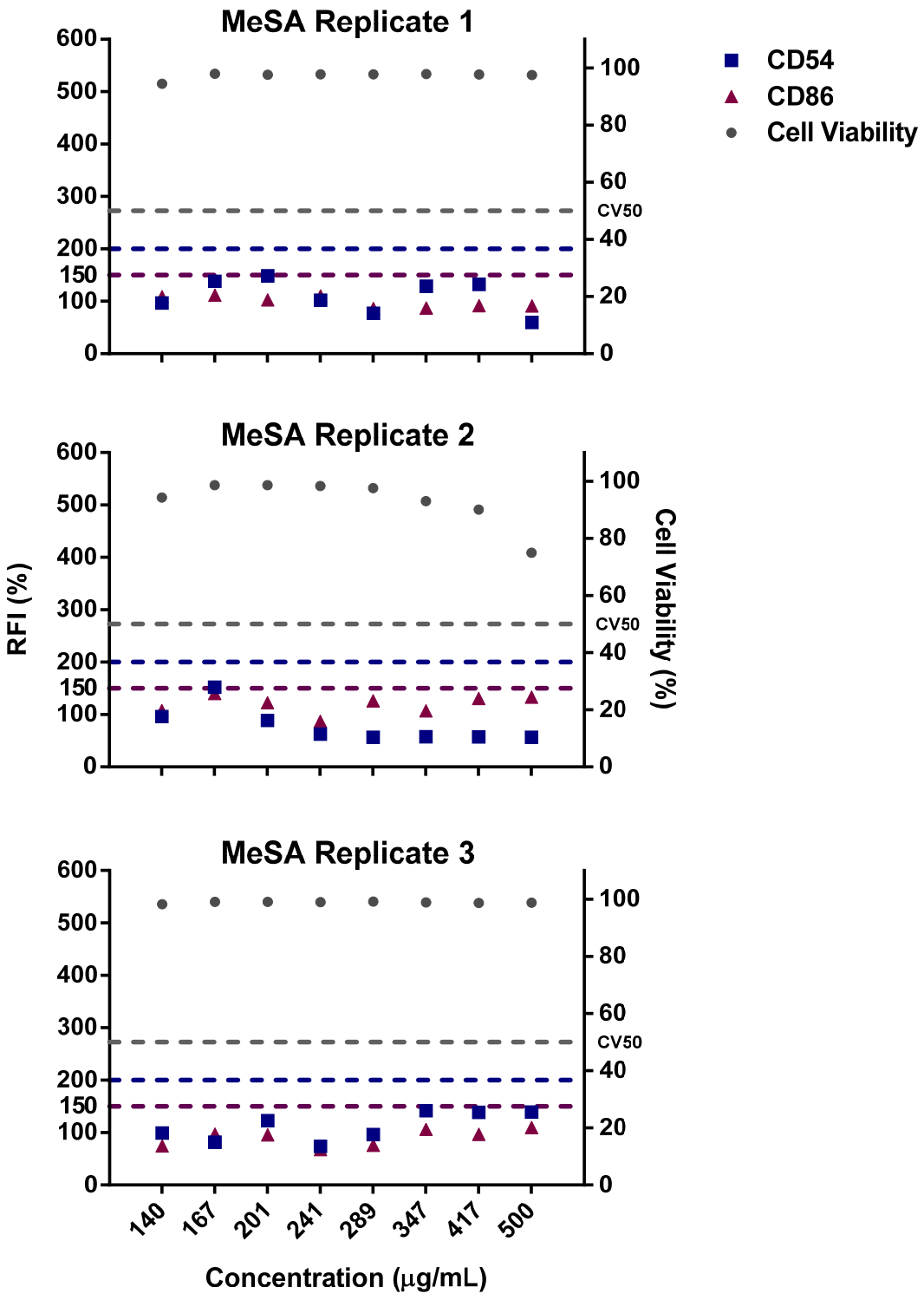


Figure 2-5: Expression levels of the cell surface molecules CD54 (■) and CD86 (▲) shown as RFI%, as well as percentage cell viability (●) of THP-1 cells incubated with 8 concentrations of 1.2-fold serially diluted methyl salicylate. The blue dotted line (---) represents the threshold value of CD54, RFI=200 while the red dotted line (---) represents the threshold value of CD86, RFI=150. Methyl salicylate was classified as a negative sensitiser.

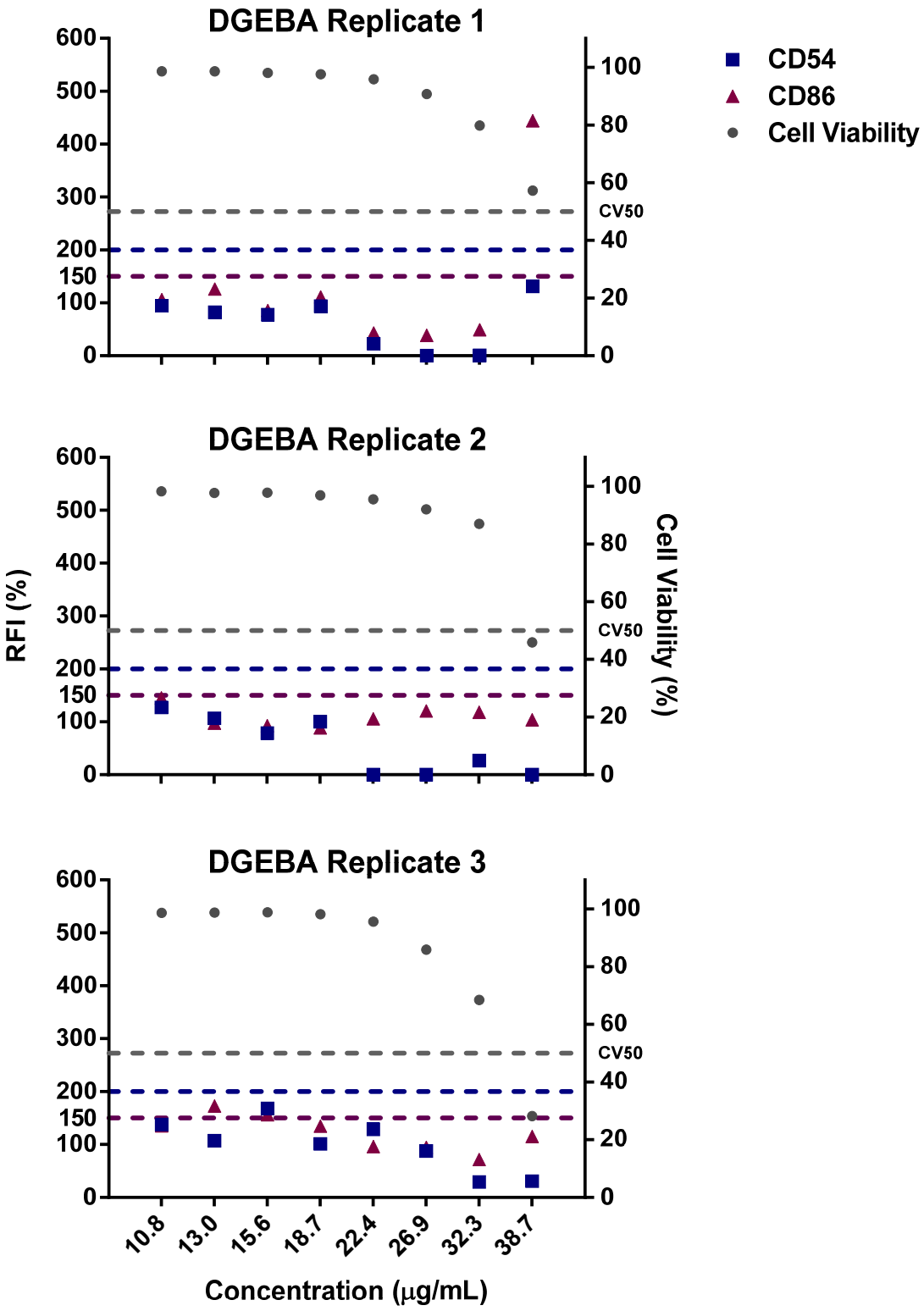


Figure 2-6: Expression levels of the cell surface molecules CD54 (■) and CD86 (▲) shown as RFI%, as well as percentage cell viability (●) of THP-1 cells incubated with 8 concentrations of 1.2-fold serially diluted DGEBA. The blue dotted line (---) represents the threshold value of CD54, RFI=200 while the red dotted line (---) represents the threshold value of CD86, RFI=150. DGEBA was classified as a negative sensitiser.

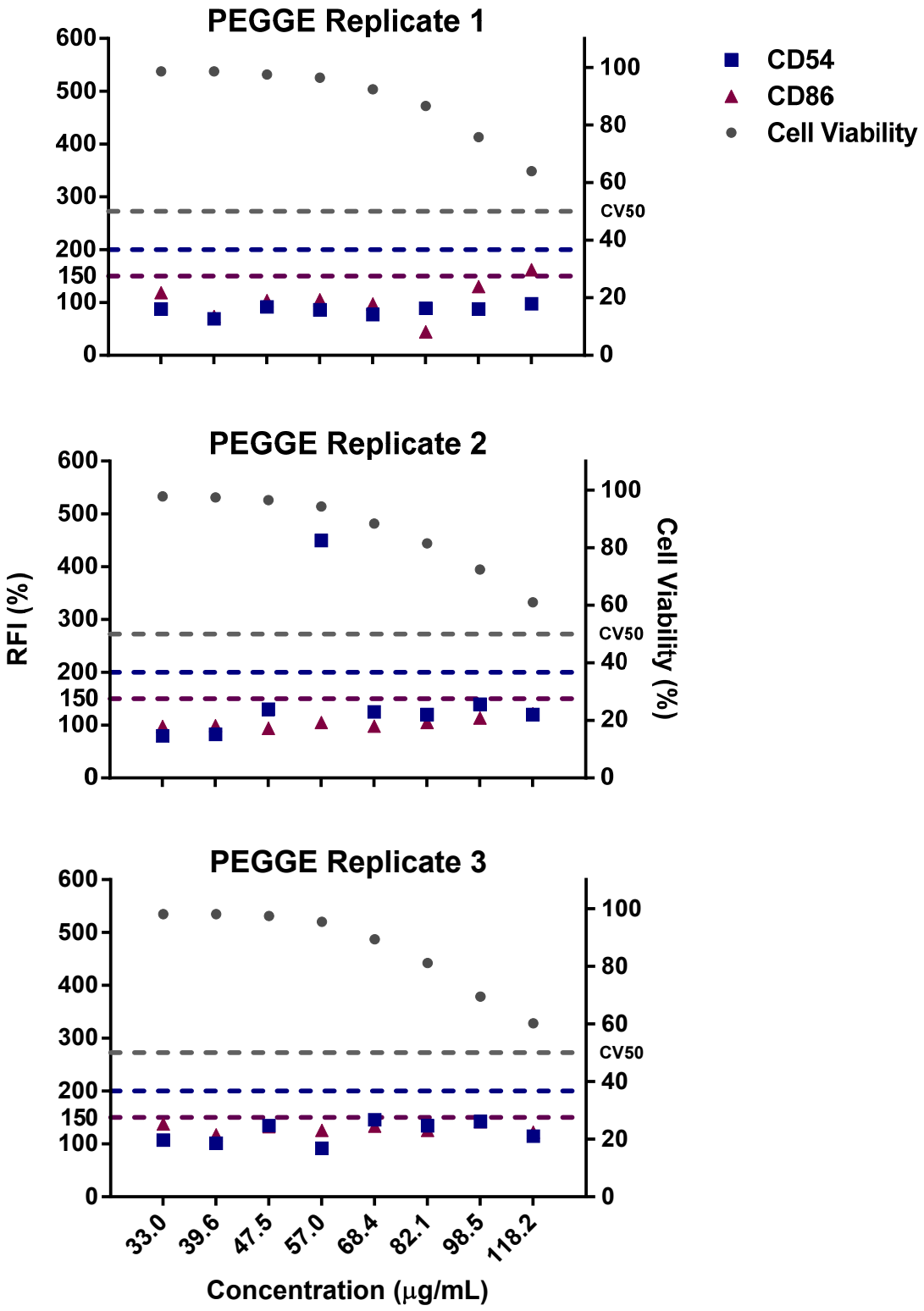


Figure 2-7: Expression levels of the cell surface molecules CD54 (■) and CD86 (▲) shown as RFI%, as well as percentage cell viability (●) of THP-1 cells incubated with 8 concentrations of 1.2-fold serially diluted PEGGE. The blue dotted line (---) represents the threshold value of CD54, RFI=200 while the red dotted line (---) represents the threshold value of CD86, RFI=150. PEGGE was classified as a negative sensitiser.

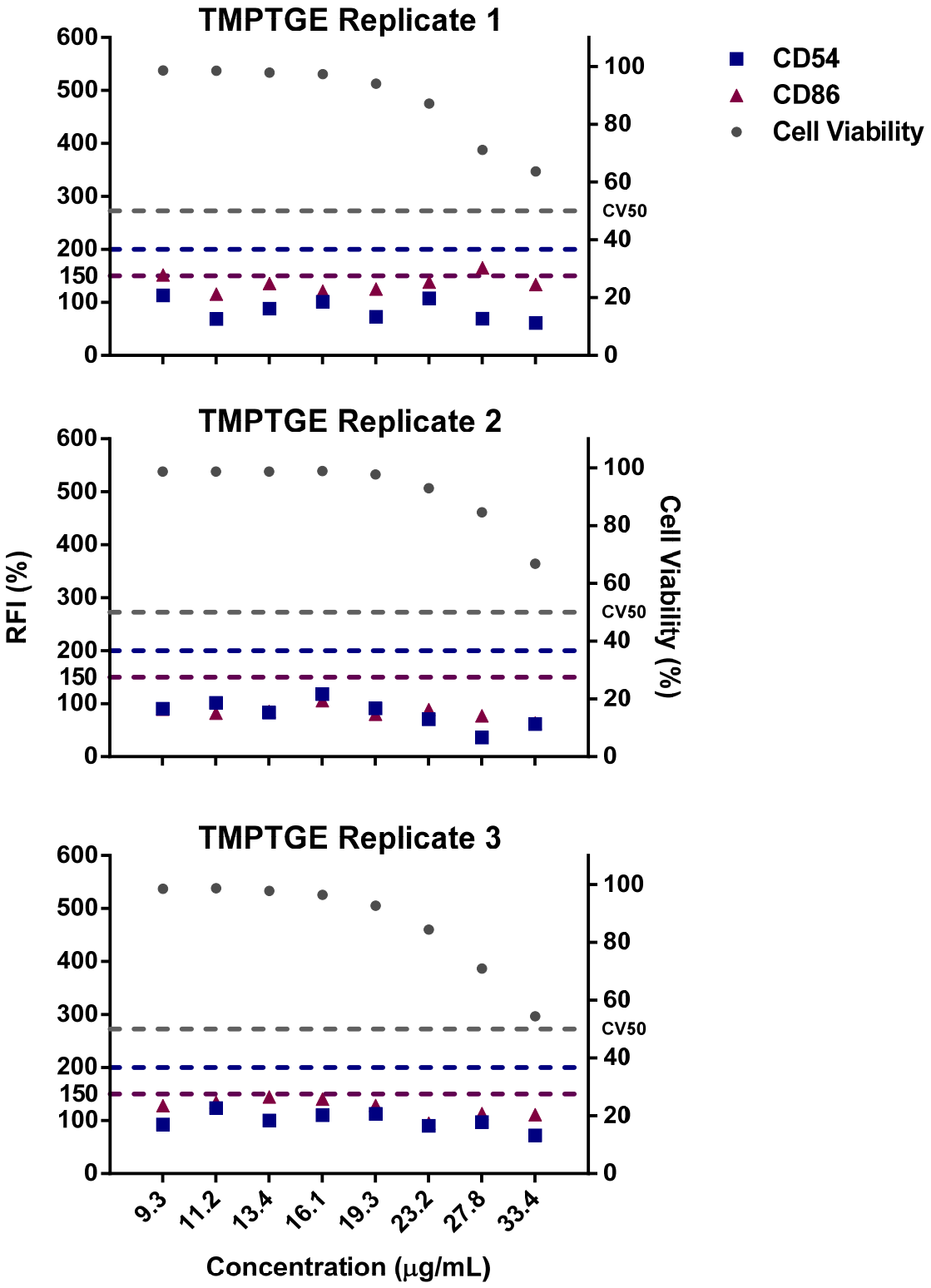


Figure 2-8: Expression levels of the cell surface molecules CD54 (■) and CD86 (▲) shown as RFI%, as well as percentage cell viability (●) of THP-1 cells incubated with 8 concentrations of 1.2-fold serially diluted TMPTGE. The blue dotted line (---) represents the threshold value of CD54, RFI=200 while the red dotted line (---) represents the threshold value of CD86, RFI=150. TMPTGE was classified as a negative sensitiser.

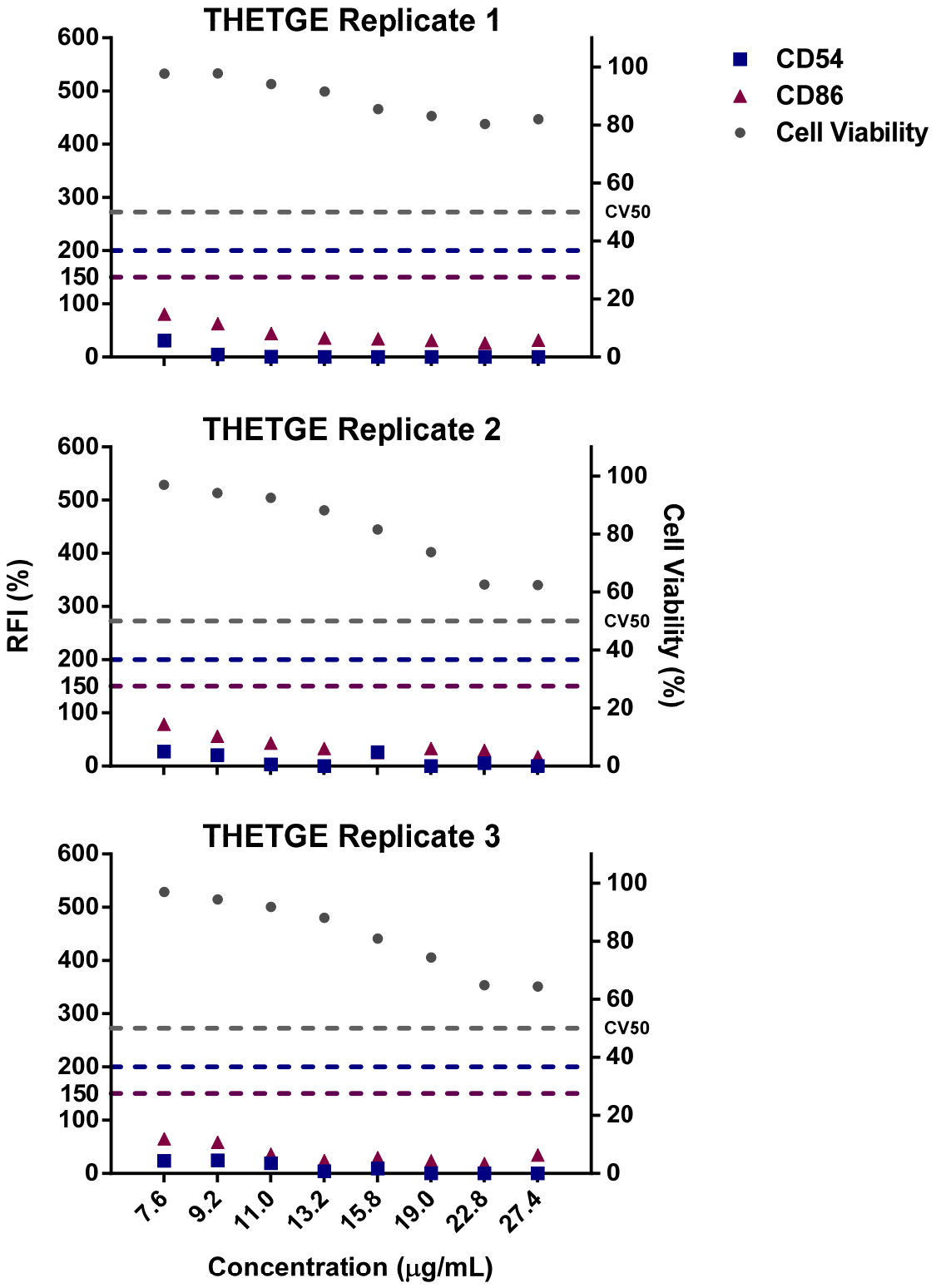


Figure 2-9: Expression levels of the cell surface molecules CD54 (■) and CD86 (▲) shown as RFI%, as well as percentage cell viability (●) of THP-1 cells incubated with 8 concentrations of 1.2-fold serially diluted THETGE. The blue dotted line (---) represents the threshold value of CD54, RFI=200 while the red dotted line (---) represents the threshold value of CD86, RFI=150. THETGE was classified as a negative sensitiser.

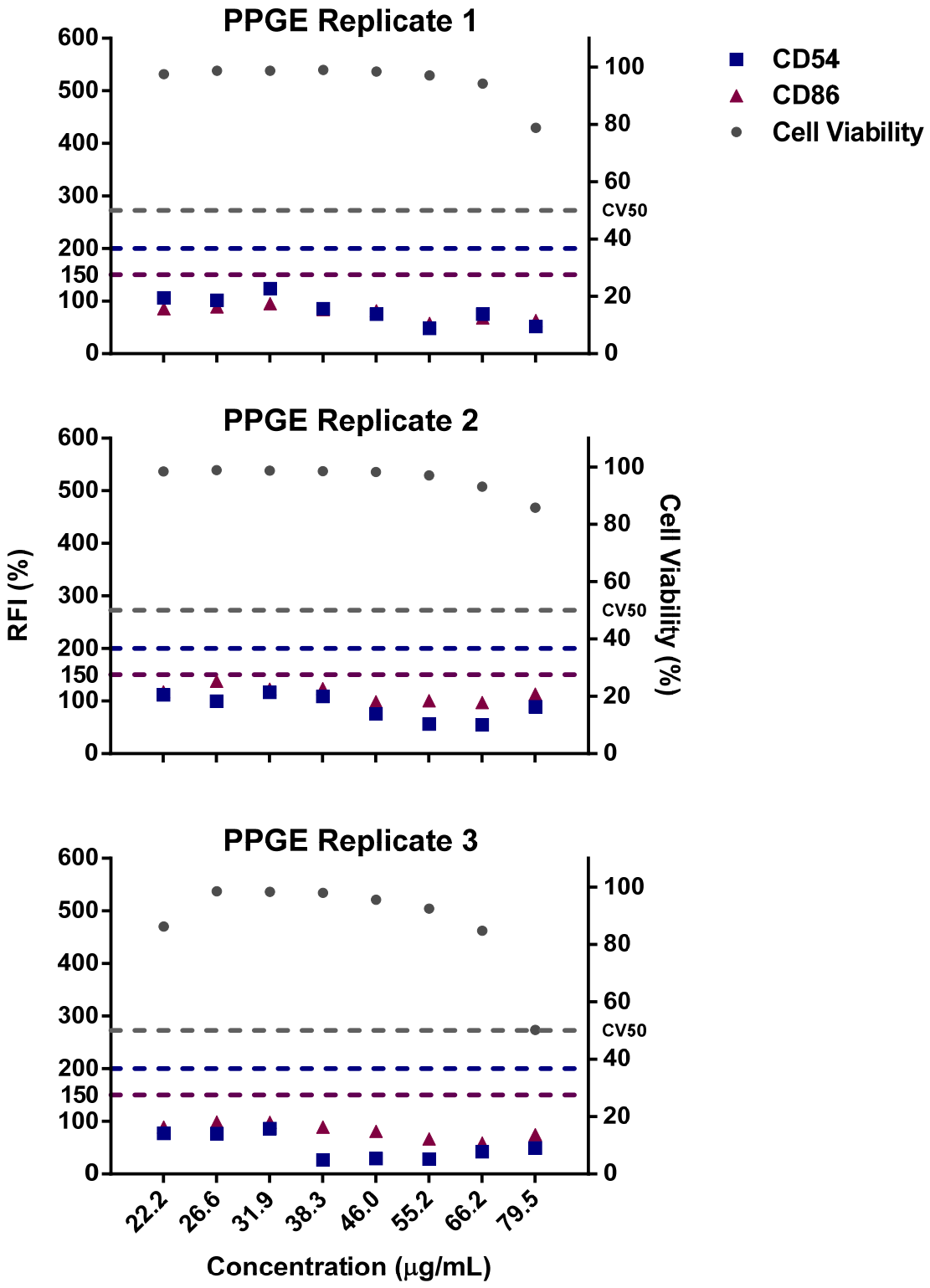


Figure 2-10: Expression levels of the cell surface molecules CD54 (■) and CD86 (▲) shown as RFI%, as well as percentage cell viability (●) of THP-1 cells incubated with 8 concentrations of 1.2-fold serially diluted PPGE. The blue dotted line (---) represents the threshold value of CD54, RFI=200 while the red dotted line (---) represents the threshold value of CD86, RFI=150. PPGE was classified as a negative sensitiser.

2.3.4. Changes in cytokine concentrations following incubation of THP-1 cells with test chemicals

Cytokine concentrations in the cell culture supernatant from THP-1 cells incubated with test chemicals in the h-CLAT assay were quantitated using the multiplex immunoassay MSD human pro-inflammatory 7-plex tissue culture kits. Three concentrations of each test chemical were selected based upon the CV75. Of the seven cytokines (IL-1β, IL-12p70, IL-6, IL-8, IL-10, TNF-α and IFN-γ) assessed, the concentration of IFN-γ in the THP-1 cell culture supernatant was below the detectable range of the multiplex assay for all of the tested chemicals evaluated (data not shown). No/minimal induction of IL-10, IL-12p70 and IL-1β were observed (Figure 2-11) for cells incubated with any of the test chemicals relative to vehicle (0.2% DMSO).

Induction of IL-6 was observed for cells incubated with DNCB and to a lesser extent with PPGE relative to vehicle-treated cells (Figure 2-11). IL-8 appeared to be up-regulated in THP-1 cells with the highest to lowest IL-8 concentrations observed in cells incubated with the test chemicals of interest, in the following order – DNCB (4213 pg/mL) > PPGE (3759 pg/mL) > THETGE (518 pg/mL) > DGEBA (205 pg/mL) > TMPTGE (98 pg/mL). No/ minimal IL-8 induction was detected for cells incubated with the epoxy resin, PEGGE, and the non-sensitiser, methyl salicylate (Figure 2-11). The TNF-α concentration (19.8 pg/mL) was markedly increased in the supernatant of THP-1 cells incubated with DNCB at 3.2 µg/mL whereas there was no/minimal induction observed for cells incubated with the other seven test chemicals evaluated (Figure 2-11).

Figure 2-12 shows the mean fold increase of the IL-6, IL-8 and TNF-α concentrations in the supernatant for THP-1 cells incubated with a range of test chemicals concentrations relative to THP-1 cells incubated with vehicle. The maximum mean fold increase in cytokine concentrations produced by each test chemical are summarised in Table 2-4. For DNCB, a known strong sensitiser, there was a 410-fold significant increase in the IL-8 concentration. The corresponding maximum increases for, IL-6 and TNF-α were 52-fold and 18-fold respectively (Table 2-4). By contrast, the corresponding extent to which the concentrations of IL-8, IL-6 and TNF-α increased were only 2.2-fold, 1.6-fold and 3.5-fold respectively for cells incubated with the non-sensitiser, methyl salicylate.

Of the five epoxy resin compounds incubated with THP-1 cells, DGEBA and THETGE increased supernatant IL-8 concentrations by up to 20- and 50-fold higher respectively

compared with vehicle-treated cells (Table 2-4). By contrast, for THP-1 cells incubated with PPGE, IL-6, IL-8 and TNF-α concentrations were increased by up to 14-, 365- and 4.9-fold relative to cells incubated with vehicle (Table 2-4). The epoxy resin compounds, PEGGE and TMPTGE, did not significantly alter supernantant cytokine concentrations for THP-1 cells relative to vehicle (Table 2-4).

2.3.5. Cytokines stability test with ELISA

2.3.5.1. Effect of incubation temperature on cytokines

Assessment of the stability and hence accuracy of the measured cytokine concentrations in the supernatant from cultured THP-1 cells incubated with the test chemicals of interest in the h-CLAT (Section 2.3.4), showed that the concentrations of IL-6, IL-8 and IL-10 remained stable at the incubation temperature of 37°C for 24 h (Table 2-6 to Table 2-8) as all of the QC samples were within ±20% of their respective nominal concentrations. By contrast, IL-1β was unstable at 37°C for 24 h and this was most marked at low and high concentrations where the deviation from the nominal concentrations was more than 20% (Table 2-5).

2.3.5.2. Effect of freeze-thaw cycle on cytokines

As the cytokine-containing cell culture supernatant samples from h-CLAT were collected and stored at -80°C prior to analysis (in Section 2.3.4) it was important to assess the effects of freeze-thaw on the cytokine stability . IL-1β, IL-8 and IL-10 were stable for up to four freeze-thaw cycles as the measured concentrations were within ±20% of their respective nominal concentrations) (Table 2-5, Table 2-7 and Table 2-8). However, the stability of IL-6 appeared to be adversely affected by four freeze-thaw cycles especially at the low QC concentrations (Table 2-6).

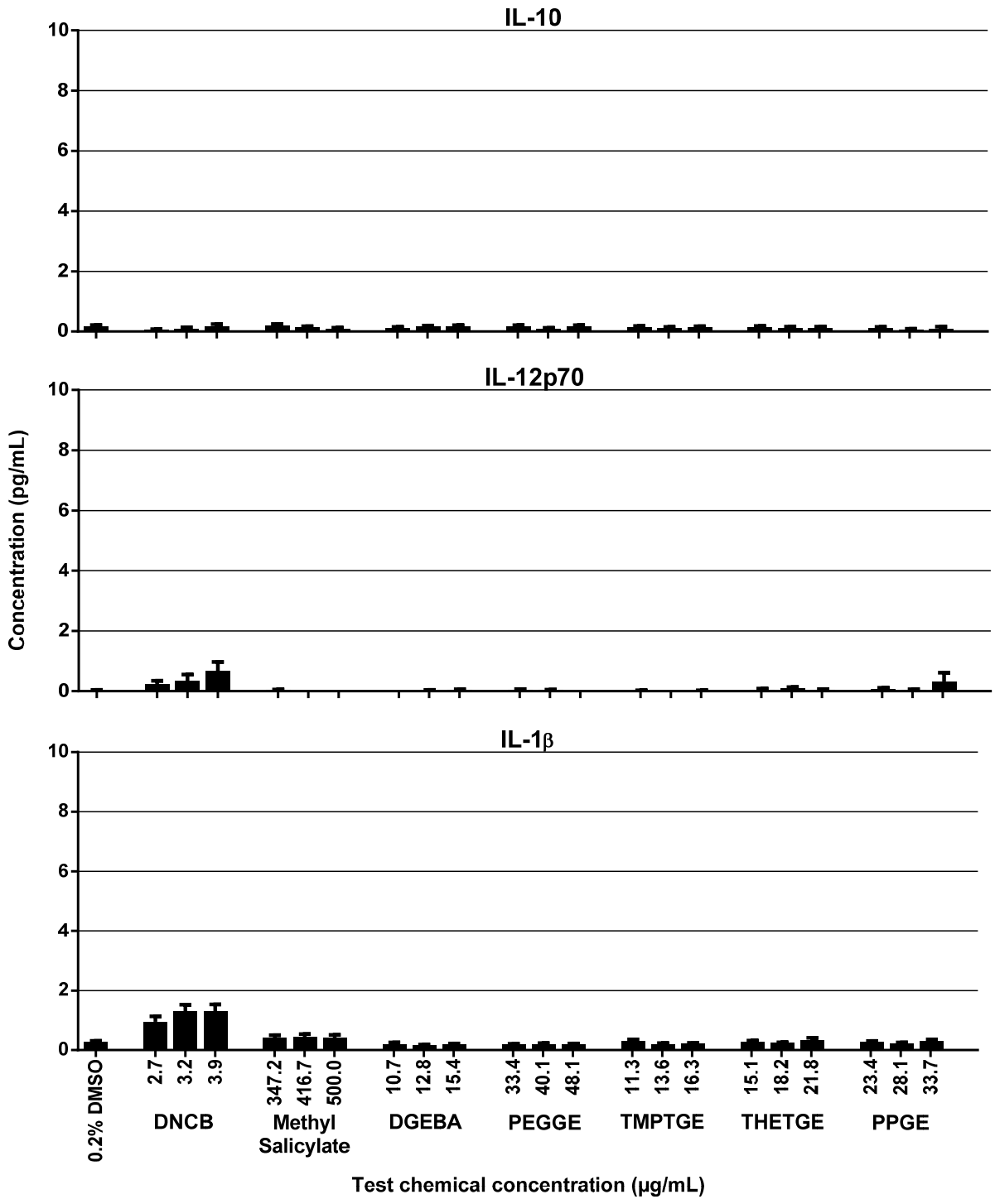


Figure 2-11: Supernatant cytokines concentrations (pg/mL ± SEM) from cultured THP-1 cells incubated with three different concentrations (µg/mL) of DNCB (strong sensitiser), methyl salicylate (non-sensitiser) and five epoxy resin compounds relative to vehicle. Cytokine assays were undertaken using MSD human pro-inflammatory 7-plex tissue culture kits.

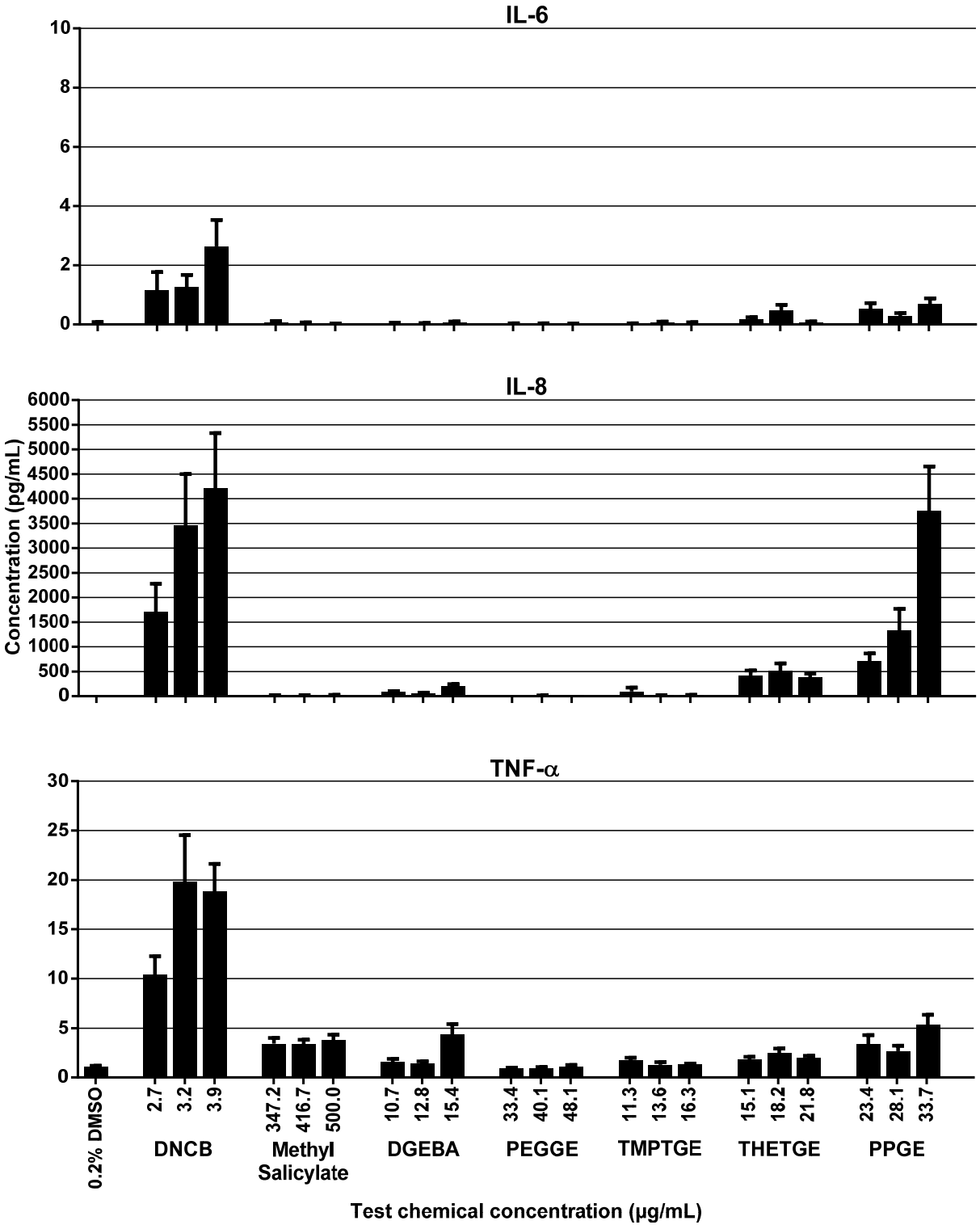


Figure 2-11: *Cont.*

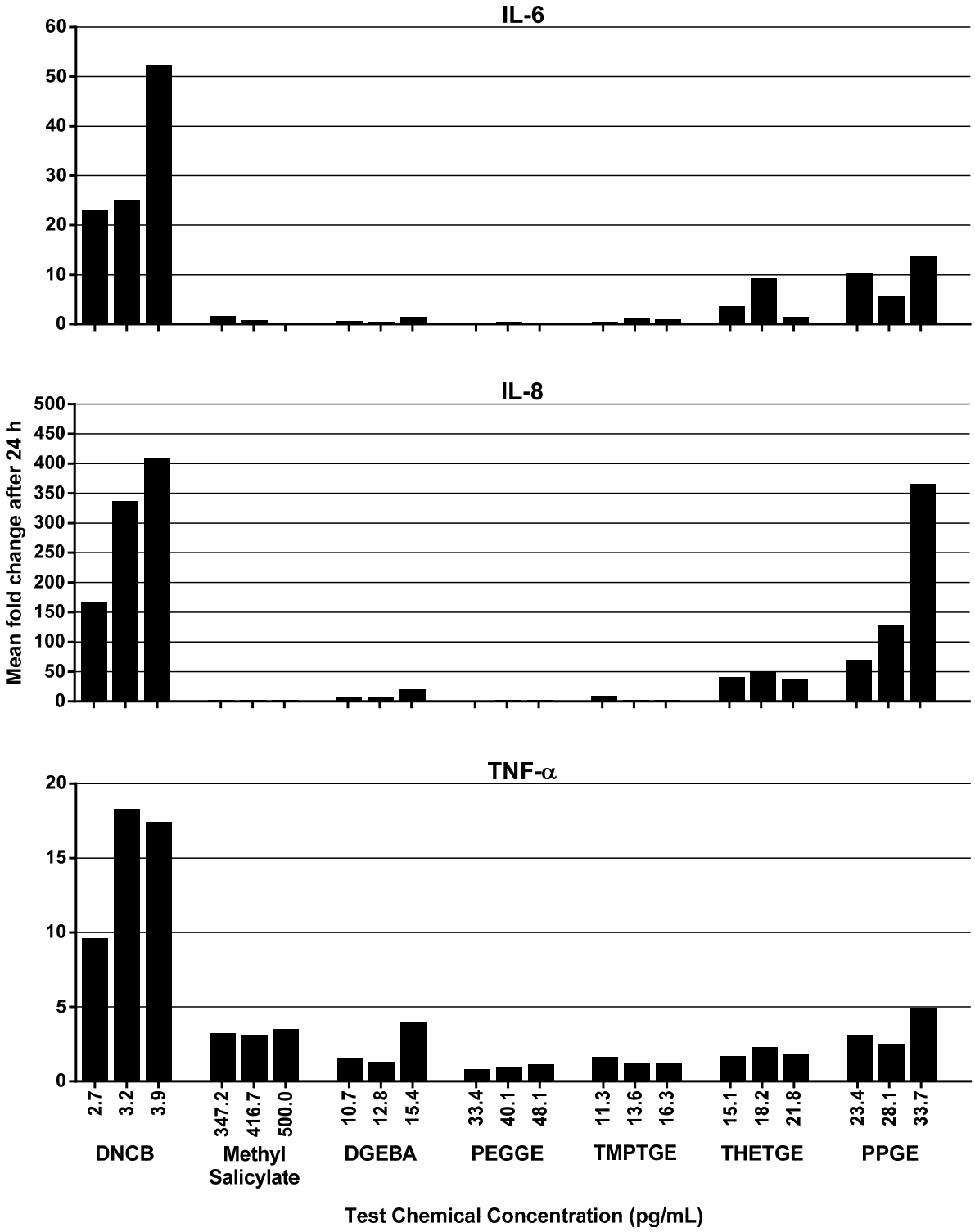


Figure 2-12: Mean fold change in IL-6, IL-8 and TNF-α concentrations in supernatant from cultured THP-1 cells incubated with three different concentrations (μg/mL) of DNCB, methyl salicylate and five epoxy resin compounds relative to vehicle-treated THP-1 cells. Cytokine assays were undertaken using MSD human pro-inflammatory 7-plex tissue culture kits.

Table 2-4: Fold-increase in the supernatant concentrations of IL-6, IL-8 and TNF-α for THP-1 cells incubated with the test chemicals of interest, relative to vehicle. The data presented below are the test chemical concentrations that induced the maximum fold increase in the cytokine concentrations.

Chemicals (μg/mL)	IL-6 (fold increase)	IL-8 (fold increase)	TNF-α (fold increase)
DNCB (3.9)	52.4	410	18.3
Methyl Salicylate (500)	1.60	2.20	3.50
DGEBA (15.4)	1.36	20.0	4.01
PEGGE (48.1)	0.441	1.82	1.06
TMPTGE (11.3)	1.14	9.47	1.61
THETGE (18.2)	9.41	50.4	2.29
PPGE (33.7)	13.7	365	4.94

Table 2-5: Summary of QC sample concentrations for IL-1β. The acceptance criterion for accuracy of QC samples was set at ±20% of the nominal concentration.

	37 °C			1x freeze-thaw			2x freeze-thaw			3x freeze-thaw			4x freeze-thaw		
QC Concentration (pg/mL)	**11.7**	**125**	**200**	**11.7**	**125**	**200**	**11.7**	**125**	**200**	**11.7**	**125**	**200**	**11.7**	**125**	**200**
Mean concentration (pg/mL)	8.50	106	160	11.5	129	179	10.1	126	181	9.90	124	174	10.2	125	170
SD	0.5	9.7	10.2	0.4	4.3	4.3	4.4	4.4	12.2	0.2	1.1	4.9	0.8	5.5	5.5
Precision	5.9	9.2	6.4	3.9	3.4	2.4	1.7	3.5	6.7	2.2	0.9	2.8	7.9	4.4	3.2
Accuracy	27.6*	15.3	20.2*	2.1	-3.2	10.7	13.8	-0.9	9.6	15.3	0.5	12.8	12.8	0.2	15.2

*failed accuracy acceptance criteria

Table 2-6: Summary of QC sample concentrations for IL-6. The acceptance criterion for accuracy of QC samples was set at ±20% of the nominal concentration.

	37 °C			1x freeze-thaw			2x freeze-thaw			3x freeze-thaw			4x freeze-thaw		
QC Concentration (pg/mL)	**14.1**	**150**	**240**	**14.1**	**150**	**240**	**14.1**	**150**	**240**	**14.1**	**150**	**240**	**14.1**	**150**	**240**
Mean concentration (pg/mL)	12.0	142	255	11.5	132	231	10.8	130	212	10.8	119	200	10.3	122	196
SD	0.7	4.6	7.7	0.2	4.3	2.9	4.0	4.0	8.6	0.3	3.7	5.9	0.2	2.1	6.7
Precision	5.6	3.3	3.0	1.7	3.3	1.3	2.3	3.1	4.0	2.8	3.1	3.0	2.1	1.8	3.4
Accuracy	14.8	5.7	-6.1	18.6	11.8	3.6	23.3*	13.1	11.6	23.5*	20.9*	16.6	26.9*	18.6	18.3

*failed accuracy acceptance criteria

Table 2-7: Summary of QC sample concentrations for IL-8. The acceptance criterion for accuracy of QC samples was set at ±20% of the nominal concentration.

	37 °C			1x freeze-thaw			2x freeze-thaw			3x freeze-thaw			4x freeze-thaw		
QC Concentration (pg/mL)	**9.38**	**100**	**160**	**9.38**	**100**	**160**	**9.38**	**100**	**160**	**9.38**	**100**	**160**	**9.38**	**100**	**160**
Mean concentration (pg/mL)	7.50	87.2	138	8.00	91.7	135	7.70	85.8	135	7.70	92.6	137	8.50	91.1	136
SD	0.2	3.5	5.4	0.2	1.3	3.7	2.6	2.6	6.2	0.2	1.6	5.2	0.4	2.1	4.0
Precision	2.5	4.0	3.9	3	1.4	2.7	4.7	3.1	4.6	2.6	1.8	3.8	4.4	2.3	3.0
Accuracy	19.7	12.8	13.7	14.9	8.3	15.5	18.6	14.2	15.9	18.3	7.4	14.1	9.5	8.9	15.1

Table 2-8: Summary of QC sample concentrations for IL-10. The acceptance criterion for accuracy of QC samples was set at ±20% of the nominal concentration.

	37 °C			1x freeze-thaw			2x freeze-thaw			3x freeze-thaw			4x freeze-thaw		
QC Concentration (pg/mL)	**23.4**	**250**	**400**	**23.4**	**250**	**400**	**23.4**	**250**	**400**	**23.4**	**250**	**400**	**23.4**	**250**	**400**
Mean concentration (pg/mL)	19.6	204	337	20.3	215	344	20.8	215	348	20.0	207	343	19.5	209	353
SD	0.6	5.1	9.6	0.6	4.5	4.6	3.8	3.8	20.0	0.9	4.2	6.6	0.3	21.3	17.7
Precision	3.0	2.5	2.9	3.1	2.1	1.3	2.3	1.7	5.8	4.4	2.0	1.9	1.7	10.2	5.0
Accuracy	16.1	18.3	15.9	13.4	14	14	11.1	13.8	12.9	14.6	17.3	14.3	16.8	16.6	11.8

2.4. Discussion

For industrial applications involving the screening of large numbers of new chemicals using the h-CLAT for assessment of skin sensitisation potential, it is essential that this test be run in high-throughput format. To address this issue, I have successfully converted the h-CLAT to 96-well plate format as a means to improve assay efficiency. Additionally, I used FITC- and PE-labelled antibodies to enable simultaneous readouts of CD54 and CD86, a step that is essential for running this method in high-throughput format. In addressing the issue of generalisability of the h-CLAT to chemicals other than small molecule fragrances used primarily in the cosmetics and toiletry industries, I found that readouts other than CD54 and CD86 are needed in order to distinguish skin sensitisers from non-sensitisers for the epoxy resin chemical class.

To improve h-CLAT efficiency, I adapted the assay from its typical 24-well format with $1x10^6$ THP-1 cells per well and FITC-labelled antibodies for quantification of the cell surface markers, CD54 and CD86, to 96-well plate format. This change in assay format required re-optimisation of the experimental conditions, including the total number of cells/well, the antibody concentrations and the parameters for flow cytometry analysis. To adapt the h-CLAT to the reduced well volume in 96-well plate format, the optimal number of cells per well were found to be $1.6x10^5$ cells in an assay volume of 160 µL. Thus the total cell concentration was maintained at $1x10^6$ cells/mL during test chemical incubation, as per the published method (Ashikaga et al., 2006). Detection efficiency for CD54 and CD86 was improved by simultaneous use of both FITC- and PE-labelled antibodies, respectively within a single well. The antibody concentrations were re-optimised the 96-well plate assay format based upon the concentrations that gave the highest signal-to-noise ratio. In my 96-well assay format, the optimal anti-CD54 and anti-CD86 concentrations were both 1/40 dilutions from the neat solution (i.e. 1.25 µL/$1.6x10^5$ cells/50µL). By contrast, the concentrations for the same clonal CD54 and CD86 antibodies previously determined were 3/25 (i.e. 6 µL/$3x10^5$ cells/50µL) for CD86 and 3/50 (i.e. 3 µL/$3x10^5$ cells/50µL) for CD54 (Ashikaga et al., 2006). Perusing the OECD recommended RFI thresholds for the h-CLAT (OECD, 2014), the positive control strong sensitiser, DNCB, was clearly identified as a sensitiser in my optimised h-CLAT method in 96-well format, akin to previous h-CLAT findings in 24-well plate format for DNCB (Ashikaga et al., 2006, Nukada et al., 2012).

Using my optimised 96-well plate format, I then assessed the generalisability of the h-CLAT for identifying skin sensitisers amongst a group of five epoxy resin compounds. Challengingly, four of the five epoxy resins compounds, DGEBA, PEGGE, THETGE and PPGE had relatively low solubility in aqueous solution. The highest technical dose (HTD) of 500 µg/mL was selected for all five epoxy resin compounds which was the maximum solubilisation of the chemical in cell culture. The final concentration of DMSO in culture did not exceed 0.2%, the threshold percentage that could be used in this cell-based assay. Importantly, the selected HTD level selected ensured that the test chemical did not precipitate out from the aqueous solution when added to the cultured THP-1 cells. It is important to recognise that the accuracy of the h-CLAT, in common with many other cell-culture based assays, is limited for test chemicals that have low aqueous-solubility. This point is emphasised by the findings of Ashikaga et al. (2010) who showed that eight of nine false negatives in the h-CLAT were for water-insoluble chemicals which limited their ability to augment CD54 and/or CD86 expression by cultured THP-1 cells.

Importantly, my novel findings suggest that use of the h-CLAT in conventional mode based primarily upon up-regulation of CD54 and CD86 expression levels on cultured THP-1 cells, is unsuitable for assessing the skin sensitisation potential of high molecular weight compounds such as epoxy resin compounds. Specifically, the h-CLAT failed to identify the tested epoxy resin compounds known to have sensitising capacity (i.e. DGEBA, TMPTGE and PPGE; refer to Chapter 4 for LLNA data) as the cell surface expression levels of the molecular markers, CD54 and CD86, were not up-regulated by these compounds. It is known that some skin sensitisers may only stimulate either CD54 or CD86 (Sakaguchi et al., 2009) or possibly other biomarkers. For instance, biodegradable polymers only induced the expression of CD54, but not CD86 by THP-1 cells (Jung et al., 2011). In other work, 1,4-phenylenediamine, diethyl sulphate and geraniol increased cell surface expression of CD86 above the RFI threshold of 150% but CD54 expression was not increased above its RFI threshold (200%) whereas propyl gallate, resorcinol and linalool only induced CD54 expression levels but not CD86 (Nukada et al., 2012, Takenouchi et al., 2013).

Apart from CD54 and CD86 as biomarkers in the h-CLAT, the potential of several other biomarkers for discriminating between sensitising and non-sensitising chemicals or compounds, have been investigated. Various quantitative and qualitative endpoints in skin sensitisation pathways, such as pro-inflammatory cytokine and chemokine readouts (e.g. IL-1β, IL-18), activation of Keap1/Nrf2/ARE pathways, protein expression and

transcriptional profiles of several genes in different cell types, have been evaluated in preliminary work and appear to be promising (Reisinger et al., 2015).

To date, *in vitro* work aimed at identifying the skin sensitising potential of epoxy resin compounds is very limited. Hence, I extended my interest in assessing and improving the generalisability of the h-CLAT to chemicals other than chemical compounds used in toiletry and cosmetic products, e.g. ERS. To this end, I evaluated secretion of pro-inflammatory cytokines by THP1-cells exposed to a range of test chemicals, as another possible assay endpoint.

Specifically, I used the commercially available multiplexed MSD human pro-inflammatory 7-plex tissue culture kit that allowed rapid and simultaneous quantification of seven cytokines, namely IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α and IFN-γ, Encouragingly, my data show for the first time that incubation of cultured THP-1 cells for 24 h with sensitising epoxy resin compounds stimulated a marked increase in the release of the pro-inflammatory cytokines, IL-8 and IL-6, into the supernatant.

Specifically, DNCB (sensitiser) but not methyl salicylate (non-sensitiser) induced a marked increase in the h-CLAT supernatant concentration of IL-8 by 410-fold. My findings are in agreement with a previous report that sensitisers but not non-sensitisers (2.2-fold), increased IL-8 expression significantly ($P<0.01$) in a cell culture assay that used THP-1 cells (Miyazawa et al., 2007, Nukada et al., 2008). Furthermore, published work by others also suggests that IL-8 may be a promising biomarker for assessing chemicals as potential skin sensitisers (Toebak et al., 2006, Python et al., 2007, Nukada et al., 2008, Takahashi et al., 2011). More recently, the stable cell line, THP-G8 was established from the THP-1-derived IL-8 reporter cell line (Takahashi et al., 2011).

For the tested epoxy resins, IL-8 was induced by DGEBA, TMPTGE and PPGE which were classified as strong, moderate and weak sensitisers respectively, based upon animal LLNA data (refer to Chapter 4 for LLNA data). In addition, human patch test studies revealed that both DGEBA and TMPTGE caused ACD in humans (Aalto-Korte et al., 2015). However, the specificity of increased IL-8 expression requires further investigation as THP-1 cells incubated with THETGE also produced an increase in IL-8 expression, although this epoxy resin compound was classified as a non-sensitiser by my LLNA data (Chapter 4).

Incubation of cultured THP-1 cells with DNCB in my present work increased the TNF-α concentration in the supernatant by 18-fold relative to vehicle in a manner similar to that reported by Miyazawa et al. (2007). However, the five epoxy resin compounds tested did not have a major impact on the release of TNF-α into the supernatant in a manner similar to that for the non-sensitiser, methyl salicylate. The low level of TNF-α production by epoxy resins may potentially explain the low levels of CD54 observed in the h-CLAT, as TNF-α stimulated the expression of CD54 in concentration-dependent manner (Miyazawa et al., 2008b). The levels of secreted TNF-α by DNCB-treated cultured THP-1 cells reportedly decreased at 24 h post-treatment compared with earlier time-points (Miyazawa et al., 2008b). This apparent reduction may possibly be underpinned by instability of TNF-α in the cell culture supernatant for a 24 h period. Hence, future work directed at measuring the secretion of TNF-α by cultured THP-1 cells incubated with epoxy resin compounds over the course of the experiment, are needed.

In addition, the concentration of IL-6 in the supernatant of cultured THP-1 cells incubated with the potent skin sensitiser DNCB for 24 h was also markedly elevated. Although my findings differ from those of Miyazawa et al. (2007) where IL-6 was not induced in cultured THP-1 cells incubated with DNCB, the most parsimonious explanation may be attributed to the different analytical methods used to measure IL-6 concentrations. An ELISA method that had an LLOQ of 3.12 pg/mL was used to measure IL-6 concentrations by Miyazawa et al. (2007). By contrast in my research herein, I used an ~25-fold more sensitive ECL-based MSD immunoassay to quantify IL-6 concentrations that had an LLOQ of 0.130 pg/mL. This more sensitive MSD immunoassay with a larger dynamic range of IL-6 detection allowed the IL-6 concentrations in my THP-1 supernatant samples to be measured (~0.3 pg/mL pre-treatment to 3.2 µg/mL post-DNCB treatment). Additionally, of the five tested epoxy resins, elevated concentrations of IL-6 were observed only for THETGE (9.4-fold) and PPGE (14-fold). These findings differ from the corresponding LLNA data as THETGE and PPGE (refer to Chapter 4) were classified by the LLNA as a non- and weak sensitiser respectively. While LLNA data are widely accepted as the gold standard against which the predictive accuracy of non-animal methods are compared, it is important to note that there is a degree of discordance between the predictive accuracy of the LLNA compared with the human maximisation and patch tests (Anderson et al., 2011, Urbisch et al., 2015). The induction of IL-6 by THETGE and PPGE may be examples of this discordance but there are no published human data on these two epoxy resin compounds with which to compare. Nevertheless, my findings serve to emphasise the risk

to accuracy with oversimplification or limitation on the number of endpoints used in non-animal methods for assessing the skin sensitisation capacity of a given chemical.

Of the four other cytokines quantified in the supernatant of cultured THP-1 cells in my 96-wel plate h-CLAT assay format, IL-1β was detected only after incubation with the strong sensitiser, DNCB. My finding is aligned with the fact that IL-1β mRNA was up-regulated by incubation of cultured human DCs with 2,4-dinitrofluorobenzene (DNFB; a skin sensitiser) in contrast to the fact that IL-6 and IL-18 mRNA were not up-regulated (Pichowski et al., 2000). Additionally, my data showed that there was an insignificant increase in the cultured THP-1 cell supernatant concentrations of IL-10, IL-12p70 and IFN-γ after incubation of these cells with the five epoxy resin compounds of interest. Previously, the metal sensitiser, nickel sulphate, was shown to stimulate the production of IL-12p40 (monomer), but not IL-12p70 (heterodimer) in human monocyte-derived DCs with the release of the latter subunit occurring only after the addition of IFN-γ (Antonios et al., 2010). In other work, there was up-regulated expression of IL-12p40 mRNA and protein as well as IL-12p70 protein in the draining lymph nodes of mice treated with the contact sensitiser, DNFB (Toichi et al., 2008). In the same study, it was also noted that UV-irradiated skin treatment in mice resulted in an up-regulation of IL-10 mRNA expression but down-regulation of IL-12p70 when mice were treated with DNFB (Toichi et al., 2008). These findings by others serve to illustrate the complexity of the immune response following exposure to a given chemical under various experimental conditions and/or the types of cells/tissues assessed. Epoxy resin compounds may differentially affect the expression of particular markers compared with nickel sulphate. For example, nickel sulphate induced IL-6, IL-8 and IL-12p40 in human DCs whereas only IL-8 expression was stimulated in DNCB-treated cells (Ade et al., 2007). This promising line of investigation warrants examination of a wider array of chemicals, particularly epoxy resin compounds, as well as a broader panel of cytokines as a means to assess whether there are particular immune profiles produced by cultured THP-1 cells exposed to various chemical classes, that may act as ‘signatures’ for skin sensitisation potential. This remains for future work beyond the scope of that described in my thesis.

From the analytical perspective, accurate quantification of cytokines in the supernatant of cultured THP-1 cells in the h-CLAT, it is important to understand the impact of the assay and sample storage conditions on the chemical stability of individual cytokines. In this chapter, I assessed the stability of four cytokines, *viz* IL-1β, IL-6, IL-8 and IL-10 with particular attention focussed on IL-6 and IL-8, as these two cytokines have been proposed

by others as biomarkers for skin sensitisation potential (Miyazawa et al., 2008a, Jung et al., 2012). Indeed, my findings showed that three of the five epoxy resin compounds tested resulted in elevated supernatant concentrations of IL-6 and IL-8 in the h-CLAT. Importantly, I also showed that IL-1β, IL-8 and IL-10 were stable for at least four freeze-thaws cycle whereas IL-6 was not stable after the second freeze-thaw cycle. Information on the stability of TNF-α and IFN-γ has been published recently by Ozbey et al. (2014) who showed that the initial cell culture supernatant concentration of TNF-α was approximately halved after the first freeze-thaw of the sample with subsequent freeze-thaw events having limited adverse impact. On the other hand, multiple freeze-thaw cycles did not appear to affect the integrity of IFN-γ, IL-6, IL-10 and IL-12 in these samples (Ozbey et al., 2014, Hosnijeh et al., 2010). Together, my findings with those of others suggest that h-CLAT supernatant samples should undergo more than two freeze-thaw cycles to ensure cytokine integrity. The small discrepancy between my findings and those of Ozbey et al. (2014) may be more of a reflection of the execution of the freeze-thaw process, such as the length of time the sample was stored frozen between each freeze-thaw cycle.

It is important to note that previous work (Ozbey et al., 2014, Hosnijeh et al., 2010) assessed the stability of cytokines in samples of cell culture supernatant, plasma or serum only, with respect to the effects of storage conditions. As Miyazawa et al. (2008b) reported an apparent decrease in the h-CLAT supernatant concentrations of TNF-α at 24 h compared with that measured at earlier time-points, these observations may be underpinned at least in part by the chemical instability of TNF-α in the collected samples over such an extended time period. In my PhD research described in this chapter, I investigated the stability of secreted cytokines in h-CLAT supernatant by the addition of known amounts of cytokines (QCs) into cell culture media, followed by an incubation for 24 h at 37°C to mimic the assay conditions. IL-6, IL-8 and IL-10 were not adversely affected by incubation at 37°C for 24 h whereas IL-1β was unstable under the same experimental conditions and study duration. Hence, the instability of IL-1β suggests that it is likely unsuitable as a biomarker for skin sensitisation in the h-CLAT. Future investigation on the temporal stability of cytokines secreted into cultured THP-1 supernatant in the h-CLAT over the 24 h incubation period is warranted to gain further insight on the accuracy of cytokine measurements in this *in vitro* test of skin sensitisation.

2.5. Conclusion

Research on the generalisability of the h-CLAT developed originally for assessment of the skin sensitisation potential of small molecules widely used in the cosmetics and toiletries industries, for assessment of a broader range of chemical classes including epoxy resin components, for their skin sensitising potential, is limited. In this chapter, the h-CLAT was optimised using a 96-well plate to improve assay efficiency. However, my PhD research also shows that the h-CLAT requires adaptation before it can be regarded as suitable for assessment of the skin sensitising potential of epoxy resin compounds. My findings on quantification of the pro-inflammatory cytokine concentrations in supernatant samples from cultured THP-1 cells after incubation with epoxy resin compounds for 24 h at 37°C, suggest that adaptation of the h-CLAT in this regard are promising. Specifically, my pilot data show that the concentrations of IL-6 and IL-8 measured in samples of cultured THP-1 cell supernatant, hold promise as quantitative endpoints for screening epoxy resin compounds for sensitisation potential. Clearly, a larger number of epoxy resin compounds need to be screened in future work to more fully evaluate the feasibility of using supernatant cytokine concentrations as quantitative endpoints in the h-CLAT for assessing the skin sensitisation potential of epoxy resin compounds. Additionally, use of MSD immunoassays provides a sensitive and rapid approach for simultaneous quantification of seven or more cytokines in h-CLAT supernatant samples. Finally, it is also important to bear in mind that cytokine quantification in cell-based assays such as the h-CLAT requires rigorous assessment of the stability of cytokines in the sample matrix under the real assay conditions. This is essential to avoid experimental artefacts from negatively impacting on the accuracy and usefulness of the assay results.

Chapter 3: Optimisation of the performance of direct peptide reactivity assay (DPRA) for assessment of the skin sensitisation potential of chemicals

3.1. Introduction[11]

Allergic contact dermatitis (ACD) is the clinically significant consequence of skin sensitisation that negatively affects approximately 15-20% of the general population (Peiser et al., 2012). At present, more than 4000 chemicals are linked to induction of ACD in humans (Cahill et al., 2012). A number of contact allergens, including fragrances, epoxy resin systems, formaldehyde, neomycin sulphate and nickel sulphate are commonly reported to induce ACD in humans (Cahill et al., 2012, Pesonen et al., 2015).

At present, the murine local lymph node assay (LLNA) is globally accepted as the 'gold standard' for screening potential haptens (contact allergens) (Wong et al., 2015). However, according to the European Cosmetic Directive (EC1223/2009), products subjected to animal testing were prohibited from being marketed in the European Union (EU) from 2013 (EU, 2009). Furthermore, implementation of the 3Rs, reduction, refinement and replacement of animal testing, has driven the need to adopt alternative non-animal skin sensitisation screening methods. Without a validated alternative method for identifying potential skin sensitisers, enforcement of the EC1223/2009 regulation has the potential to negatively affect the cosmetics and toiletries industries, in that the safety of products for end users may remain undetermined due to the absence of animal data on their toxicity, carcinogenicity and skin sensitisation potential (EU, 2009). Furthermore, the EU REACH regulation (registration, evaluation, authorization and restriction of chemicals), EC1907/2006, which came into force on 1 June 2007, imparted pressure on the testing of thousands of chemicals that had not been previously tested for skin sensitisation potential, further driving the necessity for development and implementation of alternative fast and cost effective *in vitro* screening methods (EU, 2006). To this end, multiple non-animal testing methods have been developed and evaluated (Ade et al., 2006, Ashikaga et al., 2006, Emter et al., 2010, Python et al., 2007, Sakaguchi et al., 2006, Bauch et al., 2012). The data thus generated can be incorporated into the establishment of risk assessments in the workplace, potentially leading to significant improvement in terms of public health and reduced socioeconomic costs comprising lost work days and consequent higher manufacturing costs. It was anticipated that *in vitro* methods would have the ability to

[11] This chapter of my PhD thesis has been submitted to the *Frontiers in Pharmacology*, as an original research article.

Wong C.L., Lam A, Smith MT and Ghassabian S (2015). Optimisation of the performance of the direct peptide reactivity assay (DPRA) for assessment of the skin sensitisation potential of chemicals. *Front. Pharmacol. (under review).*

screen hundreds of chemicals concurrently, which is not feasible with methods utilising animal models. Although animal models are often regarded as a superior test system for assessing skin sensitisation potential, this is not always the case as the findings do not necessarily correlate directly with humans due to inter-species differences in anatomy, physiology and biochemistry with regard to response to different chemicals (Jamei et al., 2009).

The direct peptide reactivity assay (DPRA) is accepted by the Organisation for Economic Co-operation and Development (OECD) for use in the risk assessment of chemicals as potential skin sensitisers (OECD, 2015a). The ability of haptens to bind with skin proteins is regarded as the initial key event in skin sensitisation. Hapten-protein complexes are formed via covalent modification of amino acid side chains of proteins. This process, known as haptenation, provides the scientific basis underpinning the DPRA (Gerberick et al., 2004, Gerberick et al., 2007). Most sensitising chemicals are electrophilic in nature, comprising Michael acceptors, S_NAr and S_N2 electrophiles, Schiff base formers or acylating agents and so possess the ability to react with the nucleophilic amino acid residues of skin proteins (Lalko et al., 2012, Chipinda et al., 2011a). While lysine and cysteine are more commonly found to covalently bind to these electrophiles, other residues such as histidine and methionine have been reported to react with haptens also (Gerberick et al., 2009). Irreversible covalent bond formation between haptens and amino acid residues of skin proteins is mimicked in the DPRA whereby the amount of unreacted exogenous peptide is quantified in the presence and absence of potential skin sensitising chemicals (Gerberick et al., 2004).

The sensitivity and accuracy of various amino acid combinations for simulation of skin proteins in the DPRA have been investigated. Gerberick et al. (2007) proposed a classification tree model that examined various ratios and combinations of glutathione, cysteine and lysine as a means to determine the optimum amino acid combinations for accurately identifying skin sensitisers, thereby eliminating the need for analysing a large panel of peptides to ensure reliability of the DPRA for predictive use. Based upon this classification tree model, the use of peptides containing cysteine or lysine, at a 1:10 or 1:50 molar ratio to the test chemicals of interest, respectively, gave the best predictive power for the DPRA (Gerberick et al., 2007). In addition, a synthetic peptide containing both cysteine and lysine residues (Cor1-C420) which had the added advantage of high aqueous solubility in reaction buffer, showed high reactivity towards electrophiles

(Dennehy et al., 2006, Natsch et al., 2007). Cor1-C420 heptapeptides had previously showed promising results in identifying skin sensitisers (Natsch and Gfeller, 2008).

In order to develop high-throughput approaches and minimise inter and intra-laboratory variability in results, it is important to ensure that the DPRA is robust, accurate and reproducible according to criteria that are commonly utilised in the validation of bioanalytical methods (EMA, 2011, FDA, 2011). Hence, the aims of our research described herein were to develop and optimise the performance of three LC-MS/MS bioanalytical methods for quantification of the concentrations of three heptapeptides containing lysine, cysteine and Cor1-C420, following their reaction with various test chemicals of interest. Bioanalytical method parameters optimised included accuracy, precision, carry-over, stability of peptides under various incubation temperatures, influence of solvent composition, autosampler stability, and impact of vial materials on assay performance.

3.2. Materials and methods

3.2.1. Chemicals and reagents

3.2.1.1. Peptides

Leucine enkephalin acetate salt hydrate (YGGFL) (>98%) was supplied by Sigma-Aldrich Corporation (NSW, Australia), α-N-acetyl leucine enkephalin (Ac-YGGFL) (>95%), cysteine-containing heptapeptide (Ac-RFAACAA) (>94%), lysine-containing heptapeptide (Ac-RFAAKAA) (>97%) and Cor1-C420 heptapeptide (Ac-NKKCDLF) (>98%) heptapeptides were supplied by GL Biochem (Shanghai, China).

3.2.1.2. Test chemicals

2,4-dinitrochlorobenzene (DNCB, CAS 97-00-7), cinnamaldehyde (CAS 104-55-2), ethyl acrylate (CAS 140-88-5), glutaraldehyde (CAS 111-30-8), isoeugenol (CAS 97-54-1) and methyl salicylate (CAS 119-36-8) were supplied by Sigma-Aldrich Corporation (NSW, Australia).

3.2.1.3. Reagents

Ammonium hydroxide, bovine serum albumin (BSA), DL-dithiothreitol (DTT) and deferoxamine mesylate salt were supplied by Sigma-Aldrich Corporation (NSW, Australia), HPLC grade methanol and acetonitrile were supplied by Merck (Darmstadt, Germany), sodium hydroxide and ammonium acetate were supplied by Chem-Supply (SA, Australia).

Sodium phosphate dibasic and monosodium phosphate were purchased from ThermoFisher Scientific (VIC, Australia).

3.2.2. Experimental design

3.2.2.1. LC conditions

The high performance liquid chromatography (HPLC) apparatus was a Shimadzu chromatographic system. A reversed phase C18 column (Gemini 2.0 × 150 mm, particle size 5 µm; Phenomenex, NSW, Australia) and a C18 security guard column (Gemini, Phenomenex, NSW, Australia) was used for all three heptapeptides. The column oven and autosampler temperatures were set at 40°C and 4°C, respectively. The injection volume for all samples was 5 µL. The mobile phase for the Cor1-C420 heptapeptides comprised mobile phase A (10 mM ammonium acetate, pH 9.5) and mobile phase B (acetonitrile) and the flow rate was 0.4 mL/min. The mobile phases for the heptapeptides containing cysteine or lysine comprised mobile phase A (10 mM ammonium acetate, pH 9.5) and mobile phase B (methanol) and the flow rate was 0.5 mL/min. A stepwise gradient elution program summarised in Figure 3-1 was used for each heptapeptide. The acquisition and processing of data were performed using the Applied Biosystems Sciex Analyst^{TM} software, version 1.6.1.

3.2.2.2. MS/MS conditions

Mass spectrometry (MS) detection was carried out using an Applied Biosystems Sciex API 3200 triple quadruple MS equipped with an electrospray ionisation source. The highest abundant product ions were selected for each analyte. Positive ionisation mode was chosen for all three heptapeptides and the corresponding internal standards. The first 5 min of the chromatographic run time were acquired by the MS. To tune the parameters for the heptapeptides and internal standards, molecular ions were identified by direct infusion of the solutions of interest and the parameters were automatically acquired by the Analyst software. Multiple reaction monitoring (MRM) in positive ionisation mode was used to monitor the analytes. The MS parameters for each heptapeptide and internal standard are listed in Table 3-1. The predicted fragments of three tested heptapeptides were detailed in Table 3-2 to Table 3-4. The chromatographic methods and peak area integration were performed using Analyst software version 1.6.1.

3.2.2.3. Preparation of peptide standards, calibration curves, quality control (QC) samples and test compounds with known sensitising capacity

An eight-point calibration curve for each heptapeptide (Cor1-C420 heptapeptides, 5 - 50 µM; cysteine-containing heptapeptides, 2-100 µM; lysine-containing heptapeptides 2 - 100µM) was prepared. Duplicates of three standard QC samples (three times the LLOQ, 50% of the ULOQ, 80% of the ULOQ) were prepared in 0.1 M phosphate buffer (pH7.4) for Cor1-C420 and the heptapeptide containing cysteine, whereas 0.1 M ammonium acetate buffer (pH10) was used for the heptapeptide containing lysine. The QC concentrations for Cor1-C420 were 15 µM, 25 µM and 40 µM whereas the QC concentrations for the heptapeptides containing cysteine and lysine were 6 µM, 50 µM and 80 µM. Triplicate of test chemicals with known sensitising capacity were used as total peptide depletion controls. The experiment was repeated in three independent experiments. DNCB (extreme sensitiser), isoeugenol (moderate sensitiser), cinnamaldehyde (moderate sensitiser) and methyl salicylic acid (non-sensitiser) were used to assess the stability of the Cor1-C420 and cysteine-containing heptapeptide complexes after their formation. For the lysine-containing heptapeptide, glutaraldehyde (strong sensitiser) and ethyl acrylate (weak sensitiser) were used in place of DNCB and isoeugenol. These chemicals were prepared in acetonitrile; the final percentage of organic solvent (acetonitrile) did not exceed 27% in the buffer solution. The final reaction volume was 300 µL. The molar ratio of the Cor1-C420 and cysteine-containing heptapeptides to test chemical in the incubation mixtures was 1:10. By comparison the corresponding ratio for the lysine-containing heptapeptide and the test chemicals was 1:50.

3.2.2.4. Peptide reactivity assessment

After 24 h of incubation, leucine enkephalin acetate salt hydrate (75 µL, 12 µg/mL) or α-N-acetyl leucine enkephalin (75 µL, 100 µg/mL) as internal standard, was added to the samples prior to 1 in 20 dilution for the cysteine- and lysine-containing heptapeptides, and 1 in 8 dilution for Cor1-C420 in 5% acetonitrile in water prior to final analysis. For the cysteine-containing heptapeptide, an additional step was needed to prevent dimerization of the thiol groups. Specifically, 10 µL aliquots of 16 mM DTT were added to each diluted sample (final volume 200 µL) followed by incubation for 30 min at 40°C.

3.2.2.5. Carry-over assessment and lower limit of quantification (LLOQ)

The LLOQ was assessed using the criteria that the analyte response at the LLOQ must be five times the baseline noise and it should have an accuracy of ±20% of the nominal concentration (EMA, 2011). The carry-over was assessed by injecting the highest concentration, the upper limit of quantification (ULOQ) of the analyte followed by a "blank" sample that did not contain the analyte of interest. The carry-over should not be more than 20% of the LLOQ (EMA, 2011).

3.2.2.6. Incubation temperature stability

All samples from Section 3.2.2.3 were incubated at each of three temperatures, *viz* 4°C, 25°C or 37°C for a time period of 24 (±1) h. The peptide concentrations were then assessed as per the methods described in Section 3.2.2.4.

3.2.2.7. Adsorption of heptapeptides on polypropylene and glass materials

To assess the extent to which there were adsorptive losses of each of the three heptapeptides of interest onto the vial materials over time, standard calibration curves, standard QC samples and four test chemical control samples as described in Section 3.2.2.3, were prepared in both 96-well polypropylene plate and borosilicate glass vials throughout the course of experiment. These samples were incubated at 25°C for a period of 24 (±1) h and were placed in the autosampler and injected once every 24 h for a 3 day period. The calculated concentrations on days 1, 2 and 3 were compared with that determined on day 0.

3.2.2.8. Peptide-chemical complex stability in the autosampler

Standard calibration curves, QC samples and test chemical control samples with known sensitising capacity were prepared as per the description in Section 3.2.2.3. After a time period of 24 (±1) h incubation at 25°C, the standard calibration curve samples, standard QC samples and test chemical control samples were placed in the autosampler at 4°C and the stability of the heptapeptides was monitored for 3 days post-incubation. The back-calculated concentration of the calibration standards should be within ±15% of the nominal value, except for the LLOQ for which it should be within ±20% (EMA, 2011). At least 75% of the calibration standards must fulfil these acceptance criteria for assay validation. QC sample accuracy should be within ±15% of the nominal values. At least 67% of the QC samples should comply with these criteria. If any of these criteria was not met, then the analytical batch was rejected.

3.2.2.9. Linearity

Calibration curve linearity was assessed on three separate occasions. A linear least squares regression model with 1/x weighting was applied to all calibration curves. The assay range was considered linear when the back calculated concentrations and the coefficient of variation (CV) of the calibration standards were within ±15% of the nominal concentrations, except for the LLOQ for which ±20% was acceptable. The same criteria were applied to the peptide depletion response by the reference control (i.e. 50 µM Cor1-C420 and 100 µM cysteine- or lysine- containing heptapeptides).

3.2.2.10. Data analysis

The percent heptapeptide depletion was calculated using Equation 5. Our findings were compared with the OECD TG442C for reactivity classification and DPRA prediction (OECD, 2015a). The average of total depletion of Cor1-C420 and lysine-containing heptapeptide from three independent experiments were compared against the values in Table 3-5 as Cor1-C420 contains both cysteine and lysine side chains. The average of total cysteine heptapeptide depletion from three independent experiments was compared against the values in Table 3-6.

$$\%\,\text{Depletion} = \frac{\left(\begin{array}{c}\text{Mean peptide concentration}\\ \text{in the absence of test chemical}\end{array}\right) - \left(\begin{array}{c}\text{Mean peptide concentration}\\ \text{in the presence of test chemical}\end{array}\right)}{\text{Mean peptide concentration in the absence of the test chemical}} \times 100\% \qquad \text{----- (5)}$$

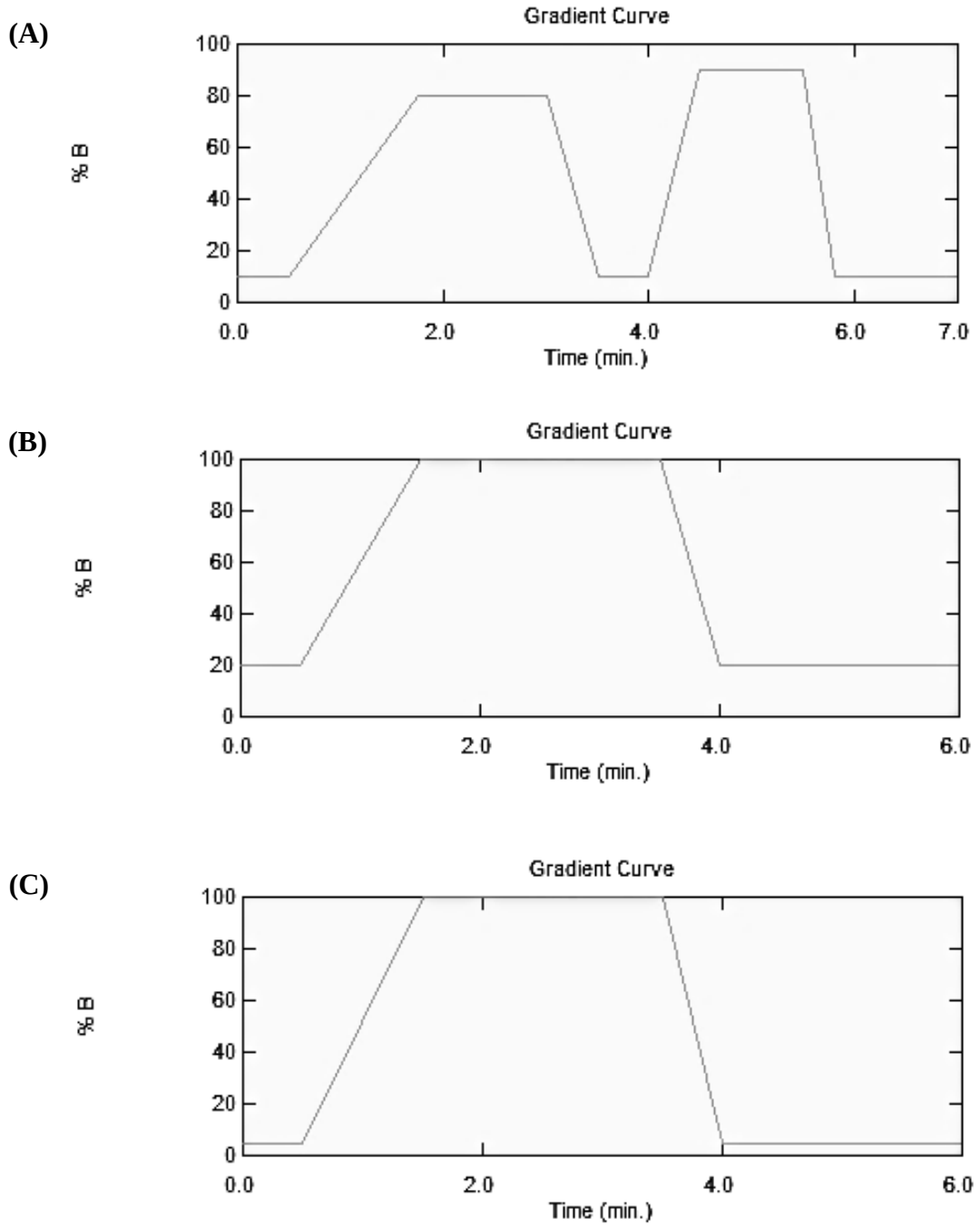
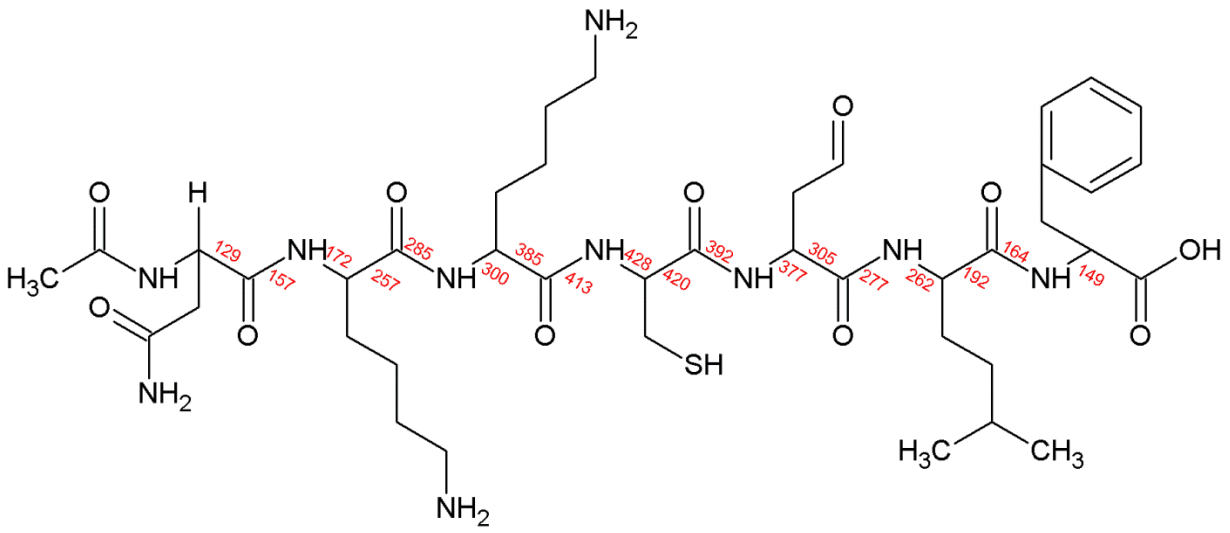


Figure 3-1: Mobile phase gradient elution of (A) Cor1-C420 (B) cysteine- and (C) lysine-containing heptapeptides.

Table 3-1: MS/MS Conditions for all analytes.

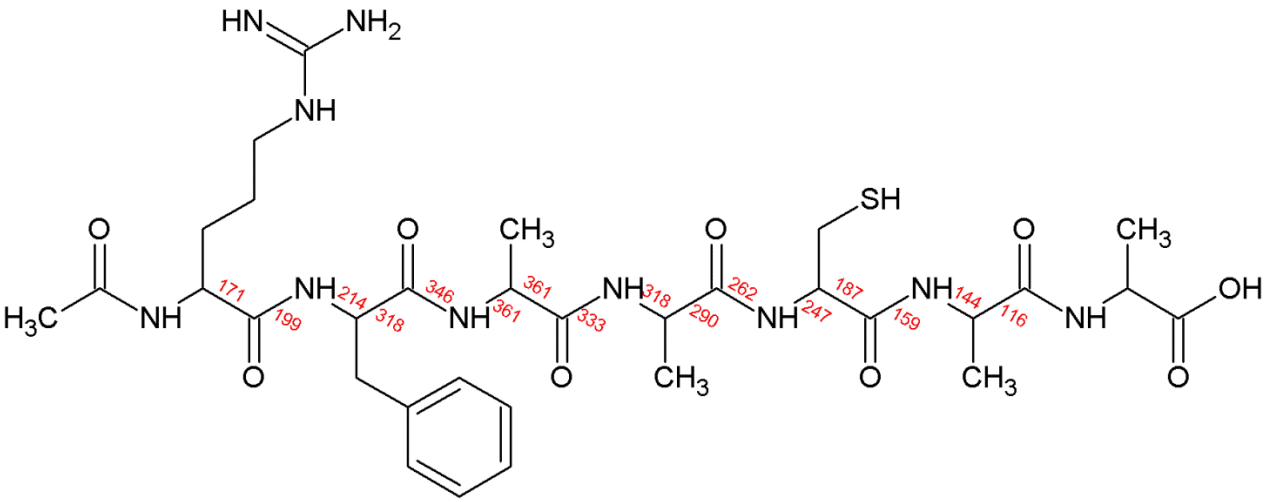
MS Condition	Cor1-C420	Cysteine-containing heptapeptide	Lysine-containing heptapeptide	α-N-acetyl leucine enkephalin	Leucine enkephalin acetate salt hydrate
Collision-induced dissociation (CAD) gas	9	5	5		
Curtain gas (CUR)	40	30	30		
Nebuliser	65	55	55		
Ion spray temperature (TEM)	550	550	550		
Collision energy (CE)	45	95	27	63	71
Collision cell exit potential (CXP)	4	4	4	4	4
Declustering potential (DP)	41	111	51	51	51
Entrance potential (EP)	7	11.5	9	9.5	9.5
MS/MS transition (Parent mass → fragment mass)	455.3 → 120.0	751.3 → 120.0	389.0 → 129.3	598.4 → 120.1	556.2 → 120.1

Table 3-2: Predicted fragments of Cor1-C420 in MS/MS. The value in red showed the predicted site of bond cleavage and predicted fragment mass.



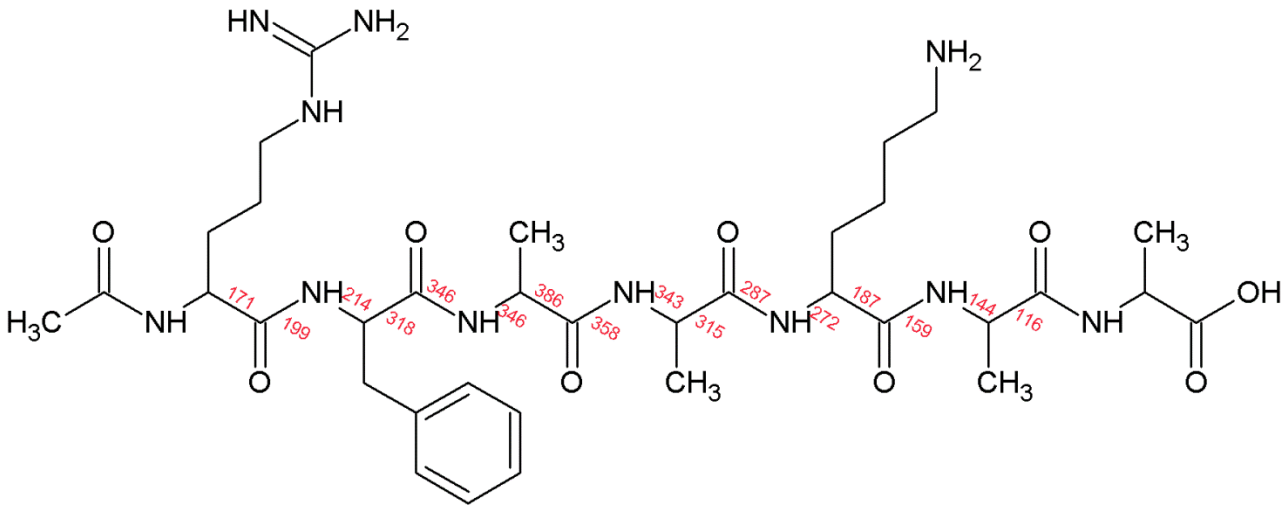
Formula	Mono. Mass	Mass Difference	Formula Difference
$C_5H_9N_2O_2$	129.0664	779.3762	$C_{35}H_{55}N_8O_{10}S$
$C_9H_9O_2$	149.0603	759.3823	$C_{31}H_{55}N_{10}O_{10}S$
$C_6H_9N_2O_3$	157.0613	751.3813	$C_{34}H_{55}N_8O_9S$
$C_9H_{10}NO_2$	164.0712	744.3714	$C_{31}H_{54}N_9O_{10}S$
$C_6H_{10}N_3O_3$	172.0722	736.3704	$C_{34}H_{54}N_7O_9S$
$C_{10}H_{10}NO_3$	192.0661	716.3765	$C_{30}H_{54}N_9O_9S$
$C_{11}H_{21}N_4O_3$	257.1614	651.2812	$C_{29}H_{43}N_6O_9S$
$C_{15}H_{20}NO_3$	262.1443	646.2983	$C_{25}H_{44}N_9O_9S$
$C_{15}H_{21}N_2O_3$	277.1552	631.2874	$C_{25}H_{43}N_8O_9S$
$C_{12}H_{21}N_4O_4$	285.1563	623.2863	$C_{28}H_{43}N_6O_8S$
$C_{12}H_{22}N_5O_4$	300.1672	608.2754	$C_{28}H_{42}N_5O_8S$
$C_{16}H_{21}N_2O_4$	305.1501	603.2925	$C_{24}H_{43}N_8O_8S$
$C_{19}H_{25}N_2O_6$	377.1713	531.2713	$C_{21}H_{39}N_8O_6S$
$C_{17}H_{33}N_6O_4$	385.2563	523.1863	$C_{23}H_{31}N_4O_8S$
$C_{19}H_{26}N_3O_6$	392.1822	516.2604	$C_{21}H_{38}N_7O_6S$
$C_{18}H_{33}N_6O_5$	413.2512	495.1913	$C_{22}H_{31}N_4O_7S$
$C_{20}H_{26}N_3O_7$	420.1771	488.2655	$C_{20}H_{38}N_7O_5S$
$C_{18}H_{34}N_7O_5$	428.2621	480.1804	$C_{22}H_{30}N_3O_7S$

Table 3-3: Predicted fragments of cysteine-containing heptapeptides in MS/MS. The value in red showed the predicted site of bond cleavage and predicted fragment mass.



Formula	Mono. Mass	Mass Difference	Formula Difference
$C_4H_{10}N_3$	100.0875	650.2608	$C_{28}H_{40}N_7O_9S$
$C_4H_6NO_3$	116.0348	634.3135	$C_{28}H_{44}N_9O_6S$
$C_6H_{10}NO_3$	144.0661	606.2822	$C_{26}H_{40}N_9O_6S$
$C_6H_{11}N_2O_3$	159.077	591.2713	$C_{26}H_{39}N_8O_6S$
$C_7H_{15}N_4O$	171.1246	579.2237	$C_{25}H_{35}N_6O_8S$
$C_7H_{11}N_2O_4$	187.0719	563.2764	$C_{25}H_{39}N_8O_5S$
$C_8H_{15}N_4O_2$	199.1195	551.2288	$C_{24}H_{35}N_6O_7S$
$C_8H_{16}N_5O_2$	214.1304	536.2179	$C_{24}H_{34}N_5O_7S$
$C_9H_{15}N_2O_4S$	247.0753	503.273	$C_{23}H_{35}N_8O_5$
$C_9H_{16}N_3O_4S$	262.0862	488.2621	$C_{23}H_{34}N_7O_5$
$C_{10}H_{16}N_3O_5S$	290.0811	460.2672	$C_{22}H_{34}N_7O_4$
$C_{12}H_{20}N_3O_5S$	318.1124	432.2359	$C_{20}H_{30}N_7O_4$
$C_{16}H_{24}N_5O_2$	318.193	432.1553	$C_{16}H_{26}N_5O_7S$
$C_{12}H_{21}N_4O_5S$	333.1233	417.225	$C_{20}H_{29}N_6O_4$
$C_{17}H_{24}N_5O_3$	346.1879	404.1604	$C_{15}H_{26}N_5O_6S$
$C_{13}H_{21}N_4O_6S$	361.1182	389.2301	$C_{19}H_{29}N_6O_3$
$C_{17}H_{25}N_6O_3$	361.1988	389.1495	$C_{15}H_{25}N_4O_6S$

Table 3-4: Predicted fragments of lysine-containing heptapeptides in MS/MS. The value in red showed the predicted site of bond cleavage and predicted fragment mass.



Formula	Mono. Mass	Mass Difference	Formula Difference
$C_4H_{10}N_3$	100.0875	675.3466	$C_{31}H_{47}N_8O_9$
$C_4H_6NO_3$	116.0348	659.3993	$C_{31}H_{51}N_{10}O_6$
$C_6H_{10}NO_3$	144.0661	631.368	$C_{29}H_{47}N_{10}O_6$
$C_6H_{11}N_2O_3$	159.077	616.3571	$C_{29}H_{46}N_9O_6$
$C_7H_{15}N_4O$	171.1246	604.3095	$C_{28}H_{42}N_7O_8$
$C_7H_{11}N_2O_4$	187.0719	588.3622	$C_{28}H_{46}N_9O_5$
$C_8H_{15}N_4O_2$	199.1195	576.3146	$C_{27}H_{42}N_7O_7$
$C_8H_{16}N_5O_2$	214.1304	561.3037	$C_{27}H_{41}N_6O_7$
$C_{12}H_{22}N_3O_4$	272.161	503.273	$C_{23}H_{35}N_8O_5$
$C_{12}H_{23}N_4O_4$	287.1719	488.2621	$C_{23}H_{34}N_7O_5$
$C_{13}H_{23}N_4O_5$	315.1668	460.2672	$C_{22}H_{34}N_7O_4$
$C_{16}H_{24}N_5O_2$	318.193	457.2411	$C_{19}H_{33}N_6O_7$
$C_{15}H_{27}N_4O_5$	343.1981	432.2359	$C_{20}H_{30}N_7O_4$
$C_{17}H_{24}N_5O_3$	346.1879	429.2462	$C_{18}H_{33}N_6O_6$
$C_{15}H_{28}N_5O_5$	358.209	417.225	$C_{20}H_{29}N_6O_4$
$C_{17}H_{25}N_6O_3$	361.1988	414.2353	$C_{18}H_{32}N_5O_6$
$C_{16}H_{28}N_5O_6$	386.204	389.2301	$C_{19}H_{29}N_6O_3$

Table 3-5: Percent peptide depletion model based upon cysteine 1:10 and lysine 1:50 (OECD, 2015a).

Mean of cysteine and lysine % depletion	Reactivity class	DPRA prediction
0% ≤ mean % depletion ≤ 6.38%	No/minimal reactivity	Negative
6.38% < mean % depletion ≤ 22.62%	Low reactivity	Positive
22.62% < mean % depletion ≤ 42.47%	Moderate reactivity	
42.47% < mean % depletion ≤ 100%	High reactivity	

Table 3-6: Percent peptide depletion model based upon cysteine 1:10 (OECD, 2015a).

Cysteine % depletion	Reactivity class	DPRA prediction
0% ≤ % depletion ≤ 13.89%	No/minimal reactivity	Negative
13.89% < mean % depletion ≤ 23.09%	Low reactivity	Positive
23.09% < mean % depletion ≤ 98.24%	Moderate reactivity	
98.24% < mean % depletion ≤ 100%	High reactivity	

3.3. Results

3.3.1. Chromatography

The MS/MS transitions and optimised MS parameters as well as the chromatograms of the peptides and internal standards are presented in Table 3-1 and Figure 3-2 respectively.

3.3.2. Carry-over assessment and LLOQ

Carry-over was observed for Cor1-C420, such that the peak area of the heptapeptide detected in the blank was 10% of the LLOQ. However, this carry-over was within the acceptance criteria of not more than 20% of the LLOQ. No carry-over was observed for cysteine- and lysine-containing heptapeptides as well as for the internal standard. The LLOQ for Cor1-C420 was 5 µM whereas the LLOQ for both the cysteine- and lysine-containing heptapeptides was 2 µM.

3.3.3. Incubation temperature stability

Statistical analysis was performed using repeated measures two-way analysis of variance (ANOVA) followed by the Bonferroni test to assess the stability of heptapeptides between incubation temperatures. Statistical analysis was carried out using the GraphPad Prism™ (Version 6.04) and the statistical significance criterion was $p<0.05$.

The standard calibration curves for Cor1-C420 that was incubated at 25°C and 37°C were significantly different ($P<0.05$ and $P<0.0001$, respectively) from that for the freshly prepared standard calibration curve. This could be due to instability of Cor1-C420 at ambient or high temperatures. No significance difference was observed for the peptide standards that were incubated at 4°C for a period of 24 (±1) h ($P>0.05$). By contrast, the cysteine- and lysine-containing heptapeptides remained stable for 24 h at 4°C, 25°C and 37°C with no significant difference ($P>0.05$) observed for each peptide at the various incubation temperatures.

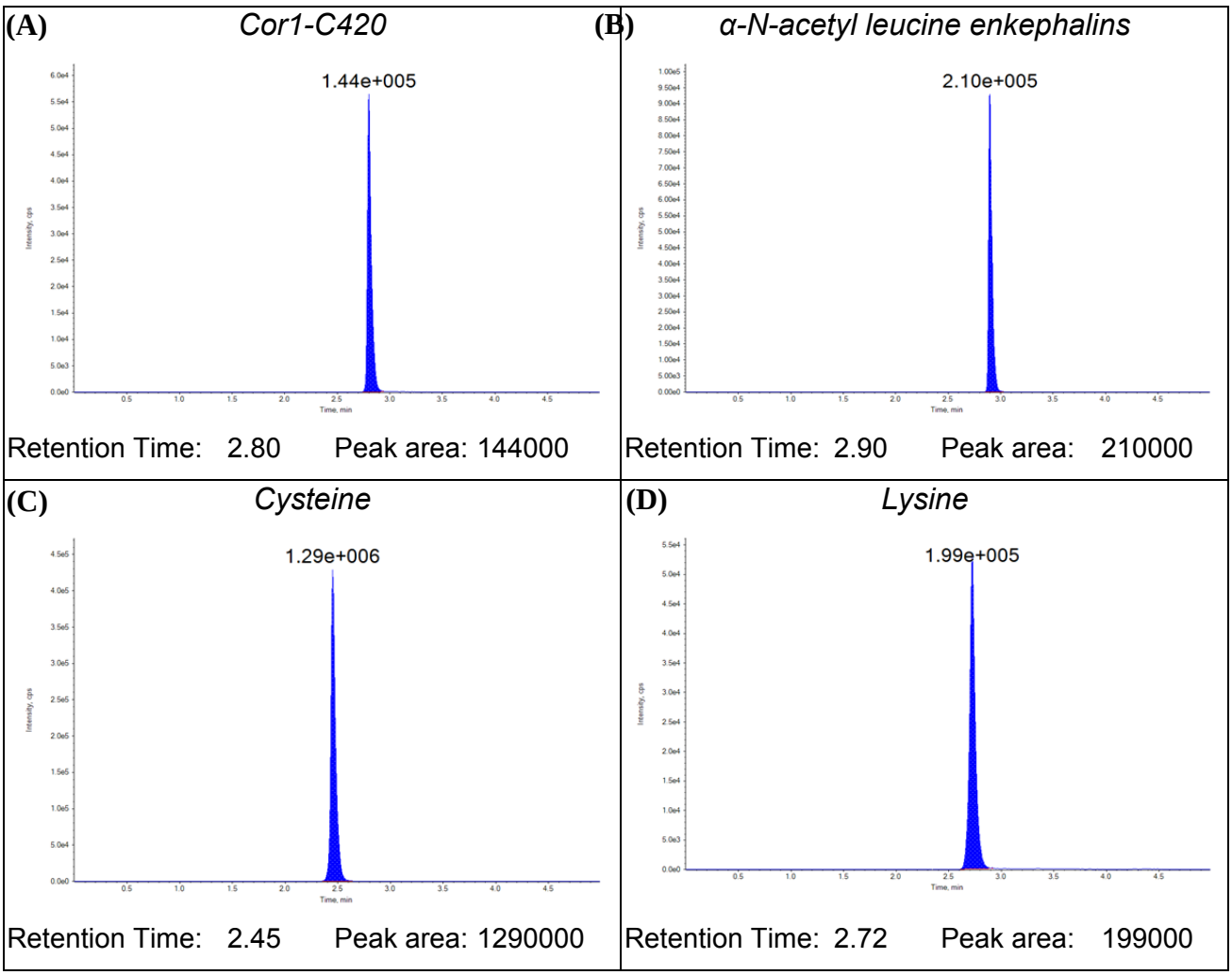


Figure 3-2: Sample chromatograms of (A) Cor1-C420 (B) internal standard (IS) α-N-acetyl leucine enkephalins (C) cysteine- and (D) lysine-containing heptapeptides.

3.3.4. Adsorption of heptapeptides onto polypropylene and borosilicate glass vessels

Peptide stability was assessed for Cor1-C420 (at 15, 25 and 40 μM) and for the heptapeptides containing lysine or cysteine (at 6, 50 and 80 μM) over 3 days in vessels made of polypropylene and borosilicate glass materials. Our data show that the Cor1-C420 concentration for QC samples prepared at low, medium and high concentrations remained unchanged in polypropylene vials (Table 3-7). The accuracy of all three Cor1-C420 QC samples across 3 days was within ±15% of their respective nominal concentrations. By contrast, all Cor1-C420 analytical batches incubated in borosilicate glass vials were rejected for days 1-3 as the repeated analyses did not meet the acceptance criteria as specified in Section 3.2.2.9 (supplementary Table 3-S1). The Cor1-C420 standard curve failed the linearity assessment and hence the accuracy of the QC samples for this peptide (in glass) was not determined.

The concentration of the cysteine-containing heptapeptide QC samples remained unchanged when stored in vessels made from polypropylene materials throughout the course of the experiment (Table 3-8). Additionally, the cysteine-containing heptapeptide QC samples remained unchanged for up to two days post incubation in borosilicate glass vials. However, the cysteine-containing heptapeptide standard curve failed the linearity assessment on day 3 (supplementary Table 3-S2). As for the lysine-containing heptapeptide, the standard curves remained unchanged in vessels made from both polypropylene (Table 3-9) and borosilicate glass (supplementary Table 3-S3) for up to three days post-incubation.

3.3.5. Peptide-chemical complex stability in autosampler

Stability of the peptide-chemical complexes stored in HPLC autosampler plates was assessed using chemicals with known sensitising capacities, *viz* DNCB, isoeugenol, cinnamaldehyde and methyl salicylate, with Cor1-C420 and the heptapeptide containing cysteine (Table 3-10 and Table 3-11 respectively). The corresponding data for glutaraldehyde, cinnamaldehyde, ethyl acrylate and methyl salicylate incubated with the heptapeptide containing lysine are shown in Table 3-12. The autosampler stability of the peptide-chemical complexes was determined to assess the feasibility of injecting a large number of samples in a single analytical experiment without adversely affecting sample integrity which would be a requirement for conducting the DPRA in high-throughput format.

Stability of the peptide-chemical complexes was assessed in polypropylene plates for the Cor1-C420 and cysteine-containing heptapeptides due to the significant adsorptive losses of both peptides onto glass materials as reported in Section 3.3.4. The total peptide depletion of chemicals with known sensitising potential for days 1-3 was compared against those determined on day 0. Following incubation of each of DNCB and cinnamaldehyde with Cor1-C420, there was a decrease in percent peptide depletion over the 3-days assessment period (i.e. an increase in peptide concentration), albeit not extensive such that the classification of these chemicals with respect to skin sensitisation capacity did not change. However, following incubation of isoeugenol and methyl salicylate with Cor1-C420, the reverse trend was observed such that there was a marked increase in peptide depletion over the 3-day assessment period (Table 3-10), that would lead to eventual misclassification of the sensitising reactivity of each of these chemicals. For example, cinnamaldehyde was initially assessed as having moderate peptide reactivity when assessed on day 0 which was in line with LLNA data, with the reactivity gradually decreasing with minimal/no reactivity by 48 hours post-chemical incubation (day 1).

Test chemicals incubated with cysteine-containing heptapeptide showed a decrease in peptide depletion over the 3-day assessment period (i.e. an increase in peptide concentration) for DNCB, isoeugenol and cinnamaldehyde (Table 3-11). In particular, the change in peptide depletion over time resulted in cinnamaldehyde initially being categorised as having moderate reactivity on day 0 but with this changing to low reactivity from day 1 onwards.

As there were no adsorptive losses of the lysine-containing heptapetide in glass or polypropylene vessels (Section 3.3.4), the stability of the formed peptide-chemical complexes for the test chemicals, glutaraldehyde, cinnamaldehyde, ethyl acrylate and methyl salicylate were assessed using vessels made from both types of materials. With the exception of cinnamaldehyde, the extent of lysine peptide depletion over the 3-day assessment period remained unchanged for glutaraldehyde, ethyl acrylate and methyl salicylate in reactions carried out in polypropylene vials (Table 3-12). However, the total lysine heptapeptide depletion by ethyl acrylate (weak sensitiser) was approximately 20% higher overall for the entire 3-day assessment period when the reaction was carried out in borosilicate glass vials (supplementary Table 3-S3) compared with the corresponding data generated using polypropylene plates (Table 3-12). This apparent difference in the extent of lysine peptide depletion between reactions carried out in polypropylene versus borosilicate glass vials was not evident for glutaraldehyde, cinnamaldehyde and methyl

salicylate as the total lysine depletion was similar (±15%) for reactions conducted in both polypropylene and borosilicate glass vials.

Overall our present data indicate that the stability of the covalent bonds formed between the test chemical and heptapeptide of interest, appears to be dependent upon the type of chemical being assessed as well as the heptapeptides utilised. Although the number of test chemicals assessed was small, our data suggest that the total elapsed time for conduct of the DPRA irrespective of the heptapeptide used, should not exceed 24 h in order to maximise assay accuracy.

3.3.6. Linearity

Calibration curves were linear and the slope, y-intercept and regression coefficient (R^2) were determined. Data showing calibration curve linearity for all three heptapeptides using polypropylene vials are summarised in Table 3-13 to Table 3-15. Our calibration data showed high precision (<10%) and high accuracy (<10%) between each replicate and days of the assay. The mean slope for Cor1-C420, cysteine- and lysine-containing heptapeptides were 0.0351, 0.0521 and 0.0306 while the mean R^2 values were 0.9876, 0.9951 and 0.9958, respectively.

Table 3-7: Summary of calculated concentrations for QC samples for the Cor1-C420 heptapeptide in 96-well polypropylene plate assessed at 24 h intervals for Days 0-3. The accuracy of QC samples was within the acceptance criterion, i.e. ±15% from the nominal concentration.

	Day 0 (n=3)			Day 1 (n=3)			Day 2 (n=3)			Day 3 (n=3)		
QC Concentration (μM)	15	25	40	15	25	40	15	25	40	15	25	40
Mean concentration (μM)	15.18	26.12	38.98	15.02	25.60	38.15	15.23	26.07	38.82	14.78	25.78	39.75
SD	0.96	1.80	3.30	0.92	1.90	1.78	1.01	2.87	5.10	1.75	3.43	4.14
Precision	6.3	6.9	8.5	6.1	7.4	4.7	6.6	11.0	13.1	11.8	13.3	10.4
Accuracy	1.20	4.47	-2.55	0.11	2.40	-4.62	1.56	4.27	-2.96	-1.47	3.12	-0.63

Table 3-8: Summary of calculated concentrations for QC samples for the cysteine-containing heptapeptide in 96-well polypropylene plate assessed at 24 h intervals for Days 0-3. The accuracy of QC samples was within the acceptance criterion, i.e. ±15% from the nominal concentration.

	Day 0 (n=3)			Day 1 (n=3)			Day 2 (n=3)			Day 3 (n=3)		
QC Concentration (μM)	6	50	80	6	50	80	6	50	80	6	50	80
Mean concentration (μM)	5.57	47.78	78.04	6.38	50.17	80.05	5.96	50.10	77.58	5.74	49.28	78.15
SD	0.37	2.65	3.60	0.48	3.81	3.59	0.29	1.79	4.15	0.42	1.74	4.41
Precision	6.7	5.5	4.6	7.4	7.6	4.5	4.9	3.6	5.4	7.4	3.5	5.6
Accuracy	-7.3	-4.4	-2.5	6.4	0.3	0.1	-0.7	0.2	-3.0	-4.4	-1.4	-2.3

Table 3-9: Summary of calculated concentrations of QC samples for the lysine-containing heptapeptide in 96-well polypropylene plate assessed at 24 h intervals for Days 0-3. The accuracy of QC samples was within the acceptance criterion, i.e. ±15% from the nominal concentration.

	Day 0 (n=3)			**Day 1 (n=3)**			**Day 2 (n=3)**			**Day 3 (n=3)**		
QC Concentration (μM)	6	50	80	6	50	80	6	50	80	6	50	80
Mean concentration (μM)	5.55	49.80	76.47	5.74	48.80	75.43	5.59	49.00	75.15	5.67	49.22	74.17
SD	0.33	1.72	4.56	0.34	0.75	4.86	0.21	1.04	6.79	0.27	0.79	8.66
Precision	5.9	3.4	6.0	6.0	1.5	6.4	3.7	2.1	9.0	4.8	1.6	11.7
Accuracy	-7.4	-0.4	-4.4	-4.4	-2.4	-5.7	-6.8	-2.0	-6.1	-5.4	-1.6	-7.3

Table 3-10: Percent depletion of the Cor1-C420 heptapeptide incubated with representative test chemicals in 96-well polypropylene plate for a period of 24 (±1) h post-incubation (n=3). Day 0 in the table denotes the first day of sample storage in an autosampler at 4°C. The mean depletion is calculated based on the data from three replicates from each of three independent experiments.

Test Chemicals	Day post incubation	Mean % depletion (±SD)	Mean Difference from Day 0	Classification of Test Chemical[12]
DNCB (Strong sensitiser)	0	97.14 (±1.0)		High reactivity
	1	93.78 (±0.7)	3.350	High reactivity
	2	92.86 (±1.5)	4.279	High reactivity
	3	92.56 (±3.4)	4.578	High reactivity
Isoeugenol (Moderate sensitiser)	0	64.08 (±1.2)		High reactivity
	1	72.96 (±2.1)	-8.881	High reactivity
	2	80.37 (±6.0)	-16.29	High reactivity
	3	82.07 (±5.0)	-17.99	High reactivity
Cinnamaldehyde (Moderate sensitiser)	0	33.83 (±8.1)		Moderate reactivity
	1	17.66 (±12.7)	16.17	Low reactivity*
	2	10.47 (±10.9)	23.36	No/minimal Reactivity*
	3	5.21 (±6.3)	28.62	No/minimal Reactivity*
Methyl salicylate (Weak sensitiser)	0	7.54 (±7.1)		No reactivity
	1	11.24 (±8.0)	-3.699	Low reactivity*
	2	14.56 (±9.4)	-7.021	Low reactivity*
	3	19.18 (±10.7)	-11.64	Low reactivity*

*change in reactivity class

[12] Category of test chemical is based on the OECD TG442C (Table 3-5)

Table 3-11: Percent depletion of the cysteine-containing heptapeptide incubated with representative test chemicals in 96-well polypropylene plate for a period of 24 h (±1) h post incubation (n=3). Day 0 in the table denotes the first day of sample storage in an autosampler at 4°C. The mean depletion is calculated based on the data from three replicates from each of three independent experiments.

Test Chemicals	Day post incubation	Mean % depletion (±SD)	Mean Difference from Day 0	Classification of Test Chemical[13]
DNCB (Strong sensitiser)	0	88.74 (±2.5)		Moderate reactivity
	1	81.36 (±1.0)	7.375	Moderate reactivity
	2	75.71 (±1.6)	13.03	Moderate reactivity
	3	75.83 (±0.9)	12.9	Moderate reactivity
Isoeugenol (Moderate sensitiser)	0	32.84 (±7.0)		Moderate reactivity
	1	29.41 (±3.9)	3.433	Moderate reactivity
	2	24.54 (±6.2)	5.328	Moderate reactivity
	3	28.73 (±7.0)	4.114	Moderate reactivity
Cinnamaldehyde (Moderate sensitiser)	0	27.40 (±2.9)		Moderate reactivity
	1	22.65 (±0.5)	4.751	Low reactivity*
	2	21.10 (±2.8)	6.306	Low reactivity*
	3	22.66 (±4.0)	4.744	Low reactivity*
Methyl salicylate (Weak sensitiser)	0	0.50 (±0.9)		No/minimal reactivity
	1	0.00 (±0.0)	1.112	No/minimal reactivity
	2	0.95 (±1.6)	0.2706	No/minimal reactivity
	3	0.84 (±1.5)	0.49	No/minimal reactivity

*change in reactivity class

[13] Category of test chemical is based on the OECD TG442C (Table 3-6)

Table 3-12: Percent depletion of the lysine-containing heptapeptide incubated with representative test chemicals in 96-well polypropylene plate for a period of 24 h (±1) h post incubation (n=3). Day 0 in the table denotes the first day of sample storage in an autosampler at 4°C. The mean depletion is calculated based on the data from three replicates from each of three independent experiments.

Test Chemicals	Day post incubation	Mean % depletion (±SD)	Mean Difference from Day 0	Classification of Test Chemical[14]
Glutaraldehyde (Strong sensitiser)	0	49.23 (±5.8)		High reactivity
	1	49.03 (±5.2)	0.1989	High reactivity
	2	49.61 (±5.3)	-0.3756	High reactivity
	3	51.25 (±6.2)	-2.019	High reactivity
Cinnamaldehyde (Moderate sensitiser)	0	7.18 (±6.7)		Low Reactivity
	1	4.89 (±5.2)	2.29	No/minimal Reactivity*
	2	4.36 (±4.7)	2.82	No/minimal Reactivity*
	3	4.08 (±4.6)	3.104	No/minimal Reactivity*
Ethyl acrylate (Weak sensitiser)	0	24.73 (±9.8)		Moderate reactivity
	1	23.54 (±7.7)	1.191	Moderate reactivity
	2	23.68 (±6.4)	1.056	Moderate reactivity
	3	23.42 (±6.6)	1.312	Moderate reactivity
Methyl salicylate (Non-sensitiser)	0	9.14 (±9.4)		Low Reactivity
	1	8.02 (±7.6)	1.119	Low Reactivity
	2	7.72 (±6.5)	1.417	Low Reactivity
	3	7.94 (±6.6)	1.203	Low Reactivity

*change in reactivity class

[14] Category of test chemical is based on the OECD TG442C (Table 3-5)

Table 3-13: Calibration curve linearity for the Cor1-C420 heptapeptide (n=3) in 96-well polypropylene plate over 3 days.

	Mean Measured Concentration (C_m)															
Nominal Conc. (μM)	**Replicate 1**				**Replicate 2**				**Replicate 3**				**Mean**	**SD**	**Precision (%)**	**Accuracy (%)**
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 0**	**Day 1**	**Day 2**	**Day 3**				
5	4.72	5.10	5.20	5.16	4.66	5.09	4.94	5.42	4.96	5.24	5.61	5.77	5.16	0.33	6.4	3.1
10	9.65	9.74	9.43	9.50	10.0	9.91	9.70	9.14	10.6	10.0*	8.93	8.59*	9.61	0.55	5.7	-3.9
15	15.4	14.9	14.9	15.6	15.1	14.6	14.2	14.4	14.3	14.2	15.1	14.8	14.78	0.46	3.1	-1.4
20	20.1	19.8	19.5	20.4	20.5	19.8	21.5	20.4	18.7	21.6	20.0	19.3	20.13	0.83	4.1	0.7
25	27.0	25.5	25.9	23.4*	26.1	25.2	26.0	25.8	25.8	22.8	22.8	21.9	24.85	1.67	6.7	-0.6
30	31.4	29.5	30.6	28.7*	30.9	30.9	29.9	29.0*	30.8	30.5*	30.8	29.9	30.24	0.85	2.8	0.8
40	39.4	41.7	40.8	37.7*	40.8	40.0	41.7	40.6	40.6	41.8	41.0	43.0	40.76	1.33	3.3	1.9
50	47.3	48.7	48.7	51.9	46.9	49.5	47.1	49.8	49.2	49.8	50.8	51.1	49.25	1.60	3.3	-1.5
a	0.0580	0.0465	0.0325	0.0336	0.0483	0.0446	0.0453	0.0453	0.0234	0.0165	0.0139	0.0138	0.0351			
b	-0.0546	-0.1400	-0.1220	-0.1490	-0.0330	-0.1250	-0.1640	-0.2050	-0.0344	-0.0383	-0.0492	-0.0597	-0.0979			
R^2	0.9800	0.9972	0.9830	0.9762	0.9938	0.9980	0.9889	0.9835	0.9881	0.9878	0.9907	0.9839	0.9876			

*denotes single data point was used

Table 3-14: Calibration curve linearity of cysteine-containing heptapeptide (n=3) in 96-well polypropylene plate over 3 days.

	Mean Measured Concentration (C_m)															
Nominal Conc. (μM)	**Replicate 1**				**Replicate 2**				**Replicate 3**				**Mean**	**SD**	**Precision (%)**	**Accuracy (%)**
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 0**	**Day 1**	**Day 2**	**Day 3**				
2	2.34	2.11*	N/A	2.15	1.84	1.81	N/A	2.03	N/A	N/A	1.87	N/A	2.02	0.19	9.6	1.1
5	4.78	4.76*	N/A	4.82	4.90	4.89	N/A	4.99	N/A	5.56*	4.97	N/A	4.96	0.26	5.2	-0.8
10	9.29	9.06*	8.98	9.62	10.5	10.4	8.91	9.64	10.9	9.99	10.1	10.9	9.85	0.71	7.2	-1.5
20	18.0	20.4	20.5	19.2	20.7	21.1	21.2	20.3	19.0	18.9	20.6	18.6	19.88	1.07	5.4	-0.6
30	30.2	29.4*	32.1	30.6	30.8	31.0	31.8	30.5	28.5	29.4	30.7	28.6	30.30	1.14	3.8	1.0
50	51.2	52.7	52.3	50.5	51.2	51.3	51.3	49.7	49.6	50.5	52.2	50.7	51.10	0.98	1.9	2.2
80	80.8	80.5	79.0	81.0	78.7	78.2	78.0	79.9	81.2	80.3	79.5	80.9	79.85	1.13	1.4	-0.2
100	100.4	97.2	97.0	99.0	98.4	98.2	98.7	99.9	101	101	97.1	100	99.06	1.55	1.6	-0.9
a	0.0291	0.0429	0.0529	0.0376	0.0432	0.0442	0.0547	0.0351	0.0489	0.0755	0.1030	0.0577	0.0521			
b	-0.0255	-0.0217	0.1920	-0.0198	0.0165	0.0262	0.2060	0.0031	-0.2070	-0.1580	0.0116	-0.2620	-0.0199			
R^2	0.9981	0.9903	0.9919	0.9979	0.9968	0.9957	0.9950	0.9993	0.9954	0.9942	0.9944	0.9922	0.9951			

*denotes single data point was used; N/A denotes the points were excluded

Table 3-15: Calibration curve linearity of lysine-containing heptapeptide (n=3) in 96-well polypropylene plate over 3 days.

	Mean Measured Concentration (C_m)															
Nominal Conc. (μM)	**Replicate 1**				**Replicate 2**				**Replicate 3**				**Mean**	**SD**	**Precision (%)**	**Accuracy (%)**
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 0**	**Day 1**	**Day 2**	**Day 3**				
2	1.96	1.93	1.92	1.85	1.87	1.95	1.96	1.97	1.84	1.92	1.86	1.92	1.91	0.05	2.5	-4.4
5	4.94	5.08	4.98	4.98	4.92	4.92	4.86	4.80	4.98	4.96	4.94	5.00	4.95	0.07	1.4	-1.1
10	9.89	10.0	9.93	9.98	10.4	10.4	10.2	10.1	10.2	10.0	10.3	9.99	10.13	0.18	1.7	1.3
20	20.4	20.4	20.8	21.1	20.9	20.4	20.6	21.0	20.8	20.5	20.8	20.3	20.67	0.27	1.3	3.3
30	30.7	29.8	30.6	31.4	31.0	30.4	30.7	30.8	31.0	30.8	30.8	31.0	30.74	0.38	1.2	2.5
50	50.7	50.3	50.7	50.9	49.3	48.8	49.2	48.4	51.0	50.6	50.2	51.0	50.09	0.92	1.8	0.2
80	79.9	79.4	78.7	78.4	77.2	76.5	77.5	78.9	78.7	79.5	79.7	79.5	78.65	1.08	1.4	-1.7
100	98.6	100	99.4	98.4	101.5	104	102	101	98.4	98.6	98.4	98.4	99.87	1.77	1.8	-0.1
a	0.0367	0.0332	0.0379	0.0378	0.0251	0.0257	0.0293	0.0302	0.0264	0.0279	0.0289	0.0280	0.0306			
b	0.0037	0.0016	0.0075	0.0098	0.0067	0.0035	0.0013	0.0033	0.0065	0.0043	0.0036	0.0007	0.0044			
R^2	0.9845	0.9984	0.9990	0.9973	0.9952	0.9968	0.9977	0.9976	0.9958	0.9950	0.9962	0.9959	0.9958			

3.4. Discussion

We used a comprehensive and systematic approach to identify the optimal experimental conditions for conducting the DPRA in 96-well plate format with LC-MS/MS quantification of the extent of peptide depletion. Specifically, the optimal assay incubation temperature was 25°C for the three heptapeptides assessed, (Cor1-C420, cysteine- and lysine-containing containing), as incubation at 37°C adversely affected Cor1-C420 peptide stability. Our data comparing the effects of using a 96-well polypropylene plate relative to borosilicate glass vials on adsorptive losses of heptapeptides as well as on the stability of peptide-chemical complexes is novel. Importantly, we found that polypropylene plates were preferable to glass vials in terms of minimising adsorptive losses of the peptides of interest even though glass vials are more commonly used for heptapeptide reactivity assessments in the DPRA. Our findings extend the existing DPRA especially for the example of the lysine-containing heptapeptide-ethyl acrylate complex where total lysine depletion was ~20% lower when the assay was conducted in polypropylene compared with glass vials under the same assay preparation conditions. Furthermore, our findings show that the DPRA may not be suitable for screening a large number of chemicals in single experiment due to the potential for instability of test chemical-peptide complexes such that the peptide concentration may change significantly when stored in an autosampler over a 3-day period.

Our present findings on the effects of varying the incubation temperature employed in the DPRA, mimicking the various temperatures used by laboratories globally, on the stability of the heptapeptides, are also novel. Natsch and Gfeller (2008) used 37°C for incubating various test chemicals with the Cor1-C420 heptapeptide, whereas Gerberick et al. (2007) and the OECD guideline, TG442C, recommend an incubation temperature of 25°C for test chemicals with the cysteine- and lysine-containing heptapeptides with an incubation period of 24 h (OECD, 2015a). Herein, we compared the effect of these two incubation temperatures (25°C and 37°C) for representative test chemicals with a range of concentrations of all three heptapeptides, *viz*, Cor1-C420, heptapeptides containing cysteine or lysine with that of freshly prepared samples as the control condition. Our findings show that an incubation temperature of 37°C may induce loss of Cor1-C420. By comparison, a temperature of 4°C did not significantly alter the stability of these three

heptapeptides. A temperature of 25°C was selected as the optimal temperature for subsequent reactions of test chemicals with each of the three heptapeptides of interest as it had a minimal effect on the stability of these heptapeptides after 24 (±1) h of incubation. Incubation time of 24 (±1) h was adopted in the experiment as previously, Gerberick et al. (2004) performed the kinetics activity of peptide depletion and showed that the optimal incubation period for obtaining high predictive power for skin sensitisers was at 24 h.

Next, we assessed the impact of the reaction vial composition (polypropylene or borosilicate glass) used for test chemical incubation reactions on apparent peptide depletion. Our data clearly show that the Cor1-C420 and cysteine-containing heptpeptides were less affected by polypropylene than by borosilicate glass as the Cor1-C420 and cysteine-containing heptapeptide QC samples did not pass the acceptance criteria for samples processed in glass vials after autosampler storage at 4°C for periods of 24 h (day 1) and 72 h (day 3) respectively in contrast to similar samples processed in polypropylene plate where the QC samples passed the assay acceptance criteria. The use of either polypropylene or glass materials for the incubation step did not appear to cause non-specific adsorptive losses of lysine-containing peptide, with the concentrations of all QC samples within the acceptance criterion of ±15% of the nominal peptide concentrations. However, incubation of ethyl acrylate (weak sensitiser) with lysine-containing heptapeptide in glass or polypropylene materials showed that the apparent total lysine depletion was 47.3% or 24.7% respectively when assessed within 24 h of test chemical addition to the peptide. However, in work by others, ethyl acrylate reportedly gave a different percentage of lysine depletion, 2.1% and 93.7% (Gerberick et al., 2007, Troutman et al., 2011), a result that would misclassify ethyl acrylate as a no/minimal to strong reactivity class, respectively. Our total lysine depletion results determined using 96-well polypropylene plates more closely reflect the classification of ethyl acrylate as weak sensitiser by the LLNA (Gerberick et al., 2005). The different observation reported for total lysine depletion with ethyl acrylate could be due to different experimental conditions employed.

Chemical reaction of amino acid residues with test chemicals involves irreversible covalent bond formation mimicking the reaction of haptens with amino acid residues

of skin proteins (Gerberick et al., 2004). However, a major challenge with the existing DPRA method is that the stability over an extended period of the covalent bond formed between heptapeptides and test chemicals, as may be required by high-throughput DPRA screening of large batches of chemicals, is unknown. In our present work, we identified the maximum period that sample analysis could be performed accurately based upon the stability of the peptide-test chemical complexes formed. Our data show for the first time that peptide-chemical complex formation appears to be partially reversible in some instances. For example, following incubation of cinnamaldehyde with Cor1-C420 or the cysteine-containing heptapeptide, apparent peptide depletion decreased by 5% and 13% respectively by day 3 following initiation of the peptide-chemical reactions. In these instances, the magnitude of these changes did not alter the skin sensitisation classifications. The stability of peptide-test chemical complex formed was assessed against standard QC samples (without test chemical) stored for the same length of time in the autosampler at 4°C. As the concentrations of the heptapeptide standard QC samples remained consistent throughout the course of experiment, this means that any changes observed in apparent levels of peptide depletion during the 3-day storage period in the autosampler to cause a change in the chemical reactivity classification of the test chemicals, were not due to instability of the heptapeptides. Instead, our findings suggest that some of the peptide-chemical complexes were held together by slowly reversible covalent bonds. Indeed, our findings are aligned with similar findings in work by others on the kinetic profiles of test chemical-peptide reactions for periods ranging from 5 min to 24 h post-incubation (Roberts and Aptula, 2014, Natsch et al., 2011b). Our findings extend previous findings to suggest that dissociation of peptide-chemical complexes appear to be more prominent for autosampler storage periods longer than 24 h.

Additionally our data indicate that the peptide-chemical complex dissociation rate is chemical-specific. For example, change in apparent peptide depletion was prominent for the Cor1-C420-cinnamaldehyde complex such that during the first 24 h of complex formation, it was classified correctly as a moderate sensitiser. However, it would have been incorrectly classified as a non-sensitiser if assessed only on day 3 post-incubation. By contrast, the extent of peptide depletion determined following incubation of DNCB with the Cor1-C420 differed by ≤5% over several days of

storage at 4°C in an autosampler. Work involving assessment of the kinetic reactivity profiles of test chemicals with the cysteine-containing heptapeptide showed that the extent of cysteine depletion was dependent upon both the test chemical concentration and the incubation time, thereby potentially affecting the chemical potency classification (Roberts and Natsch, 2009, Natsch et al., 2015). Although future investigation is required to characterise the dissociation rate kinetics of peptide-chemical complex formation for a broad range of chemicals, we recommend based upon our present findings showing time-dependent changes in apparent peptide depletion by a range of heptapeptides and chemicals, that all DPRA samples be analysed within 24 h of initiation of incubation (at 25°C) between the heptapeptides of interest and a test chemical.

Our present research highlights the importance of optimising the reaction conditions in a systematic and comprehensive manner when evaluating the applicability of an assay such as the DPRA for assessing a wide range of chemical classes. It is crucial to determine the choice of peptide for DPRA as not all sensitisers will react with thiol and/or amine side chains. For instance, DNCB is thiol reactive and therefore it binds with the thiol side chain of Cor1-C420 and cysteine-containing heptapeptide which will then activate the nuclear factor erythroid-derived 2-related factor 2 (Nrf2)-ARE pathway in cells that is a well-known toxicity pathway activated by skin sensitisers (Natsch, 2010). In contrast, DNCB did not bind with the amine group in lysine and therefore it was replaced with glutaraldehyde, a lysine reactive compound in our lysine depletion assay. Due to the nature of the chemical reactivity of compounds, peptides with different side chains should be included in the DPRA.

We used MS/MS herein rather than an ultraviolet (UV) detector as per the OECD TG442C (OECD, 2015a), because MS/MS is more sensitive and selective compared with UV-based detection systems (Natsch and Gfeller, 2008). Use of MS/MS detection enabled us to adapt the DPRA to a smaller reaction volume prepared in 96-well plate format. This 96-well assay format improved assay efficiency with the potential to be further developed into a high-throughput assay.

Overall, our findings show that the optimal peptide-chemical incubation conditions for the DPRA are a 25°C incubation temperature using polypropylene plates/vials. Observations of adsorptive loss of heptapeptides onto the surface of vial/plate

materials in the DPRA are novel, and not hitherto reported. This effect may not have been significant in the classical DPRA method which used a higher peptide concentration (Gerberick et al., 2004) with analyte adsorption more significant at lower concentrations (Goebel-Stengel et al., 2011). In particular, peptide adsorption was noted for Cor1-C420 (15, 25 and 40 μM) in glass vials, with the measured QC sample concentrations falling outside the acceptance criterion of ±15% of the nominal concentration.

3.5. Conclusion

In summary, our present work, we investigated systematically a number of critical aspects of the DPRA that may potentially confound the accuracy and reproducibility of the data generated by the DPRA. Use of three different heptapeptides in the DPRA has the potential to increase assay specificity for detection of skin sensitisers that may bind more favourably to a particular amino-acid on one peptide rather than another. Hence optimisation of the assay protocol to provide favourable assay conditions for both peptide and the chemical class being assessed is recommended to ensure that accurate and meaningful data are obtained from the DPRA. Additionally, our findings show that conduct of the DPRA in large batch sizes may result in inaccurate data due to instability of chemical bond formation between heptapeptides and some compounds. These observations further highlight the difficulty in adapting *in vitro* methods to high-throughput formats for screening of large numbers of chemicals whilst ensuring that the data produced are both accurate and reproducible.

3.6. Supplementary Data

Table 3-S1: Percent depletion of the Cor1-C420 heptapeptide incubated with representative test chemicals in boroslicate glass vials for a period of 24 (±1) h post-incubation (n=3). Day 0 in the table denotes the first day of sample storage in an autosampler at 4°C. The mean depletion is calculated based on the data from three replicates from each of three independent experiments.

Test Chemicals	Day post incubation	Mean % depletion (±SD)	Mean Difference with Day 0	Classification of Test Chemical[15]
DNCB (Strong sensitiser)	0	98.18 (±1.6)		High reactivity
	1	N/A		
	2	N/A		
	3	N/A		
Isoeugenol (Moderate sensitiser)	0	70.07 (±5.5)		High reactivity
	1	N/A		
	2	N/A		
	3	N/A		
Cinnamaldehyde (Moderate sensitiser)	0	35.76 (±5.8)		Moderate reactivity
	1	N/A		
	2	N/A		
	3	N/A		
Methyl salicylate (Weak sensitiser)	0	8.84 (±5.4)		Low reactivity
	1	N/A		
	2	N/A		
	3	N/A		

*change in reactivity class
N/A denotes the batch failed acceptance criteria

[15] Category of test chemical is based on the OECD TG442C (Table 3-5)

Table 3-S2: Percent depletion of the cysteine-containing heptapeptide incubated with representative test chemicals in boroslicate glass vials for a period of 24 h (±1) h post incubation (n=3). Day 0 in the table denotes the first day of sample storage in an autosampler at 4°C. The mean depletion is calculated based on the data from three replicates from each of three independent experiments.

Test Chemicals	Day post incubation	Mean % depletion (±SD)	Mean Difference with Day 0	Classification of Test Chemical[16]
DNCB (Strong sensitiser)	0	85.13 (±1.7)		Moderate reactivity
	1	73.83 (±2.6)	11.3	Moderate reactivity
	2	64.09 (±2.7)	21.04	Moderate reactivity
	3	N/A		
Isoeugenol (Moderate sensitiser)	0	38.77 (±6.9)		Moderate reactivity
	1	35.90 (±0.5)	2.872	Moderate reactivity
	2	30.99 (±2.5)	7.778	Moderate reactivity
	3	N/A		
Cinnamaldehyde (Moderate sensitiser)	0	35.26 (±2.3)		Moderate reactivity
	1	24.34 (±7.5)	10.91	Moderate reactivity
	2	16.82 (±6.7)	18.44	Low reactivity*
	3	N/A		
Methyl salicylate (Weak sensitiser)	0	5.94 (±3.3)		No/minimal reactivity
	1	0.45 (±4.5)	5.486	No/minimal reactivity
	2	1.72 (±0.9)	4.223	No/minimal reactivity
	3	N/A		

*change in reactivity class
N/A denotes the batch failed acceptance criteria

[16] Category of test chemical is based on the OECD TG442C (Table 3-6)

Table 3-S3: Percent depletion of the lysine-containing heptapeptide incubated with representative test chemicals in boroslicate glass vials for a period of 24 h (±1) h post incubation (n=3). Day 0 in the table denotes the first day of sample storage in an autosampler at 4°C. The mean depletion is calculated based on the data from three replicates from each of three independent experiments.

Test Chemicals	Day post incubation	Mean % depletion (±SD)	Mean Difference with Day 0	Classification of Test Chemical[17]
Glutaraldehyde (Strong sensitiser)	0	55.33 (±4.0)		High reactivity
	1	58.68 (±3.7)	-3.349	High reactivity
	2	61.98 (±4.8)	-6.654	High reactivity
	3	66.51 (±7.4)	-11.18	High reactivity
Cinnamaldehyde (Moderate sensitiser)	0	9.89 (±6.1)		Low Reactivity
	1	6.28 (±2.4)	3.608	No/minimal Reactivity*
	2	4.88 (±1.7)	5.008	No/minimal Reactivity*
	3	4.40 (±0.8)	5.492	No/minimal Reactivity*
Ethyl acrylate (Weak sensitiser)	0	47.28 (±7.7)		High reactivity
	1	43.40 (±5.9)	3.882	High reactivity
	2	42.05 (±6.1)	5.229	Moderate reactivity*
	3	42.18 (± 6.2)	5.099	Moderate reactivity*
Methyl salicylate (Non-sensitiser)	0	3.52 (±5.5)		No/minimal Reactivity
	1	2.89 (±1.7)	0.6289	No/minimal Reactivity
	2	1.52 (±0.4)	1.997	No/minimal Reactivity
	3	1.90 (±0.7)	1.618	No/minimal Reactivity

*change in reactivity class
N/A denotes the batch failed acceptance criteria

[17] Category of test chemical is based on the OECD TG442C (Table 3-5)

Chapter 4: Comparative evaluation of *in vitro* approaches for hazard assessment of epoxy resin compounds relative to the *in vivo* local lymph node assay (LLNA)

4.1. Introduction

Early diagnosis of allergic contact dermatitis (ACD) is crucial for prevention and treatment of disease (Jacob and Steele, 2006a). At present, there is no effective treatment for ACD other than application of skin creams containing corticosteroids to reduce local inflammation and relieve itch (Cohen and Heidary, 2004). For this reason, skin sensitiser screening methods were introduced in the early 1940s such that individuals suspected of having ACD would undergo patch testing to identify the causative allergens. However, a major shortcoming of this approach is that the human patch tests were conducted only after symptoms of ACD had occurred. To address this issue, animal models were introduced for à priori detection of potential skin sensitisers in raw ingredients of consumer products. The currently accepted standalone *in vivo* method is the murine local lymph node assay (LLNA) where the sensitising capacity of skin allergens was found to be proportional to the extent of T-cell proliferation in the local lymph nodes of the tested mice (Basketter et al., 2002). Nevertheless, due to animal welfare concerns, the implementation of non-animal testing has been required in accordance with the ethical principles of the 3Rs.

The mechanism underpinning development of ACD is underpinned by four key biological events that form the Adverse Outcome Pathway (AOP) for skin sensitisation (OECD, 2012a). These four events are protein binding between the sensitising chemical and skin proteins (haptenation), keratinocyte activation, dendritic cell (DC) activation and proliferations of hapten-specific T-cells (OECD, 2012a). Multiple non-animal approaches have been designed to address each of the four AOP key events. To date, the DPRA (Gerberick et al., 2004), KeratinoSens™ (Emter et al., 2010) and h-CLAT (Ashikaga et al., 2006) that map to the first three AOP events respectively, have undergone validation by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM). While several methods hold great potential, it is recognised by most experts in the field that a single *in vitro* method is inadequate to represent the complex mechanisms underpinning ACD development. Hence, the acceptance of an *in vitro* method for à priori screening of chemicals as potential skin sensitisers will be dependent upon important factors that include accuracy, sensitivity, specificity and cost-effectiveness of the assay.

The generalisability of these *in vitro* methods for accurately identifying skin sensitisers from a broad range of chemical classes including epoxy resin compounds is as yet

unclear. This is because evaluation of the performance of most *in vitro* methods developed to date used chemicals that are primarily utilised in the manufacture of cosmetic and toiletry products. However, a recent 9-year retrospective assessment on occupational contact dermatitis in 11 European countries has identified epoxy resin compounds as the second most common allergen type for inducing occupational-related ACD (Pesonen et al., 2015). With the increasing global demand for epoxy resin compounds for industrial applications, it is imperative to address the knowledge gap with regards to the generalisability of the current *in vitro* skin sensitisation tests for hazard assessments of these chemicals.

The findings of my doctoral research program described in Chapters 2 and 3 of this thesis, have brought innovation into and new insights on the limitations of two *in vitro* methods, the h-CLAT and DPRA, that were developed by others to assess the skin sensitisation potential of chemical compounds.

My work described in this chapter, evaluated the predictive accuracy of my optimised h-CLAT method for assessing the skin sensitisation potency of five epoxy resin compounds relative to the corresponding data produced by the LLNA and/or Organisation of Economic Co-operation and Development (OECD) quantitative structural activity relationship (QSAR) toolbox herein. For ethical reasons, the sensitising potency of the five representative epoxy resin compounds assessed herein were drawn from either previous published animal or human data and/or existing information from the OECD QSAR toolbox. The QSAR toolbox, outlined in Chapter 1 Section 1.8.3, is a software application that uses interpolation to fill data gaps regarding assessment of chemical hazards. The database in the OECD QSAR toolbox contains data from experimental studies as well as accumulated knowledge on structural alerts (characteristics) that can be used to identify chemical hazards and to interpolate/extrapolate experimental values by using read-across analysis, trend analysis and QSAR models. For the epoxy resin compounds where the skin sensitising potency data did not exist, they were assessed using the murine LLNA to determine their sensitising capacity.

Additionally, I evaluated the accuracy of my optimised DPRA method described in Chapter 3 for assessment of the skin sensitisation potency of a set of 19 molecules that are widely used in the cosmetics and toiletries industries. Additionally, I evaluated the feasibility of the DPRA to accurately identify and classify the sensitising potential of five epoxy resin compounds.

4.2. Materials and methods

4.2.1. Chemicals and reagents

4.2.1.1. Test chemicals

Bisphenol A diglycidyl ether (DGEBA, CAS 1675-54-3), trimethylolpropane triglycidyl ether (TMPTGE, CAS 3454-29-3, technical grade), poly(ethylene glycol) diglycidyl ether (PEGGE, CAS 72207-80-8), tetraphenylolethae glycidyl ether (THETGE, CAS 7328-97-4), poly[(phenyl glycidyl ether)-co-formaldehyde] (PPGE, CAS 28064-14-4), 2,4-dinitrochlorobenzene (DNCB, CAS 97-00-7), 2-mercaptobenzothiazole (CAS 149-30-4), 3-methylcatechol (CAS 488-17-5), 5-amino-*o*-cresol (CAS 2835-95-2), benzalkonium chloride (CAS 63449-41-2), benzocaine (CAS 94-09-7), cinnamaldehyde (CAS 104-55-2), cinnamyl alcohol (CAS 104-54-1), ethyl acrylate (CAS 140-88-5), eugenol (CAS 97-53-0), geraniol (CAS 106-24-1), glutaraldehyde (CAS 111-30-8), glycerol (CAS 56-81-5), imidazolidinyl urea (CAS 39236-46-9), isoeugenol (CAS 97-54-1), isopropanol (CAS 67-63-0), lactic acid (CAS 50-21-5), methyl salicylate (CAS 119-36-8), resorcinol (CAS 108-46-3), salicylic acid (CAS 69-72-7), α-hexylcinnamaldehye (CAS 101-86-0) were purchased from Sigma-Aldrich Corporation (NSW, Australia).

4.2.1.2. Reagents

Acetone, olive oil, trichloroacetic acid were supplied by Sigma-Aldrich Corporation (NSW, Australia). Tritium thyimidine (^{3}HTdR) and Ultima Gold^{TM} liquid scintillation cocktail were purchased from PerkinElmer (MA, USA). Phosphate buffer saline (PBS) was obtained from Life Technologies Invitrogen (VIC, Australia).

4.2.2. Experimental design

4.2.2.1. OECD QSAR toolbox

Before using the QSAR toolbox, the Chemical Abstract Service (CAS) registration numbers and simplified molecular-input line-entry system (SMILES) of the test chemicals used in my work described this chapter were obtained from the website ChemSpider (http://www.chemspider.com/) and this information is listed in Table 4-1. This information was used as the input data into the OECD QSAR Toolbox version 3.3.2 (downloadable from http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm) to generate the main characteristics of each test chemical utilised herein, according to several predefined classification schemes, namely mechanisms or modes of action (MOA) as well as observed and/or simulated metabolites of each chemical. More specifically, the profiling methods selected within the MOA classification for characterising these potential skin sensitisers were DPRA cysteine peptide depletion, DPRA lysine peptide depletion, protein binding by OASIS v1.3 (Laboratory of Mathematical Chemistry (LMC), Bourgas, Bulgaria), protein binding by the OECD (European Chemicals Agency (ECHA); OECD), keratinocyte gene expression (Laboratory of Mathematical Chemistry (LMC), Bourgas, Bulgaria) and protein binding alerts for skin sensitisation by OASIS v1.3.

The profiling methods/options selected in the QSAR to generate the chemical features based upon observed/simulated metabolites of the queried chemical, included data on *in vivo* metabolism in the rat, rat liver S9 metabolism data, as well as data from the autoxidation simulator, autoxidation simulator (alkaline medium), rat liver S9 metabolism simulator and skin metabolism simulator. The retrieval of existing information and data from experimental studies for the queried test chemicals were sourced from the various OECD QSAR databases, such as the chemical reactivity COLIPA (European Cosmetic Association), DC COLIPA, GSH experimental RC50 (Unilever, International QSAR Foundation, University of Tennessee, Knoxville, USA), keratinocyte gene expression Givaudan (Givaudan International AG, Switzerland), skin sensitisation (Unilever; Procter & Gamble; ExxonMobil; OECD) and skin sensitisation ECETOC (ECETOC Belgium) databases. If no experimental data were available, the chemical was placed into one of the available chemical categories, namely protein binding by OASIS v1.1 as recommended by the OECD (2012b). For chemicals without predefined skin sensitisation information, a read-across analysis was performed as a means to predict the sensitisation capacity of the given chemical.

Table 4-1: List of chemicals, the Chemical Abstract Service (CAS) registration and the molecular-input line-entry system (SMILES) used in the OECD QSAR Toolbox

Chemical Name	Molecular Weight (g/mol)	Purity (%)	SMILE	CAS Number
2,4-dinitrochlorobenzene (DNCB)	202.55	99.8	ClC1=CC=C([N+]([O-])=O)C=C1[N+]([O-])=O	97-00-7
2-Mercaptobenzothiazole	167.25	99.9	SC1=NC2=CC=CC=C2S1	149-30-4
3-Methylcatechol	124.14	99.6	OC1=CC=CC(C)=C1O	488-17-5
5-amino-*o*-cresol	123.15	99.7	OC1=CC(N)=CC=C1C	2835-95-2
Benzalkonium Chloride	364.60	64.8	CCCCCCCCCCCCCC[N+](C)(C)C1=CC=CC=C1.O.[Cl-]	63449-41-2
Benzocaine	165.19	100	NC1=CC=C(C(OCC)=O)C=C1	94-09-7
Bisphenol A Diglycidyl Ether (DGEBA)	340.41	-	CC(C1=CC=C(OCC2CO2)C=C1)(C)C3=CC=C(OCC4CO4)C=C3	1675-54-3
Cinnamaldehyde	132.16	98.4	O=C/C=C/C1=CC=CC=C1	104-55-2
Cinnamyl alcohol	134.18	98.7	OC/C=C/C1=CC=CC=C1	104-54-1
Ethyl Acrylate	100.12	100	O=C(C=C)OCC	140-88-5
Eugenol	164.20	99.4	OC1=CC=C(CC=C)C=C1OC	97-53-0
Geraniol	154.25	99.0	CC(C)=CCC/C(C)=C/CO	106-24-1
Glutaraldehyde	100.12	25.0	O=CCCCC=O	111-30-8
Glycerol	92.09	-	OC(CO)CO	56-81-5

"-" denotes purity was not specified in the certificate of analysis and 100% purity was used in calculation.

Table 4-1: *cont.*

Chemical Name	Molecular Weight (g/mol)	Purity (%)	SMILE	CAS Number
Imidazolidinyl urea	388.29	-	O=C(N1)N(CO)C(NC(NCNC(NC(C(N2)=O)N(CO)C2=O)=O)=O)C1=O	39236-46-9
Isoeugenol	164.20	99.0	OC1=CC=C(/C=C/C)C=C1OC	97-54-1
Lactic Acid	90.08	90.2	CC(O)C(O)=O	50-21-5
Methyl Salicylate	152.15	99.4	OC1=CC=CC=C1C(OC)=O	119-36-8
Poly(ethylene glycol) diglycidyl ether (PEGGE)	526.00	-	C1(CO1)COCCOCC2CO2	72207-80-8
Poly[(phenyl glycidyl ether)-co-formaldehyde] (PPGE)	570.00	-	C=O.C1(OCC2CO2)=CC=CC=C1	28064-14-4
Resorcinol	110.11	99.8	OC1=CC=CC(O)=C1	108-46-3
Salicylic Acid	138.12	99.4	OC1=CC=CC=C1C(O)=O	69-72-7
Tetraphenylolethane glycidyl ether (THETGE)	622.70	-	C1(C(C(C(C=C2)=CC=C2OCC3OC3)C4=CC=C(C=C4)OCC5OC5)C(C=C6)=CC=C6OCC7CO7)=CC=C(C=C1)OCC8CO8	7328-97-4
Trimethylolpropane triglycidyl ether technical grade (TMPTGE)	302.36	-	CCC(COCC1CO1)(COCC2CO2)COCC3CO3	3454-29-3
α-Hexylcinnamaldehye	216.32	97.8	O=C/C(CCCCCC)=C/C1=CC=CC=C1	101-86-0

4.2.2.2. Murine local lymph node assay (LLNA)

Approval was obtained from the Animal Ethics Committee of The University of Queensland for the murine LLNA. For ethical reasons, the LLNA was performed only for those epoxy resin compounds (PEGGE, THETGE and PPGE) where there was no published LLNA data available and there was not enough data within QSAR to make an informed prediction on skin sensitisation potency. The murine LLNA was performed according to the OECD Test Guideline 429 (OECD, 2010a) (Figure 4-1). Briefly, female mice of the CBA strain were chosen for the LLNA. Before performing the LLNA, all existing toxicological information, including acute toxicity and dermal irritation as well as structural and physicochemical information on the test chemicals were taken into account for dose selection. In the absence of such information, a pre-screen test was carried out for dose selection.

The maximum concentration of each test chemical was used as the initial concentration for topical application. Maximum concentration is defined as the maximum solubility of a chemical in vehicle. Acetone:olive oil (AOO, 4:1, v/v) was selected as the solvent/vehicle for all the tested chemicals. Briefly, pre-screen tests involved topical application of three concentrations of a test chemical (i.e. starting from the highest concentration, 100%, 50%, 25%, 10%,…etc) on the dorsal surface of both mouse ears for three consecutive days (n=2 mice/test item). The topical application of test chemicals was performed in a blinded manner to avoid inadvertent bias in scoring erythema three days later (day 6) as shown in Table 4-2. Clinical signs and behaviour of these mice were monitored throughout the test. The assessment of lymph nodes was not performed in the pre-screen test.

The main LLNA test was carried out similarly to the pre-screen test for the first 5 days (Figure 4-1). Three concentrations of each test chemical, starting with the concentration that did not induce erythema to the mouse ears in the pre-test were applied topically (n=4). Additionally, a 25% solution of α-hexylcinnamaldehyde (weak sensitiser) in AOO and vehicle AOO (negative control) were also applied to the ears of mice in parallel with the test chemicals in each cohort. After a two-day rest period, the mice were administered a single bolus intravenous dose (150 µL dose volume) of 20 µCi radiolabelled thymidine (^{3}HTdR) in sterile 1x PBS via tail injection. The mice were euthanised at 5 h post-injection and the auricular draining lymph nodes were excised (Figure 4-2). The auricular lymph nodes from mice within the same treatment group were pooled (n=4) in 1 mL of iced cold 1x PBS and the lymph nodes were mechanically disaggregated using a 35µm nylon mesh

cell strainer. The cells were then washed with 3 mL of 1x PBS and centrifuged at 200 xg for 10 min at 4°C. The supernatant was discarded and the cells were given two times washes with 3 mL of 1x PBS at 4°C. The washed cells were then resuspended in 3 mL of 5% (v/v) trichloroacetic acid in deionised water and were incubated at 4°C for 18 h. After incubation, the cells were centrifuged at 200 xg for 10 min at 4°C. The supernatant was discarded and resuspended in 1 mL of 5% trichloroacetic acid. The resuspended cells from each treatment group were transferred into individual glass scintillation vials, followed by the addition of 10 mL of Ultima Gold™ liquid scintillation cocktail in all vials. The radioactivity level of ^{3}HTdR was determined using a LS6500 multi-purpose scintillator counter (Beckman Coulter, Fullerton, CA, USA). The data were captured using the LS6000 Data Capture/Network Software Version 2.11 and expressed as disintegrations per minute (dpm).

4.2.2.3. SI and EC3 values determination for LLNA

Using dpm data from the scintillator counter, the results of each treatment were calculated and expressed as a stimulation index (SI), which was a ratio of the T-cell proliferation in the treated group to that in the corresponding vehicle-treatment negative control group (Equation 6). A sensitiser was defined as a chemical with one or more doses with an SI≥3. The potency of a skin sensitiser was expressed as an EC3 value (as a percentage), which was the estimated chemical concentration that was able to induce a 3-fold increase in T-cell proliferation, i.e. SI=3. EC3 values were calculated as per Equation 7. The level of ^{3}HTdR incorporated into the DNA of auricular draining lymph nodes was proportional to the potency of the hapten which facilitated further sub-division of a hapten as a non-sensitiser, weak, moderate, strong or extreme sensitising agent (Table 4-3).

$$SI = \frac{Radioactivity\ dpm\ of\ pooled\ nodes\ administered\ with\ test\ item - Background\ in\ dpm}{Radioactivity\ dpm\ of\ pooled\ nodes\ administered\ with\ vehicle - Background\ in\ dpm} \quad \text{------- (6)}$$

$$EC3\ (\%) = c + \frac{(3-d)}{(b-d)} \times (a - c) \quad \text{------- (7)}$$

where

a is the dose concentration in percentage immediately above SI=3

b is the SI immediately above 3

c is the dose concentration in percentage immediately below SI=3

d is the SI immediately below 3

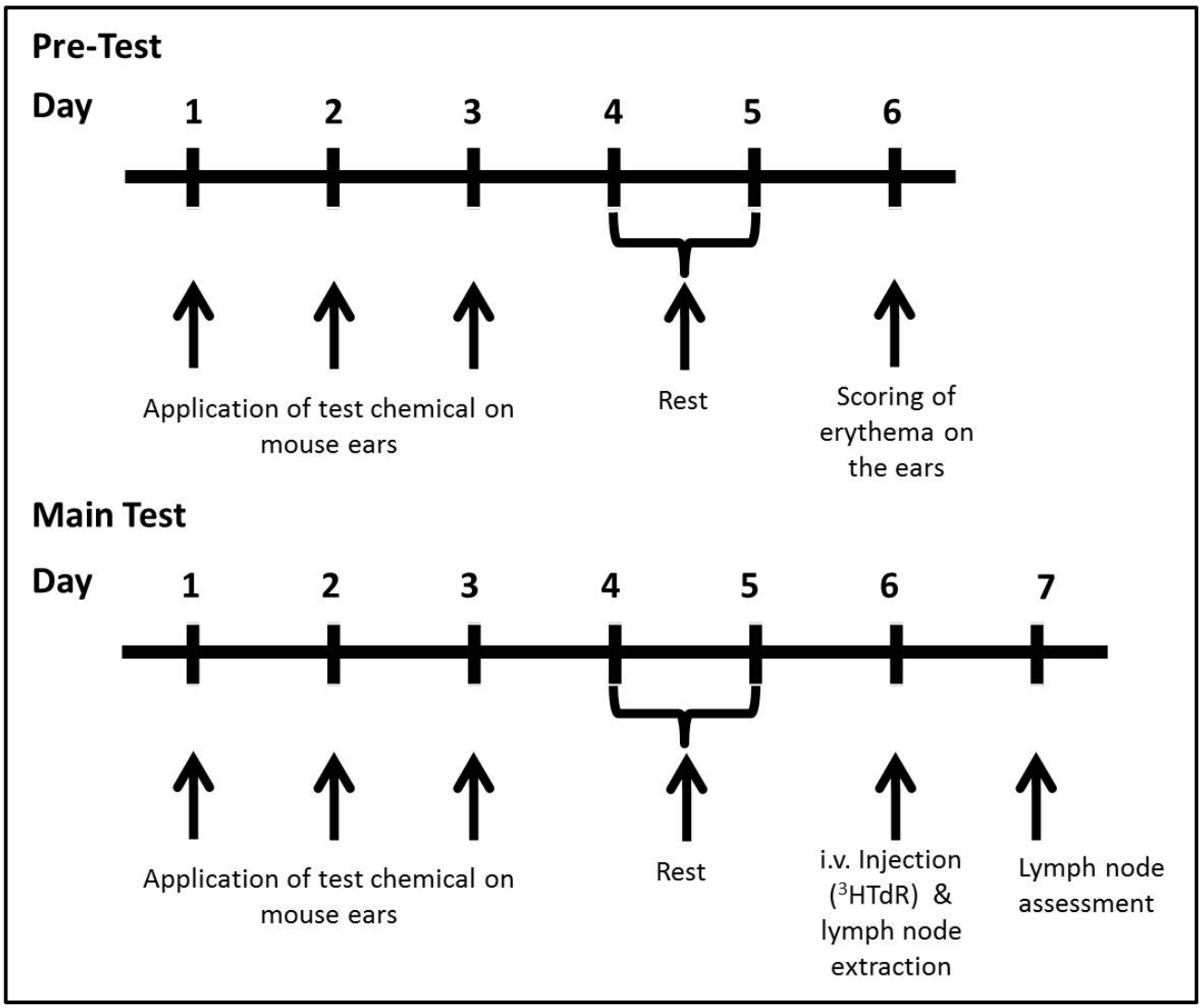


Figure 4-1: Pre-test and main test of the murine LLNA

Table 4-2: Erythema scores for the pre-test in the murine LLNA (OECD, 2010a).

Observation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4

Table 4-3: Potency classification using the mouse LLNA (Kimber et al., 2003)

EC3 Value (%)	Potency Classification
≥10 - ≤100	Weak
≥1 - <10	Moderate
≥0.1 - < 1	Strong
<0.1	Extreme

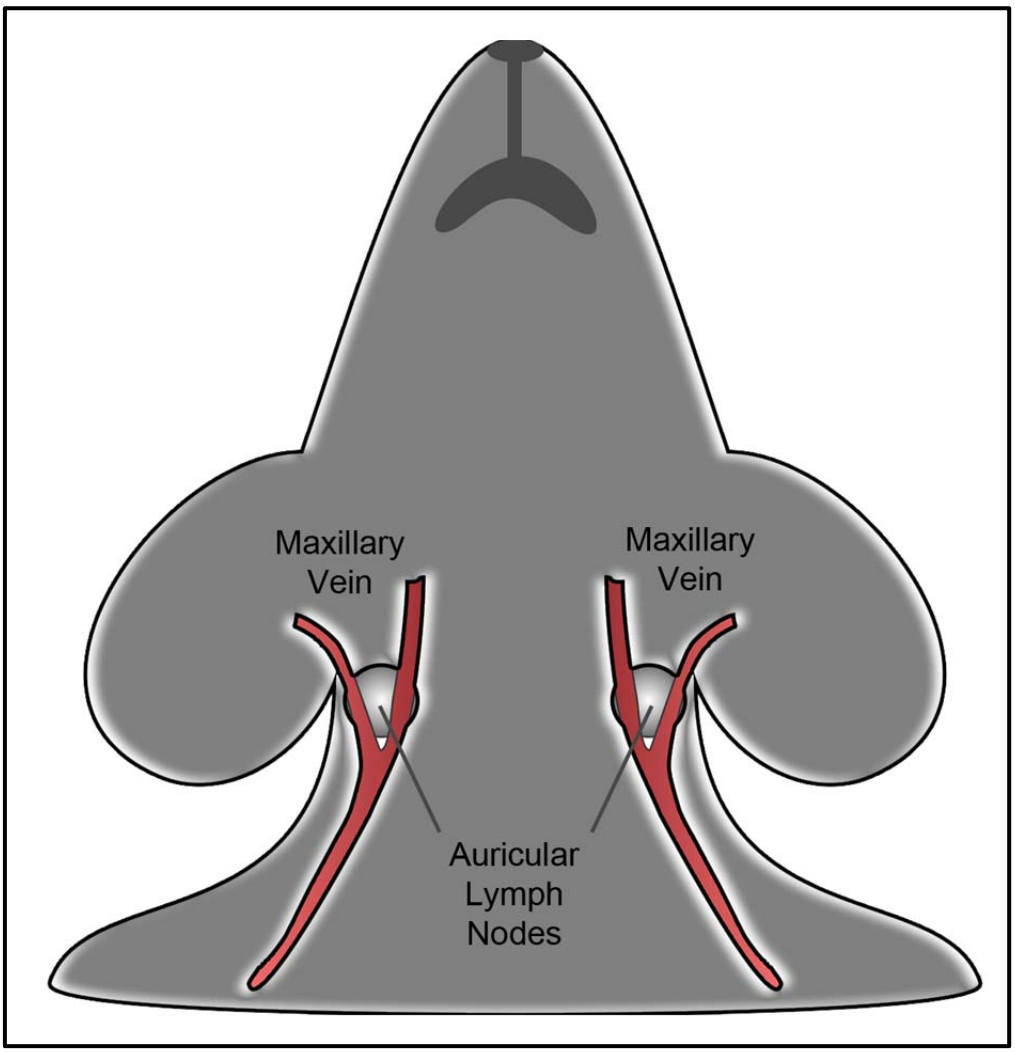


Figure 4-2: Ventral dissection of the mouse. The auricular lymph nodes were extracted on the sixth day after the initial application of a test chemical to the ears.

4.2.2.4. Human cell line activation test (h-CLAT)

My optimised h-CLAT method was performed on five representative epoxy resin compounds using the methods described in Chapter 2 Section 2.2.2.2 and Section 2.2.2.4.

4.2.2.5. Direct peptide reactivity assay (DPRA)

My optimised DPRA method (described in Chapter 3) was used to assess the skin sensitisation potential of five representative epoxy resin compounds. Preliminary assessment showed that further optimisation work was required in order to assess these epoxy resin compounds particularly aimed at addressing the issues of solvent selection for dissolution and minimising adsorptive losses of the heptapeptides used in the DPRA on vessel walls.

4.2.2.5.1. Solvent selection for epoxies

A range of solvents were evaluated for their effects on depletion of heptapeptides in the peptide-epoxies test system. The DPRA using the Cor1-C420 peptide was performed using a range of solvents and their combinations, such as acetonitrile, methanol, 1:1 Acetonitrile:Methanol, 1:1 Acetonitrile:Methanol with 1% *tert*-butanol, 1:1 Acetonitrile: Methanol with 1% methylbutanol, and dimethylsulfuxide (DMSO). These solvents were mixed with phosphate buffer (pH7.4) at varying ratios (v/v), specifically 50%, 25%, 12.5%, 6.25% and 3.125% solvent in phosphate buffer (pH7.4).

4.2.2.5.2. Effect of bovine serum albumin (BSA) on Cor1-C420 heptapeptide

The effect of including BSA, a commonly employed blocking agent in assays to reduce adsorptive losses of peptides onto vessel walls, on DPRA performance was also assessed. Peptide reactivity using 1:10, 1:5 and 1:2.5 (peptide:test chemical ratio) of the Cor1-C420 heptapeptide with DGEBA (strong sensitiser), TMPTGE (moderate sensitiser) and PEGGE (non-sensitiser), respectively, were determined in the presence and absence of 1% BSA during the 24 (±1) h incubation period.

4.3. Results

4.3.1. OECD QSAR toolbox

Based upon the information in the databases of the OECD QSAR toolbox, DGEBA has a measured EC3 value of 1.5% and so it is classified as a moderate sensitiser. Both TMPTGE and THETGE were predicted to be sensitisers with an EC3 value of 16% according to the read across methodology. The prediction results were based upon four measured (experimental) LLNA values from structural analogues. Hence, to assess the accuracy of the predicted value for these two epoxy resins, LLNA data were required (see next section). The sensitisation capacity of the two epoxy resin compounds, PEGGE and PPGE could not be predicted by QSAR due to the lack of existing training datasets within the QSAR databases. The QSAR predicted/measured values for each of the five epoxy resin compounds which were the EC3 value in LLNA and EC1.5, EC2 and EC3 of the KeratinoSens^{TM} assay are summarised in Table 4-4.

4.3.2. Murine local lymph node assay (LLNA)

4.3.2.1. Pre-test

The pre-screen test was performed using PEGGE, THETGE and PPGE to determine the appropriate doses for the main LLNA test. For ethical reasons, TMPTGE was not tested in the LLNA as published data indicate that this compound was a moderate sensitiser (Gamer et al., 2008). Three concentrations of each of PEGGE, THETGE and PPGE were applied to mice ears (n=2 per compound), with the highest concentration equal to the maximum solubility of the test chemical (Table 4-5). AOO was used as the vehicle for all test chemicals. The severity of erythema on both ears was scored on day 0 (pre-dose application) and Day 6 post-application.

During the LLNA, there was a small decrease in body weights of mice treated with test chemicals up to 4 days post-dose application. Overall, the maximum body weight loss was ≤10% for mice treated with THETGE which is acceptable (Figure 4-3). The body weights of all mice increased on days 5 and 6 post-application of test compound.

Using the erythema scoring paradigm shown in Table 4-2, the ears of mice in the LLNA were assessed on day 6. Importantly, the tissue on and around the ears appeared healthy and there was no evidence of erythema prior to test chemical application (Figure 4-4(A)).

For mice where vehicle was applied to the ears, erythema was not observed at the site of application (Figure 4-4(B)). For mice treated with 25% and 50% of PEGGE, The ears of one of two mice per group treated were inflamed and dried test chemical was observed on the surface of the ears (Figure 4-4(C)). The mice also displayed pain behaviour when touched gently on the ear skin and so were assigned a score of 3. The mice treated with the highest dose of 33.3% PPGE tested, there was well-defined erythema and alopecia on the ears and so they were assigned a score of 2 (Figure 4-4(D)). For the mice administered 50% THETGE on the ears, there was well-defined erythema (score = 1) observed. A summary of erythema scores for mice in the LLNA test is shown in Table 4-5.

For the main LLNA test, the selected chemical concentration range was reduced if any of the previously tested concentrations in the pre-screen test gave an erythema score of 3 and above. Based upon the scores shown in Table 4-5, the test chemical concentrations for the main test were 50% (v/v), 25% and 10% for THETGE; 12.5%, 5% and 2.5% for PEGGE; 33.3%, 16.7% and 8.3% for PPGE.

Table 4-4: Predicted skin sensitisation potency of five epoxy resin compounds obtained from the OECD QSAR toolbox.

Chemicals	QSAR Predicted/measured Potency[18]	Comments
DGEBA	Moderate sensitiser	-
TMPTGE	Weak sensitiser	• Read-across prediction of EC3, based upon 3 values of the structurally similar compounds • The log K_{ow} (-0.5) for the target chemical is outside the range of the values for compounds (1.08-7.79)
PEGGE	N/A[19]	• There is not enough data to make a prediction or build a model
THETGE	Weak sensitiser	• Read-across prediction of EC3, based upon 3 values from structurally similar compounds
PPGE	N/A[19]	• There is not enough data to make a prediction or build a model

[18] QSAR predicted/measured potency was based upon the predicted/measured value of EC3 of LLNA and EC1.5, EC2 and EC3 of KeratinoSens™

[19] N/A denotes data not able to be predicted in the database

Table 4-5: LLNA: Erythema scores on mice ears (n=2).

Treatment(s)	Chemical Concentration (%)	Erythema score	
		Day 0	Day 6
PEGGE	50.0	0	3
		0	1
	25.0	0	3
		0	1
	12.5	0	1
		0	1
PPGE	33.3	0	2
		0	2
	16.7	0	1
		0	1
	8.3	0	0
		0	0
THETGE	50.0	0	2
		0	1
	25.0	0	0
		0	0
	10.0	0	0
		0	0

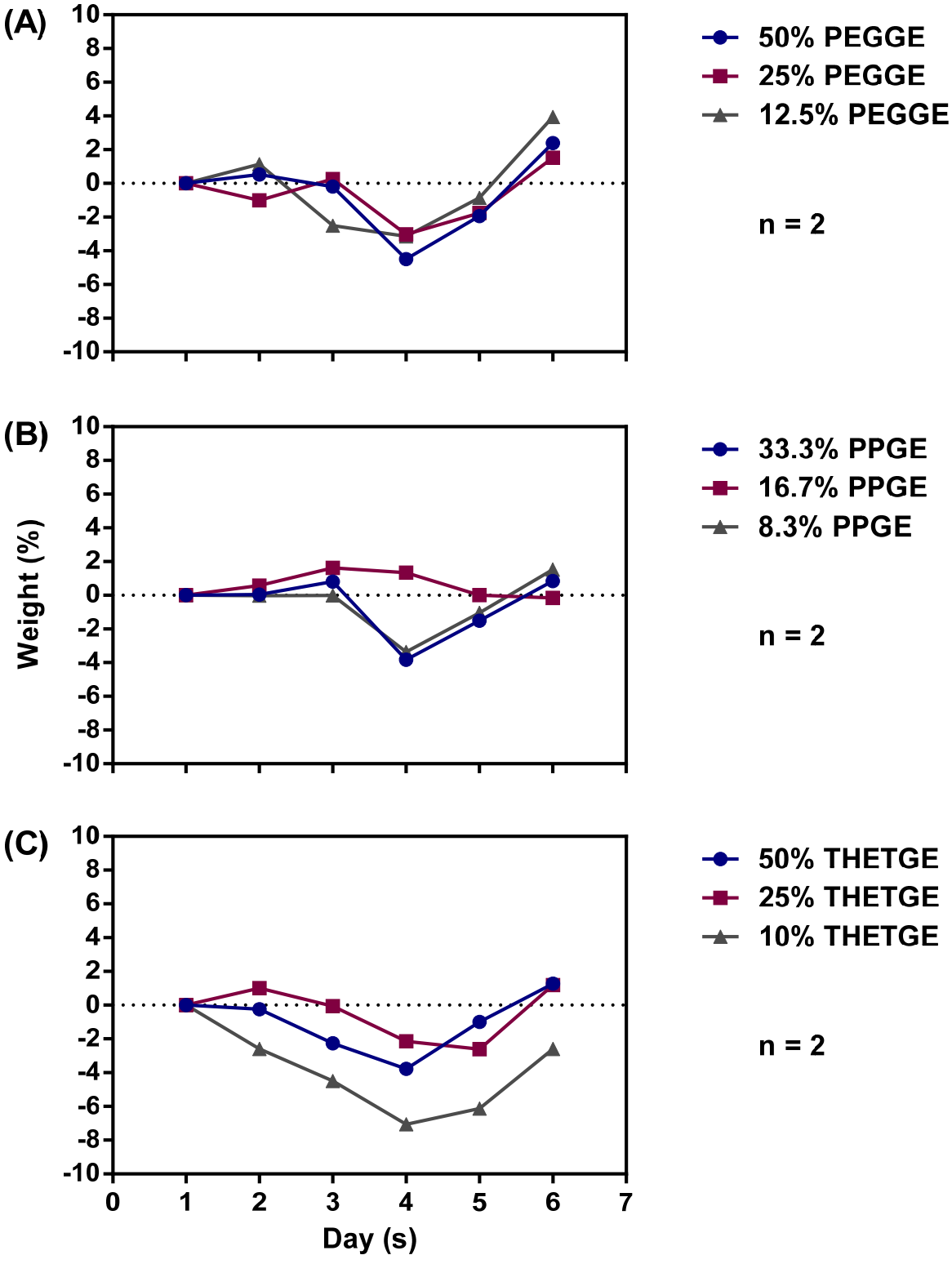


Figure 4-3: Mean percentage weight gain or loss in mice (n=2) from the pre-dose group. The following compounds (A) PEGGE, (B) PPGE and (C) THETGE were topically applied to both ears. Weight loss was <10% in all mice treatment groups.

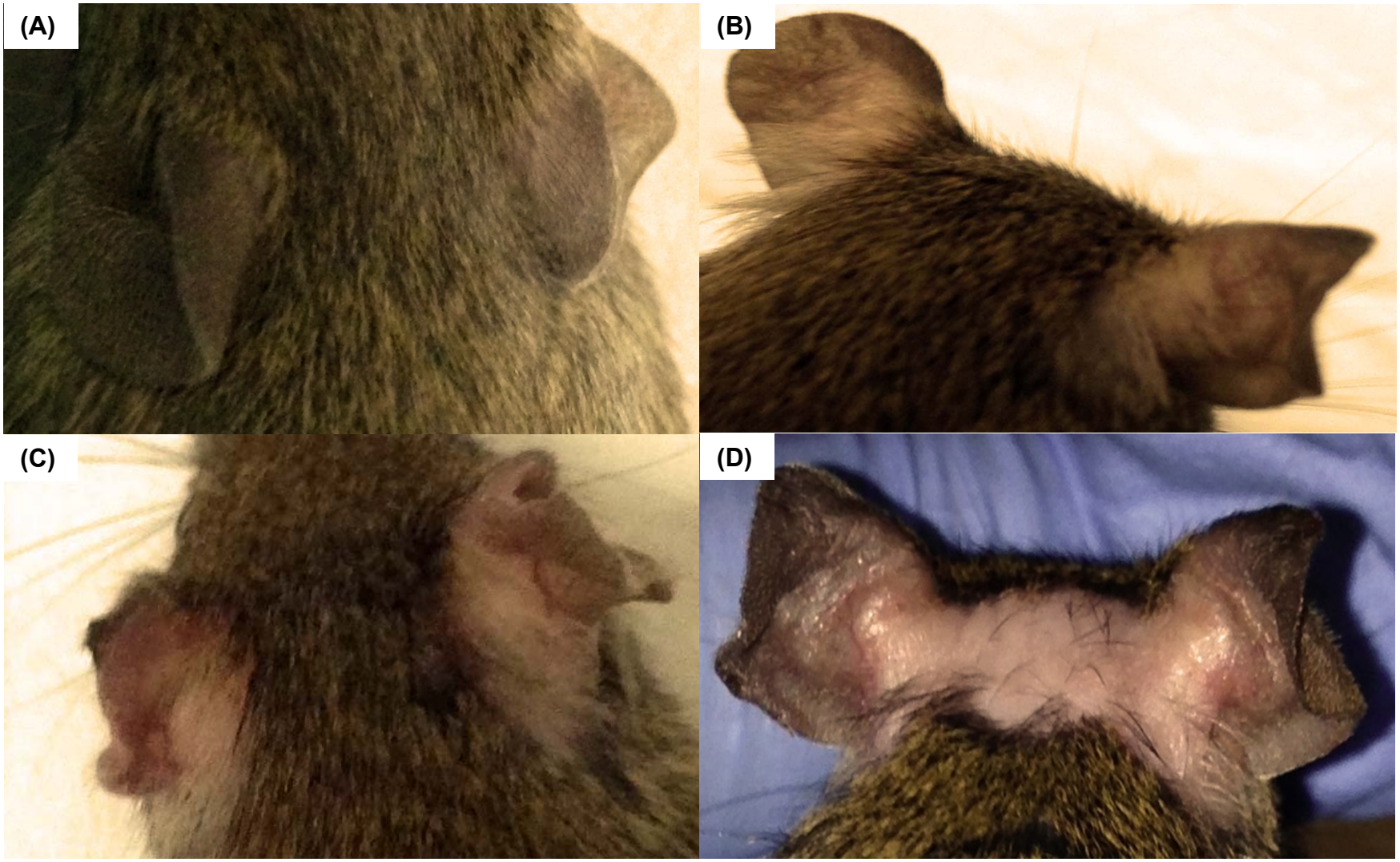


Figure 4-4: Representative images from the ears of mice at (A) day 0 before any treatment and day 6 after treatment with (B) 25% THETGE: no erythema observed, score = 0 (C) 50% PEGGE: well-defined erythema observed and pain behaviour when touched lightly, score = 3 (D) 33.3% PPGE: slight erythema and alopecia were observed, score = 2.

4.3.2.2. Main test

Three concentrations for each test chemical (as described in Section 4.3.2.1) were applied to both ears of mice (n=4/concentration). The highest selected concentration was gave an erythema score of ≤2. Similar to the pre-test, there was a gradual decrease in body weight of ~ 4 – 6% in the first 4 days for mice treated with test chemicals, followed by body weight gain on the fifth and sixth day (Figure 4-5). On the sixth day, the body weights of mice were in the range of 16.5 – 24.0 g. Hence, the volume of 20 µCi ^{3}HTdR injected was 150 µL, i.e. did not exceed 1% of body weight.

Based upon the ^{3}HTdR radioactivity level (expressed in dpm) from the extracted lymph nodes, the SI values for the positive control (25% α-hexylcinnamaldehyde) from three independent experiments were 3.4, 4.4 and 3.7, respectively (Figure 4-6), thereby fulfilling the criteria (SI≥3) for classification as a sensitiser and verifying the validity of the experiment. The SI for all three tested concentrations for PEGGE (Figure 4-6(A)) and THETGE (Figure 4-6(C)) were well below the threshold of SI≥3, and were thus classified as non-sensitisers. Conversely, two of the three tested PPGE concentrations (16.7% and 8.3%) resulted in SI values of 3.6 and 2.1 respectively, with a calculated EC3 of 13.3% and hence was designated as a weak sensitiser (Figure 4-6(B)). A summary of the LLNA potency classification of the five epoxy resin compounds assessed in my PhD thesis is shown in Table 4-6.

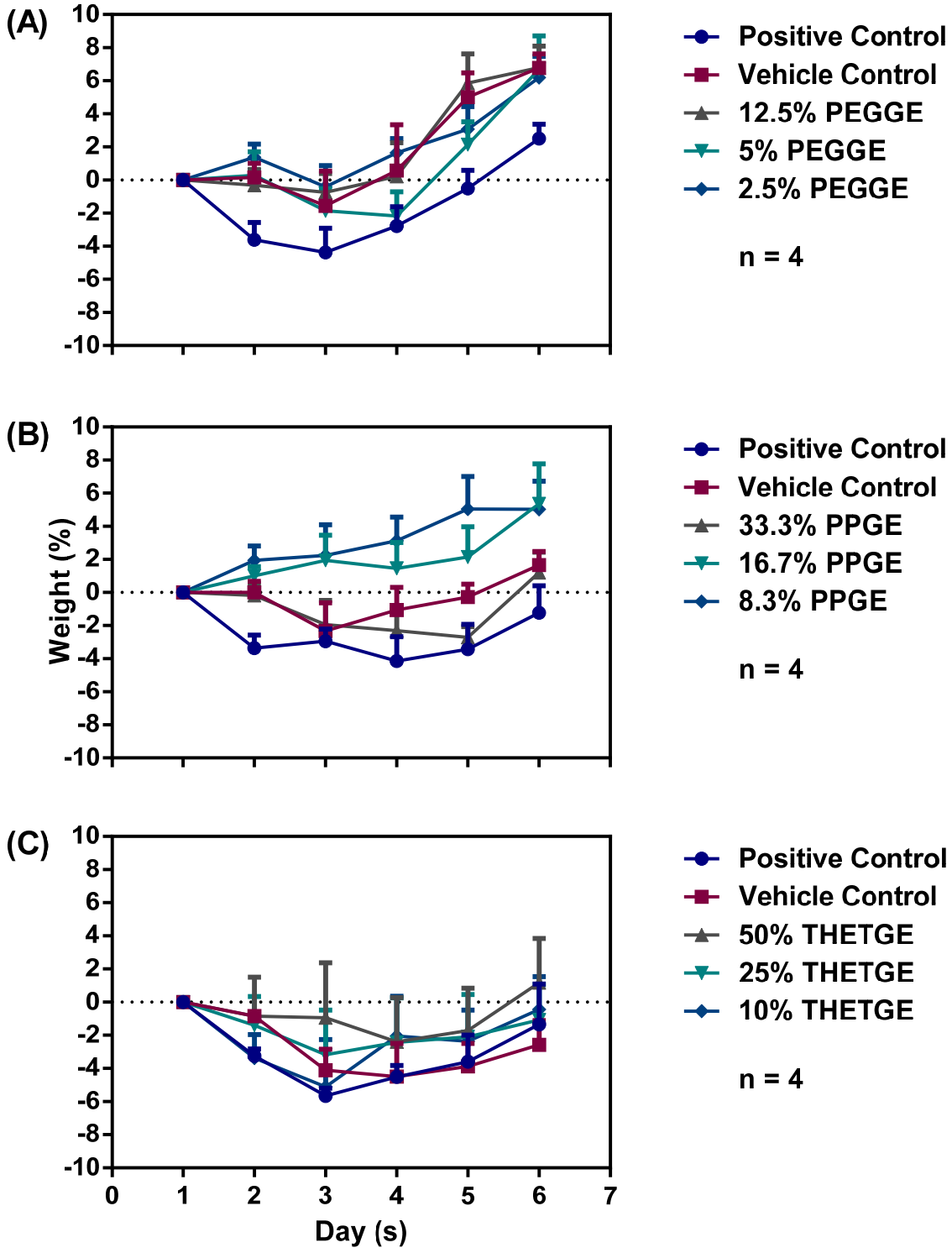


Figure 4-5: Percentage body weight gain or loss (mean ± SEM) for LLNA mice (n=4) in the initial pre-dose experiment. Mice were received topical application to the ears of three different concentrations of (A) PEGGE, (B) PPGE and (C) THETGE. The positive control was mice (n=4) treated with 25% α-hexylcinnamaldehyde while the vehicle control was mice (n=4) treated with 4:1 AOO. There was insignificant weight loss in all mice groups.

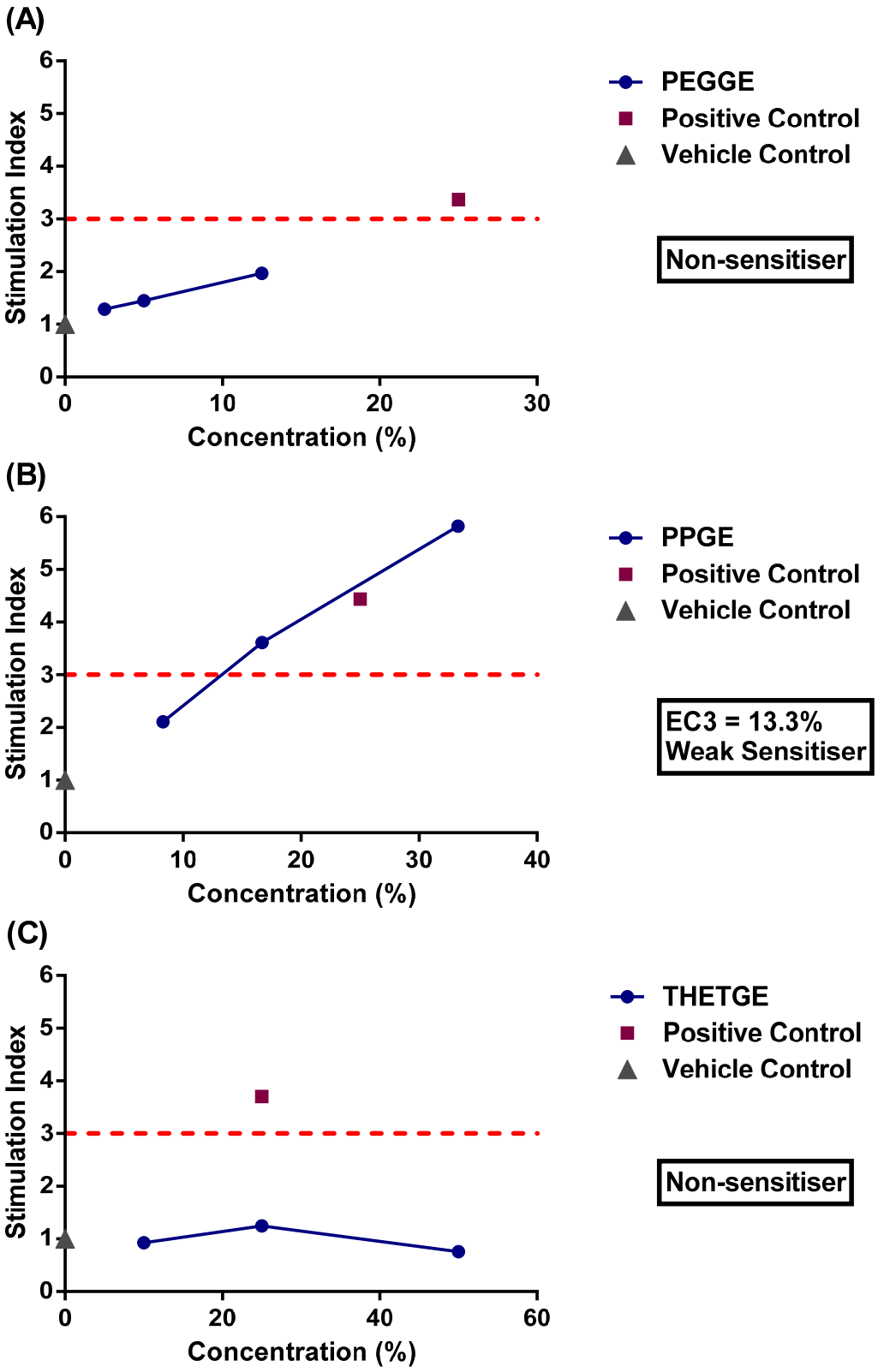


Figure 4-6: SI and EC3 values of (A) PEGGE, (B) PPGE and (C) THETGE-treated mice (n=4). In each experiment, there was a positive control group (n=4) that received 25% α-

hexylcinnamaldehyde applied topically to the ears and a corresponding vehicle-treated control group (n=4) that received 4:1 AOO applied topically to both ears.

Table 4-6: LLNA potency classification of five representative epoxy resin compounds

Chemicals	Measured Potency	Reference
DGEBA	Strong sensitiser	Gamer et al. (2008)
TMPTGE	Moderate sensitiser	Gamer et al. (2008)
PEGGE	Non-sensitiser	In this study (Figure 4-6)
THETGE	Non-sensitiser	In this study (Figure 4-6)
PPGE	Weak sensitiser	In this study (Figure 4-6)

4.3.3. Human cell line activation test (h-CLAT)

The h-CLAT results for the five representative epoxy resin compounds as well as DNCB and methyl salicylate were presented in Chapter 2, Figure 2-4 to Figure 2-10, and Table 2.4. These data are compared with the corresponding LLNA data described in this chapter.

4.3.4. DPRA

4.3.4.1. Heptapeptides (Cor1-C420, cysteine and lysine) depletion

The peptide depletion data for the 19 chemicals and the five representative epoxy resin compounds using Cor1-C420, cysteine and lysine heptapeptides in the optimised DPRA methods described in Chapter 3, Sections 3.2.2.1 to 3.2.2.4, are shown in Table 4-7. Due to time constraints, DPRA analyses with the cysteine heptapeptide for 17 chemicals were not performed, and so these data were sourced from previously published work by others (Gerberick et al., 2009, Gerberick et al., 2007, Natsch and Gfeller, 2008).

My findings showed that DNCB reacts with the cysteine and Cor1-C420 heptapeptides and not the lysine heptapeptide where 69% and >96% depletion was observed for cysteine and Cor1-C420 heptapetides, respectively, but no/minimal depletion was detected with the lysine heptapetide (Table 4-7). Conversely, no/minimal peptide reactivity was observed for all three heptapeptides with salicylic acid (non-sensitiser). It is interesting to note that ethyl acrylate, designated by LLNA as a weak sensitiser showed >96% Cor1-C420 depletion (Table 4-7). Overall, my results for the 16 out of 19 chemicals showed total Cor1-C420 and cysteine heptapeptides depletion that correlated to previously published LLNA data.

Following assessment of the five epoxy resin compounds with all three heptapeptides in the DPRA, two of these, *viz* PEGGE and TMPTGE, showed moderate peptide reactivity with all three heptapeptides. The remaining three epoxy resins, *viz* DGEBA, THETGE and PPGE had low reactivity with each of the three heptapeptides. This lack of peptide reactivity for the known skin sensitiser, DGEBA, was unexpected. Hence, further work was undertaken to optimise the assay conditions for the DPRA with respect to the assessment of epoxy resin compounds with a particular focus on solvent composition and the use of BSA to block nonspecific adsorptive heptapeptide losses.

Table 4-7: DPRA Results using 27% acetonitrile in phosphate buffer (pH 7.4) as solvent.

Chemicals	Total % depletion (±SD)		
	Cor1-C420	**Cysteine**	**Lysine**
Extreme/Strong sensitiser			
2,4-dinitrochlorobenzene	>96	69.07 (±0.7)	0.25 (±0.8)
3-Methylcatechol	>96	80.3 (±2.5)[20]	31.97 (±1.2)
Glutaraldehyde	95.67 (±2.9)	30.2 (±0.5)[2]	63.15 (±1.5)
Moderate sensitiser			
2-Mercaptobenzothiazole	97.8 (±3.7)[21]	97.5 (±4.2)[22]	1.59 (±2.2)
5-amino-*o*-cresol	38.62 (±7.1)	ND	4.38 (±1.2)
Cinnamaldehyde	47.9 (±0.9)[1]	70.6 (±1.0)[2]	5.97 (±1.7)
Isoeugenol	64.91 (±1.5)	78.5 (±4.2)[1]	1.61 (±2.0)
Resorcinol	8.38 (±3.5)	1.6 (±5.6)[2]	7.02 (±3.3)
Weak Sensitiser			
Benzocaine	1.10 (±2.2)	ND	2.23 (±2.5)
Ethyl Acrylate	>96	96.4 (±0.3)[2]	17.10 (±2.4)
Eugenol	19.76 (±3.1)	13.0 (±4.8)[3]	0.13 (±0.2)
Geraniol	ND	2.4 (±4.4)[3]	0.96 (±1.0)
Imidazolidinyl Urea	97.9 (±3.7)[1]	52.3 (±6.0)[2]	2.06 (±1.7)
α-Hexylcinnamaldehye	2.3 (±3.5)	-0.3 (±1.2)[2]	1.42 (±1.7)
Non-sensitiser			
Benzalkonium Chloride	11.85 (±4.5)	20.4 (±2.5)[2]	ND
Glycerol	3.40 (±2.5)	-3.8 (±5.2)[2]	3.28 (±1.3)
Lactic Acid	0.09 (±0.2)	-0.9 (±0.3)[2]	1.15 (±1.5)
Methyl Salicylate	7.69 (±6.5)	0.86 (±1.6)	2.87 (±1.3)
Salicylic Acid	0.96 (±2.2)	5.3 (±5.5)[2]	3.10 (±2.8)
Epoxy resin compounds classification to be determined in this chapter			
DGEBA	1.86 (±2.5)	0.63 (±1.3)	0.16 (±0.5)
PEGGE	28.61 (±4.3)	21.28 (±1.7)	41.16 (±3.6)
TMPTGE	39.79 (±3.7)	30.13 (±3.2)	27.83 (±4.7)
THETGE	14.75 (±2.3)	0.37 (±0.6)	5.04 (±4.1)
PPGE	3.85 (±3.0)	0.00 (±0.0)	2.26 (±3.4)

[20] Data obtained from Gerberick et al. (2009)
[21] Data obtained from Natsch et al. (2008)
[22] Data obtained from Gerberick et al. (2007)
Note: 25% acetonitrile was used as the final solvent concentration in Gerberick et al. (2007, 2009) and Natsch et al. (2008)

4.3.4.2. Solvent selection for epoxy resins

Solvents comprising acetonitrile:methanol (1:1) with 1% methylbutanol, and DMSO were unsuitable for use in DPRA as there was Cor1-C420 peptide depletion at all solvent concentrations assessed (Figure 4-7). The four other solvents evaluated, *viz* acetonitrile, methanol, acetonitrile:methanol (1:1) and acetonitrile:methanol (1:1) with 1% *tert*-butanol, had minimal effects on total Cor1-C420 depletion, especially when used at concentrations ≤25% in the DPRA (Figure 4-7). However, acetonitrile, methanol, acetonitrile:methanol (1:1) were unsuitable as solvents for PEGGE, THETGE and PPGE because although these epoxy resins initially dissolved in acetonitrile and/or methanol, they precipitated out of solution after aliquots were added into 0.1 M phosphate buffer (pH7.4) during the DPRA incubation step. The most suitable solvent composition was acetonitrile:methanol (1:1) with 1% *tert*-butanol at a final assay concentration of ~25% for the five representative epoxy resin compounds. Importantly, the solvent alone had a minimal effect on peptide depletion and dissolution of the epoxy resin compounds was maintained during the DPRA.

Total peptide depletion data for all three heptapeptides in the DPRA for the five epoxy resins dissolved in acetonitrile:methanol (1:1) with 1% *tert*-butanol, are summarised in Table 4-8. Peptide reactivity was classified according to the criteria specified in Table 4-9 for the Cor1-C420 peptide and the lysine heptapeptide, as well as Table 4-10 for the cysteine heptapeptide. Using the peptide reactivity criteria for Cor1-C420, the five epoxy resin compounds were classified as sensitisers because total peptide depletion for was greater than 6.38%. Additionally, as DGEBA, PEGGE and TMPTGE induced total cysteine depletion greater than 13.89%, they were designated sensitisers. By contrast, THETGE and PPGE were classified as non-sensitisers due to their no/minimal cysteine peptide reactivity. Based upon the total lysine depletion data and the reactivity criteria presented in Table 4-9, only PEGGE was categorised as a sensitiser but with low peptide reactivity of 12% depletion. By contrast, the remaining four tested epoxy resin compounds were classified as negative sensitisers because they showed no/minimal reactivity with the lysine heptapeptide.

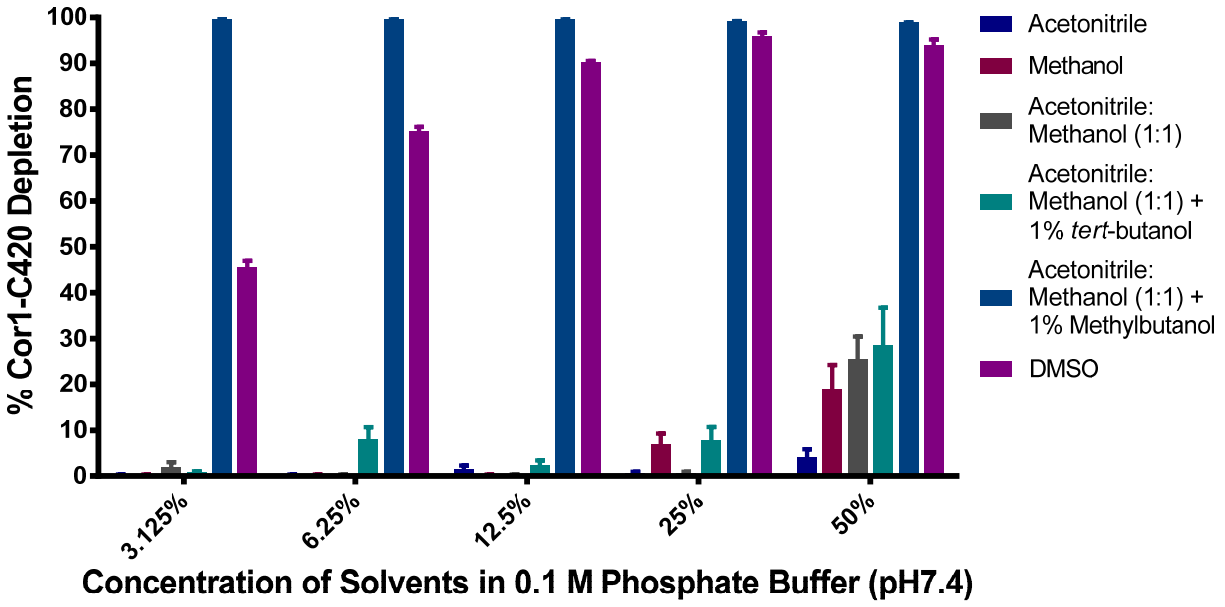


Figure 4-7: Effect of solvent composition on Cor1-C420 heptapeptide depletion.

Table 4-8: Percent heptapeptide depletion using a solvent comprising 27% acetonitrile:methanol (1:1) with 1% *tert*-butanol and their respective buffers and reaction ratio.

Test chemicals	% Depletion (±SD) (Reactivity Class[23])		
	Cor1-C420	Cysteine	Lysine
DGEBA	40.3 (±1.9) (Moderate reactivity)	44.6 (±8.5) (Moderate reactivity)	1.7 (±1.3) (No/minimal reactivity)
PEGGE	28.9 (±6.3) (Moderate reactivity)	49.2 (±4.4) (Moderate reactivity)	12.0 (±3.5) (Low reactivity)
TMPTGE	37.7 (±3.5) (Moderate reactivity)	31.9 (±2.4) (Moderate reactivity)	6.4 (±3.9) (No/minimal reactivity)
THETGE	12.4 (±1.9) (Low reactivity)	2.6 (±4.3) (No/minimal reactivity)	4.1 (±3.3) (No/minimal reactivity)
PPGE	34.1 (±8.2) (Moderate reactivity)	10.9 (±5.8) (No/minimal reactivity)	1.3 (±1.0) (No/minimal reactivity)

[23] Reactivity class of heptapeptides was based upon the criteria listed in Table 4-9 for Cor1-C420 and lysine and Table 4-10 for cysteine.

Table 4-9: Percent peptide depletion model based upon heptapetide: test chemical ratios of 1:10 for cysteine and 1:50 for lysine (OECD, 2015a).

Mean of cysteine and lysine % depletion	Reactivity class	DPRA prediction
0% ≤ mean % depletion ≤ 6.38%	No/minimal reactivity	Negative
6.38% < mean % depletion ≤ 22.62%	Low reactivity	Positive
22.62% < mean % depletion ≤ 42.47%	Moderate reactivity	
42.47% < mean % depletion ≤ 100%	High reactivity	

Table 4-10: Percent peptide depletion model based upon a heptapetide: test chemical ratio of 1:10 for cysteine (OECD, 2015a).

Cysteine % depletion	Reactivity class	DPRA prediction
0% ≤ % depletion ≤ 13.89%	No/minimal reactivity	Negative
13.89% < mean % depletion ≤ 23.09%	Low reactivity	Positive
23.09% < mean % depletion ≤ 98.24%	Moderate reactivity	
98.24% < mean % depletion ≤ 100%	High reactivity	

4.3.4.3. Effect of BSA on Cor1-C420 heptapeptide

BSA at a concentration of 1% was added into each well of the polypropylene assay plate prior to performing the Cor1-C420-chemical reactions using three concentrations of each of DGEBA, TMPTGE and PEGGE. Inclusion of 1% BSA alone (in the absence of any test chemical) in the DPRA increased Cor1-C420 depletion to 17% compared with only 3% depletion in the absence of BSA (Figure 4-8). There was also a similar trend for the Cor1-C420-epoxy resin reactions where in the presence of 1% BSA, peptide depletion was higher than for the corresponding samples without the addition of BSA. These findings suggested that BSA was binding to the Cor1-C420 leading to apparently higher Cor1-C420 peptide depletion in the DPRA and so 1% BSA was not included in the assay.

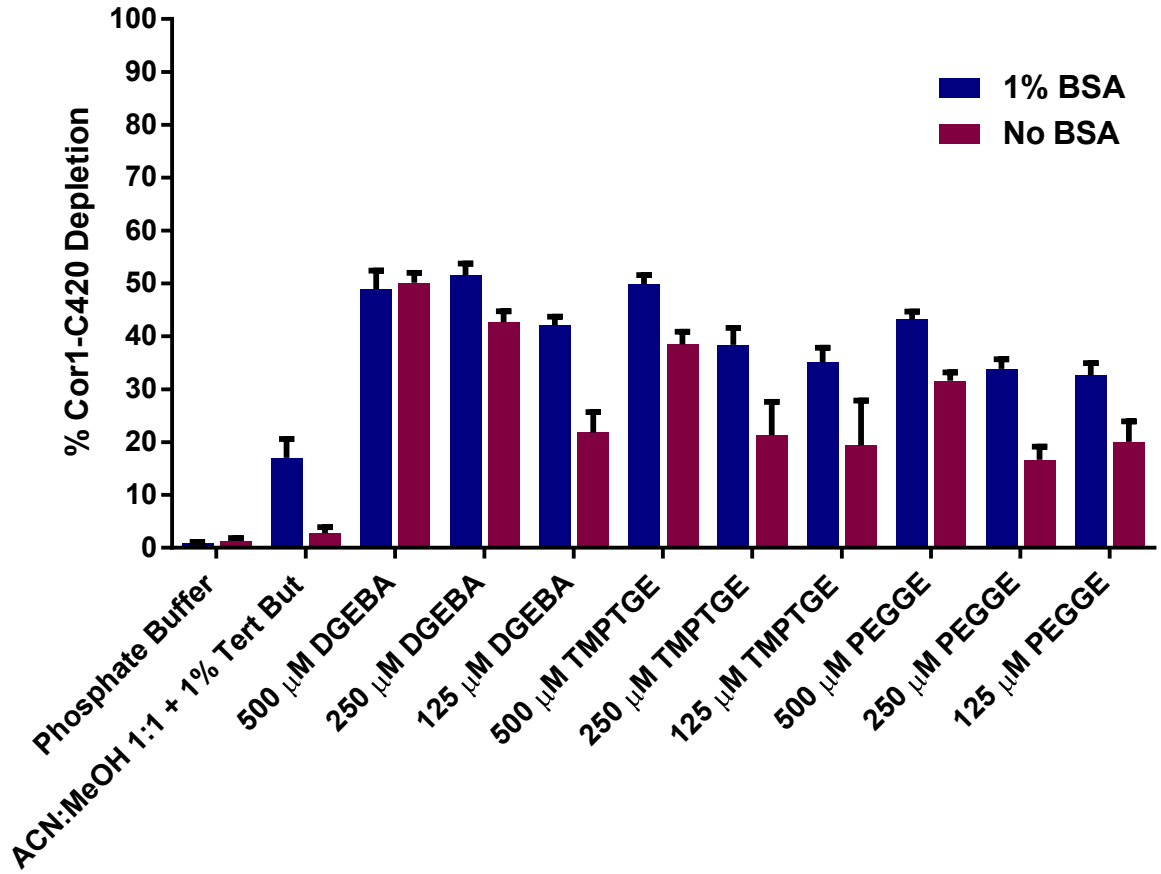


Figure 4-8: Effect of addition of 1% BSA to the DPRA incubation mixture on total depletion of the Cor1-C420 heptapeptide.

4.4. Discussion

My findings described in this chapter show that the h-CLAT correctly identified the sensitising capacity of four out five epoxy resins compounds based upon the extent to which the release of the cytokines, IL-6 and IL-8, were increased in cultured THP-1 cell supernatant. By contrast, use of the h-CLAT in classical mode to quantify expression levels of the cell surface markers, CD54 and CD86, failed to correctly classify the five epoxy resin compounds evaluated.

In this chapter, my DPRA data generated using the optimised experimental conditions developed in Chapter 3, correctly identified the sensitising potential of 16/19 chemicals tested, thereby demonstrating that conversion of the DPRA to a 96-well format to increase assay throughput, did not adversely affect assay accuracy. Additionally, my optimised DPRA method correctly classified the skin sensitising potential of three from five epoxy resin compounds using acetonitrile:methanol (1:1) with 1% *tert*-butanol as the test solvent. My novel findings collectively illustrate that the current h-CLAT and DPRA methods need to be adapted to enhance the accuracy of these methods when applied to assessment of the skin sensitisation potential of chemical classes such as epoxy resin compounds that differ markedly from the chemicals that were used to originally validate these methods.

The sensitising potency of the five representative epoxy resin compounds was assessed initially using information in the OECD QSAR toolbox that is drawn from existing experimental *in vivo* and *in vitro* datasets (Cronin, 2010). Using this toolbox, DGEBA gave an EC3 of 1.5% (moderate sensitiser) based upon published data (Gerberick et al., 2005). In a separate study, the EC3 value for DGEBA was estimated at 0.1% and hence it was classified as a strong sensitiser (Gamer et al., 2008). This classification discrepancy may potentially be underpinned by the difference in chemical grades of DGEBA used and/or dissolution solvents utilised as Gerberick et al. (2005) and Gamer et al. (2008) used AOO and acetone as solvents respectively. The solvent used in the LLNA may alter the solubility and/or the volatility of the test compounds as well as the penetration of the compound into the stratum corneum, thus affecting the potency classification of a chemical (Jowsey et al., 2008, Anderson et al., 2011).

Herein, DGEBA was classified as a moderate to strong sensitiser. On the other hand, TMPTGE and THETGE were predicted as weak sensitisers based upon QSAR read-across analysis of four structurally similar compounds whereas no prediction could be made for PEGGE and PPGE due to the lack of available information in the QSAR

databases. By comparison, TMPTGE was categorised as a moderate sensitiser based upon LLNA data (Gamer et al., 2008). In a retrospective analysis of 79 patients suspected of ACD who underwent patch-testing, only one patient reacted positively to TMPTGE (Aalto-Korte et al., 2015). This low incidence rate may reflect the fact that this epoxy resin diluent is not encountered as commonly as DEGBA. Nevertheless, this showed that the QSAR prediction of sensitisation potential for lesser studied epoxy resin components may not be accurate due to the sparse experimental information on their mode of action and/or on structurally similar compounds within the database. Of the 120 tested chemicals with established *in vivo* skin sensitisation data, the read-across algorithm in the OECD QSAR Toolbox had a predictive accuracy of 77% (Strickland et al., 2015).

Due to the lack of animal and human data on PEGGE and PPGE, and the inability of the QSAR method to predict their skin sensitisation potential, the LLNA was performed on these compounds, TMPTGE, whilst designated by QSAR as a weak sensitiser was also tested in the LLNA as TMPTGE was outside the prediction range for its log octanol-water partition coefficient (log K_{ow}) of 1 – 7 raising the likelihood that the QSAR prediction may not be accurate. As published LLNA data were available for the other two epoxy resin compounds, this work was not repeated so as to reduce the total number of animals used for testing in accordance with the 3Rs principles. PPGE was classified in the LLNA as a weak sensitiser with an EC3 value of 13.3% whereas both THETGE and PEGGE were non-sensitisers. The LLNA data for the five epoxy resin components either generated herein or from previously published work by others was used for comparison with my corresponding results generated using my optimised h-CLAT and DPRA methods.

Using my optimised 96-well plate-based DPRA method described in Chapter 3, a dataset of 19 chemicals with published skin sensitisation data were tested to assess the validity of my newly optimised method. All chemicals with the exception of benzocaine, α-hexylcinnamaldehyde and benzalkonium chloride were correctly identified using the Cor1-C420 and cysteine heptapeptides when compared with the mouse LLNA data (Table 4-7). For chemicals that appear to give incorrect results, care should be taken with respect to potential over-reliance on comparisons with the LLNA data. This is because although there is high concordance between the LLNA and human patch test data (Urbisch et al., 2015), assignation of the skin sensitising potential of a given chemical should include all available information including human patch test data including on structurally related compounds.

For example, for oxalic acid that was classified by the LLNA as a weak sensitiser, there are no reported cases affecting humans to date (Gerberick et al., 2007).

Using my optimised DPRA method (Chapter 3), there was no peptide depletion induced by the five representative epoxy resin compounds dissolved in acetonitrile, for all three heptapeptides utilised (Table 4-7). This result was surprising as DGEBA is one of the most common sensitising epoxy resin compounds identified, as it has tested positive in human patch testing of many individuals (Aalto-Korte et al., 2015), as well as being classified as a sensitiser when tested in both *in vivo* and *in vitro* testing (Gamer et al., 2008, Ponten et al., 2009, Majasuo et al., 2012, Natsch et al., 2013). In previous work by Natsch et al. (2013), DGEBA dissolved in acetonitrile depleted cysteine and lysine heptapeptides by 42.5% and 1.1% respectively. The major difference between my results for DGEBA and those of Natsch et al (2013) may be due to the technical modifications in my assay. For example, the final concentration of cysteine and Cor1-C420 heptapeptides within the individual wells of the 96-well plate DPRA method was lower than that used by others (Gerberick et al., 2007, Natsch and Gfeller, 2008). However, my DPRA reaction ratio (peptide:test chemical) at 1:10 for Cor1-C420 and cysteine heptapeptides, and 1:50 for the lysine heptapeptide were similar to those used previously by others (Gerberick et al., 2004, Gerberick et al., 2007, Natsch and Gfeller, 2008). Nevertheless, it is possible that the lower concentrations of both peptides and test chemicals used in my DPRA reactions may have decreased the overall sensitivity of the assay.

Another difference is that my assay was performed using a polypropylene 96-well plate instead of the borosilicate glass vials utilised by Gerberick et al. (2007) and Natsch and Gfeller (2008). My results described in Chapter 3 showed that neither polypropylene nor glass materials appeared to affect the extent of peptide depletion for either cysteine or Cor1-C420, when analysed after the 24 h incubation period. However, it is important to note that the effect of vessel materials on peptide depletion by chemicals was determined only with a small subset of chemicals that did not include epoxy resin compounds. For example, lysine heptapeptide depletion within the 24 h incubation period was consistent across borosilicate glass and polypropylene for all tested chemicals with the exception of ethyl acrylate which induced more pronounced peptide depletion in borosilicate glass (47%) than in polypropylene (25%) (Chapter 3). Further work to evaluate peptide depletion by epoxy resin compounds in the DPRA undertaken in polypropylene plates relative to borosilicate glass vials requires future investigation.

Three of the five epoxy resin compounds evaluated in my research had low aqueous solubility such that PEGGE, THETGE and PPGE precipitated out of solution when added to the DPRA reaction buffer. As such, a series of solvents of varying composition were assessed to ensure that that the most suitable solvent system was selected to optimise solubility equilibrium between the epoxy resin compounds and the assay reaction buffer. Of the five solvent systems tested, only acetonitrile:methanol (1:1) with 1% *tert*-butanol was acceptable for all five epoxy resin compounds as this solvent avoided the precipitation issue for the three aforementioned epoxy resin compounds. Most importantly the solvent comprising acetonitrile:methanol (1:1) with 1% *tert*-butanol alone, did not induce significant depletion of the heptapeptides. Using acetonitrile:methanol (1:1) with 1% *tert*-butanol as a solvent, Cor1-C420 peptide depletion by the epoxy resin compounds, DGEBA, TMPTGE, PPGE and PEGGE, was 40%, 37%, 34% and 29% respectively. Total depletion of THETGE was 12% and so it was classified as having low peptide reactivity. Interestingly, cysteine and lysine heptapeptide depletion with DGEBA using acetonitrile:methanol (1:1) with 1% *tert*-butanol as solvent were 44.6% and 1.7% respectively. These findings were similar to the findings of Natsch et al. (2013) whereby total cysteine and lysine heptapeptide depletion by DGEBA were 42.5% and 1.1% respectively.

Overall, the epoxy resin compounds assessed appeared to react primarily with the Cor1-C420 and/or cysteine heptapeptides, with limited or no reactivity with the lysine heptapeptide. This is likely due to the fact that epoxy resin compounds bind to the thiol side chain of Cor1-C420 and cysteine. More recently, DPRA analysis of several epoxy resin compounds with a hexapeptide, PHCKRM showed promising results such that several epoxy resin compounds covalently bound to the cysteine and proline residues of PHCKRM (Niklasson et al., 2009, O'Boyle et al., 2012, O'Boyle et al., 2014). However, the peptide reactivity assay using the PHCKRM hexapeptide may not be cost effective for large sample analysis or adapted to high-throughput as it requires a continuous stream of expensive argon gas to displace air from the reaction mixture to prevent oxidation of the DMSO used as the test chemical solvent in this assay. In the absence of argon gas, DMSO was found to react with the PHCKRM peptide leading to a false positive result (N O'Boyle 2014, pers. comm., 19 March) Further investigation in using other solvents in this test system may eliminate the requirement for argon gas. Nevertheless, these data highlight the importance of appropriate peptide selection for use in the DPRA such that the reactive peptide residues including cysteine (-SH), histidine (=N-), lysine ($-NH_2$), methionine (-S-), and tyrosine (-OH) are chemical-specific (Lepoittevin and Leblond, 1997,

Vocanson et al., 2009). Skin sensitisers that contain distinctive mechanistic domains can influence the type and strength of adducts formed for downstream processing which subsequently affects their allergenic potency (Chipinda et al., 2011a).

In my work herein, I have used three different heptapeptides to gauge their ability to accurately assess the skin sensitisation potential of epoxy resin compounds. I propose that chemicals which show reactivity to two out of three heptapeptides should be classified as positive skin sensitisers whereas those that show no/minimal reactivity for two out of three heptapeptides, should be categorised as non-sensitisers. Using this proposed classification approach, three of the epoxy resin compounds, DGEBA, TMPTGE and THETGE were accurately identified relative to the corresponding LLNA data. For the other two epoxy resin compounds (PEGGE and PPGE), one was a false positive and the other a false negative respectively (Table 4-11).

By comparison, my optimised h-CLAT method described in Chapter 2 performed poorly as all five epoxy resin compounds including the known sensitiser DGEBA, were classified as non-sensitisers as they failed to up-regulate cell surface expression levels of CD54 and CD86 above the OECD threshold criteria of RFI≥200 and RFI≥150 respectively (OECD, 2014). Conversely, in recent work by others, DGEBA was reported as a positive sensitiser based upon up-regulated expression of CD54 and CD86 in the h-CLAT (Takenouchi et al., 2013). It is noted however, that the CV75 for DGEBA used in my h-CLAT experiment was 15.4 µg/mL whereas that used by Takenouchi et al. (2013) was 36 µg/mL. Hence, this difference is a possible factor contributing to these between-laboratory differences. It is also of note that between-laboratory differences in the extent to which expression levels of CD54 and/or CD86 are up-regulated by the same chemical, e.g. DNCB, have been reported (Ashikaga et al., 2006, Jung et al., 2011).

To address the failure of the h-CLAT method to correctly classify the skin sensitisation potential of any of the five epoxy resin compounds evaluated, I then assessed the potential utility of increased secretion of the pro-inflammatory cytokines IL-6 and IL-8 by cultured THP-1 cells, as alternative assay endpoint measures. Using a >5-fold induction of pro-inflammatory cytokine secretion by a test chemical relative to vehicle as a positive assay readout, DGEBA, PEGGE, TMPTGE and PPGE were accurately identified whereas THETGE was a false positive (Table 4-11).

Although the generalisability of my results on epoxy resin compounds is limited by the relatively small number of compounds utilised, my findings nevertheless indicate that both

the h-CLAT and DPRA have the potential to be adapted to identify sensitisers amongst this chemical class. Clearly, future work on a broader range of epoxy resin compounds is needed to determine the appropriate assay endpoints and/or the technical specifications to optimise the predictive accuracy of h-CLAT and DPRA for this chemical class. Although false results were obtained for both the h-CLAT and DPRA, these instances appeared to be predominantly for compounds classified by the LLNA as weak or non-sensitisers. Of particular interest, the LLNA reportedly has poor predictive accuracy for detecting low to moderate sensitisers and therefore the LLNA data should be used prudently to avoid misclassification of sensitisers (ECVAM, 2013). Additionally, the predictive accuracy of the LLNA is only 82% compared with human data (Urbisch et al., 2015) showing that the LLNA does not always reflect the actual human response to skin allergens.

It is acknowledged that the limited number of epoxy resin compounds tested in my PhD research is insufficient to propose an integrative strategy for skin sensitisation potential of epoxy resin compounds. Nevertheless, my preliminary data provide insight into the considerations required in the application of *in vitro* methods to a chemical class dissimilar to those used to develop and validate the method in the first place. Future work directed to assess generalisability of the currently utilised in vitro methods of skin sensitisation assessment is an area of research endeavour that requires concerted attention.

4.5. Conclusion

ACD involves the activation of a series of complex signalling pathways in human skin. Cross-talk between these multiple pathways in ACD cannot be replaced by a single *in vitro* method. Hence, several non-animal testing methods directed at specific key events in the development of skin sensitisation have been developed and further optimised by my work described in this thesis. In this chapter, the feasibility of using the h-CLAT and DPRA methods for evaluating the skin sensitising potential of epoxy resin compounds was investigated. Using my optimised DPRA method and an alternative quantitative endpoint in the h-CLAT (cytokine secretion), the utility of these *in vitro* test methods for assessing the skin sensitising potential of epoxy resin compounds, was encouraging. However, future work involving assessment of a large number of epoxy resin compounds is required to more thoroughly evaluate these *in vitro* assays for screening the skin sensitising potential of epoxy resin compounds.

Table 4-11: Compilation of data from human, animal and *in vitro* methods for classifying representative skin sensitisers of interest.

Chemicals	Human Data	QSAR	LLNA	DPRA[24]				h-CLAT[25]				
								Expression		Fold increase		+/-[26]
				Cor1-C420	Cysteine	Lysine	+/-	CD54 (RFI≥200%)	CD86 (RFI≥150%)	IL-6 (≥5-fold)	IL-8 (≥5-fold)	
DNCB	Sensitiser	Strong sensitiser	Strong sensitiser	High reactivity	High reactivity	No/minimal reactivity	+	Yes	Yes	Yes	Yes	+
Methyl Salicylate	Non-sensitiser	Non-sensitiser	Non-sensitiser	No/minimal reactivity	No/minimal reactivity	No/minimal reactivity	-	No	No	No	No	-
DGEBA	Sensitiser	Moderate sensitiser	Moderate/ strong sensitiser	Moderate reactivity	Moderate reactivity	No/minimal reactivity	+	No	No	No	Yes	+
PEGGE	N/A[27]	N/A[9]	Non-sensitiser	Moderate reactivity	Moderate reactivity	Low reactivity	+	No	No	No	No	-
TMPTGE	Sensitiser	Weak sensitiser	Moderate sensitiser	Moderate reactivity	Moderate reactivity	Low reactivity	+	No	No	No	Yes	+
THETGE	N/A[9]	Weak sensitiser	Non-sensitiser	Low reactivity	No/minimal reactivity	No/minimal reactivity	-	No	No	Yes	Yes	+
PPGE	N/A[9]	N/A[9]	Weak sensitiser	Moderate reactivity	No/minimal reactivity	No/minimal reactivity	-	No	No	Yes	Yes	+

[24] DPRA: Chemical is classified as a sensitiser (+) if reactivity is observed in two out of three heptapeptides, otherwise is classified as non-sensitiser (-).
[25] h-CLAT: "Yes" represents the data above threshold value of CD54 and CD86 expression as well as cytokine fold-induction, otherwise categorised as "No".
[26] h-CLAT: Chemical is classified as a sensitiser (+) if 'yes' is observed in either CD54, Cd86, IL-6 or IL-8, otherwise is classified as non-sensitiser (-).
[27] N/A denotes that data is not available from the respective test.

Chapter 5: Summary, conclusions and future directions

5.1. Summary and conclusions

With the growth of global demand for epoxy resins, the existing composite resin systems do not meet current and forthcoming manufacturing requirements as they are hazardous to both the environment and public health. The prevalence of allergic contact dermatitis (ACD) in industrial workers dealing with epoxy resin systems (ERS) has increased considerably over the years and it is one of the most common occupational contact allergies reported (Niklasson et al., 2009). At present, the 'gold standard' method for assessing the skin sensitisation liability of chemical compounds is the mouse local lymph node assay (LLNA). This stand-alone *in vivo* method is accepted widely for identifying and classifying chemicals for their potency as skin sensitisers. In spite of this, ethical concerns have been raised on the use of animal testing for this purpose. To address these concerns, multiple validated non-animal test methods have been introduced for incorporation to identify and classify chemicals with skin sensitisation potential and for incorporation into risk assessments (Goebel et al., 2012).

The mechanism underpinning ACD has been divided into four key biological events that form the Adverse Outcome Pathway (AOP) for skin sensitisation (OECD, 2012a). These four mechanistic events comprise the binding of the sensitising chemical with skin proteins (haptenation), keratinocyte activation, dendritic cell (DC) activation and the proliferation of hapten-specific T-cells (OECD, 2012a). While many *in vitro* methods have considerable potential for identifying and classifying chemicals as skin sensitisers (Wong et al., 2015), a single *in vitro* method is inadequate for representing the complex interplay and cross-talk between mechanisms involved in the development of ACD in humans.

My PhD research was directed to bringing innovation into two of these *in vitro* methods, *viz* the direct peptide reactivity assay (DPRA) which represents the initial interaction between skin sensitisers and skin proteins, and the human cell line activation test (h-CLAT) that mimics the maturation of Langerhans cells (LCs) to DCs. The DPRA and h-CLAT together with the KeratinoSens^{TM} have been subjected to formal validation. Test guidelines for use of all three of these *in vitro* methods for assessment of skin sensitisation potential have been promulgated by the OECD (OECD, 2014, OECD, 2015a, OECD, 2015b). Recently, evaluation of the performance of a panel comprising all three of these methods for assessing the skin sensitisation potential of various test chemicals showed that it had an accuracy of 90% compared with the corresponding LLNA data (Urbisch et al., 2015). However, a significant limitation of these *in vitro* assays is that they were developed for

screening small molecules that are typically used in products manufactured by the cosmetics and toiletries industries. Hence, there are knowledge gaps with respect to their generalisability beyond small molecules. To date, reports on the applicability of the DPRA and the h-CLAT *in vitro* methods for assessing the skin sensitising potential of ERS, are limited. Hence, another major aim of my PhD research was to address this knowledge gap and assess the generalisability of the DPRA and the h-CLAT for evaluating the skin sensitisation potential of various components of ERS. As already noted, during the course of my PhD research, I brought innovation into these methods by improving and optimising their throughput and efficiency for the *in vitro* screening of large numbers of new chemical compounds. The data generated using my optimised h-CLAT and DPRA for assessing the skin sensitising potential of five representative epoxy resin compounds were compared with the corresponding *in vivo* LLNA data as well as both experimental and/or predicted data from the databases in the OECD QSAR toolbox.

With regard to bringing innovation into the h-CLAT method for assessing skin sensitisation potential of test chemicals that comprised the first aspect of my doctoral research, I re-designed and re-optimised this method to improve its throughput, efficiency and cost-effectiveness. I then used my re-configured h-CLAT method to evaluate its generalisability for hazard assessment of ERS chemical components. To increase the efficiency of the h-CLAT, I converted the assay from the typical 24-well plate format into a 96-well format. When performed in 24-well plate format, the h-CLAT typically has a total of $1x10^6$ THP-1 cells per treatment per well and it uses FITC-conjugated antibodies for quantification of both of the following cell surface markers, CD54 and CD86 (Ashikaga et al., 2006).

In my research aimed at improving the h-CLAT, I showed that the optimal number of THP-1 cells/well in 96-well format was $1.6x10^5$ and that the optimal antibody concentrations for targeting the CD54 and CD86 cell surface molecules were both 1/40 dilution of the manufacturer supplied antibody solutions (1.25 µL/$1.6x10^5$ cells/50µL). Additionally, I introduced use of FITC- and PE-labelled antibodies to facilitate simultaneous quantification of the cell surface expression levels of CD54 and CD86. Importantly, the positive control strong sensitiser, DNCB, showed induction of the cell surface expression levels of CD54 and CD86 above the relative fluorescence intensity (RFI) thresholds of 200% and 150% respectively, and the negative control, methyl salicylate, was accurately identified as a non-sensitiser. My results correspond with previously published data generated using both the LLNA and h-CLAT (Ashikaga et al., 2008). Taken together, my findings show that use of fewer THP-1 cells together with FITC-labelled antibodies for simultaneous quantification

of CD54 and CD86 expression levels to bring innovation into the h-CLAT by adapting it to 96-well plate format to improve efficiency and throughput, retained the ability of this method to discriminate between representative small molecules known to be strong and non-sensitisers.

My PhD research directed at evaluation of the generalisability of the conventional h-CLAT based upon up-regulation of CD54 and CD86 expression levels on cultured THP-1 cells, show that it is unsuitable for assessing the skin sensitisation potential of epoxy resin compounds. Specifically, of the five representative epoxy resin compounds assessed, the h-CLAT failed to identify those known to have skin sensitising capacity, *viz* DGEBA, TMPTGE, and PPGE, as expression levels of the THP-1 cell surface molecular markers, CD54 and CD86, were not up-regulated by these compounds.

Several other biomarkers have been proposed as possibilities for discriminating between sensitising and non-sensitising compounds. Quantitative and qualitative endpoints proposed include pro-inflammatory cytokine and chemokine (e.g. IL-1β, IL-18) readouts, activation of Keap1/Nrf2/ARE cell signalling pathways, as well as transcriptional profiles and protein expression encoded by genes expressed by various cell types relevant to skin sensitisation (Reisinger et al., 2015). These have been evaluated in preliminary work and appear to be promising (Reisinger et al., 2015). Hence, in the next part of my PhD research, I evaluated the extent to which there were changes in the secretion of pro-inflammatory cytokines by THP1-cells in the h-CLAT, to determine the feasibility of this approach for use as an h-CLAT assay endpoint for epoxy resin compounds.

Specifically, I used a Meso Scale^{TM} Discovery (MSD) system and highly-sensitive MSD human pro-inflammatory 7-plex tissue culture kits to quantify the concentrations of seven cytokines, namely IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α and IFN-γ, simultaneously, in aliquots of h-CLAT supernatant. Encouragingly, my findings showed for the first time that incubation of epoxy resin compounds with cultured THP-1 cells for 24 h increased the concentrations of IL-8 and IL-6 in the h-CLAT cell culture supernatant. Specifically, the epoxy resin compounds, DGEBA, TMPTGE, THETGE and PPGE, induced a marked increase in the release of IL-8 and to a lesser extent, IL-6, in aliquots of supernatant from cultured THP-1 cells in the h-CLAT. My findings are reminiscent of work by others, whereby incubation of cultured human osteoblastic (U2OS) cells with epoxy resin-based root canal sealants stimulated IL-8 and to a lesser extent IL-6 mRNA production by these cells (Huang et al., 2005).

Following incubation of cultured THP-1 cells in 96-well plate format with the strong skin sensitiser, DNCB for 24 h in my optimised h-CLAT method, there was a 410-fold increase in the IL-8 concentration in aliquots of cell culture supernatant whereas incubation with the non-sensitiser, methyl salicylate for 24 h, produced only low levels of IL-8 release from the cultured THP-1 cells. My findings are similar to those of a previous report whereby incubation of cultured THP-1 cells with DNCB but not methyl salicylate, increased the IL-8 concentration significantly ($P<0.01$) in supernatant aliquots (Miyazawa et al., 2007, Nukada et al., 2008). For the epoxy resins tested, IL-8 release into the THP-1 cell culture medium was stimulated by the epoxy resin compounds, DGEBA, TMPTGE and PPGE, that were classified by LLNA data as strong, moderate and weak sensitisers respectively (Gamer et al., 2008). However, future investigation on the specificity of elevated IL-8 secretion by cultured THP-1 cells incubated with various epoxy resin compounds is required, as THETGE, classified as a non-sensitiser by my LLNA data, also increased the secretion of IL-8 by THP-1 cells. However, it is also plausible that the LLNA data may be inaccurate in this instance as it has been documented that the LLNA data accuracy relative to human data is only 82% (N=111) (Urbisch et al., 2015).

With regard to my findings on IL-6, incubation of cultured THP-1 cells in my optimised h-CLAT method in 96-well plate format with the strong sensitiser, DNCB for 24 h, increased the IL-6 concentration in aliquots of cell culture supernatant in contrast to previous work by others who reported that the IL-6 concentration was not increased in the supernatant of similarly treated cultured THP-1 cells (Miyazawa et al., 2007). However, these apparently opposing findings likely reflect the fact that I used a highly sensitive MSD 7-plex cytokine assay for quantification of IL-6 whereas Miyazawa et al. (2007) used an ELISA method. The MSD 7-plex cytokine immunoassay has a larger dynamic range for quantification of IL-6 concentrations in the range ~0.3 pg/mL to 3.2 µg/mL whereas the LLOQ for the ELISA used by Miyazawa et al. (2007) was 3.2 µg/mL. Interestingly, increased concentrations of IL-6 in aliquots of supernatant from cultured THP-1 cells in my optimised h-CLAT method were only observed after incubation of cells with THETGE and PPGE for 24 h.

Encouragingly, the cytokine induction profiles (Chapter 4; Table 4-11), showed that four of five tested epoxy resin compounds were accurately identified in my optimised h-CLAT method in 96-well plate format. Hence, future work aimed at characterising the cytokine induction profiles for a broad range of epoxy resin compounds as well as other chemical classes, is warranted to further assess the potential value of this method for accurate identification of epoxy resin components with skin sensitisation potential.

In my h-CLAT experiments described in Chapter 2, I also investigated the effects of the assay and storage conditions on the accuracy of the cytokine measurements. Cytokine instability in various sample matrices under conditions of high temperature and/or multiple freeze-thaw events has been reported previously (Ozbey et al., 2014, Zhou et al., 2010). Hence, it was imperative to assess the stability of the four cytokines of interest, *viz* IL-1β, IL-6, IL-8 and IL-10 using known concentrations of cytokines (QCs) added to aliquots of cell culture medium incubated at 37°C for 24 h in order to gauge the accuracy of the data generated in my h-CLAT experiments. Additionally, I assessed the impact of four freeze (-80°C)-thaw cycles on cytokine stability in cell culture medium. Importantly, my data show that the concentrations of IL-6, IL-8 and IL-10, but not IL-1β, in aliquots of cell culture medium remained stable following incubation at 37°C for 24 h. Additionally, following four freeze-thaw cycles, the concentrations of IL-1β, IL-8 and IL-10 remained unchanged whereas that of IL-6 decreased markedly, particularly at the lower QC concentrations. This information is key to affirming the accuracy of the large increases in the IL-8 concentrations measured in aliquots of supernatant from cultured THP-1 cells incubated with skin sensitisers at 37°C for 24 h in the h-CLAT and then stored at -80°C prior to analysis. These data on cytokine integrity under usual assay and sample storage conditions underpin evaluation of the usefulness of increased cytokine concentrations in the h-CLAT as a potential biomarker for determining skin sensitisation potency.

In work by others, investigation of the temporal profile of TNF-α secreted into cell culture medium by THP-1 cells incubated with each of nickel sulphate and DNCB, showed that the maximum TNF-α concentration was found at 12 h with a lower concentration observed at 24 h of incubation, in contrast to IL-8 (Miyazawa et al., 2008b). This apparent decrease in the TNF-α concentration in aliquots of cell culture supernatant, may reflect instability of this cytokine under the assay conditions at 37°C; however this remains for future investigation.

In Chapter 3, I used a comprehensive and systematic approach to identify the optimal experimental conditions for conducting the DPRA in 96-well plate format with LC-MS/MS quantification of the extent of peptide depletion. My findings on the effects of varying the incubation temperature employed in the DPRA, mimicking the various temperatures used by laboratories globally, on the stability of the heptapeptides, are novel. Natsch and Gfeller (2008) used 37°C for incubating various test chemicals with the Cor1-C420 heptapeptide (Ac-NKKCDLF), whereas Gerberick et al. (2007) and the OECD guideline, TG442C (OECD, 2015a) recommend an incubation temperature of 25°C for test chemicals with the cysteine (Ac-RFAACAA) and lysine heptapeptides (Ac-RFAAKAA) with an incubation

period of 24 h. Herein, I found that 25°C was the optimal incubation temperature between heptapeptides and test chemicals as there was minimal impact on the concentrations of these three-heptapeptides.

Next, I investigated the effect of different types of vial materials used for the DPRA incubation step as work by others had shown this to be an important consideration with regard to apparent heptapeptide depletion through adsorptive losses onto surfaces (Goebel-Stengel et al., 2011). In overview, my findings showed that both polypropylene and borosilicate glass vials were suitable for use in the DPRA provided that the peptide: test chemical reactivity assessment was performed within 24 h after incubation at 25°C. Adsorptive losses of Cor1-C420 and cysteine heptapeptides appeared to be minimal for polypropylene vials compared with borosilicate glass such that the QCs of each peptide failed to meet the acceptance criteria when samples were stored in glass vials in an autosampler at 4°C for periods of 24 and 72 h. Conversely, neither polypropylene nor glass materials appeared to be associated with adsorptive losses of the lysine heptapeptide during the incubation step, as there were no significant differences in QC sample concentrations after storage in an autosampler at 4°C for periods of up to 3 days.

Overall, my novel findings show that polypropylene vials are preferable to glass vials in terms of minimising adsorptive losses of these three peptides in the DPRA, and this is particularly so if the DPRA analysis is extended for periods longer than 24 h. This major finding has important implications if the DPRA is to be for extended periods of time in high-throughput format in the future.

In the next series of experiments, I investigated the feasibility of converting the DPRA to a higher throughput assay by analysing a large number of samples in polypropylene 96-well plates as a single analytical experiment as a means to assess the extent to which sample integrity was affected. To that end, the stability of the peptide-chemical complex in the autosampler at 4°C was investigated by measuring the concentration of free heptapeptides in the reaction buffer over 3 days post-chemical incubation at 25°C. My data showed that total depletion of the Cor1-C420 heptapeptide following reaction with each of DNCB and cinnamaldehyde appeared to decrease over the 3-day assessment period, characterised by an increase in free peptide concentration. Conversely, following incubation of the Cor1-C420 heptapeptide with isoeugenol or methyl salicylate, there was marked increase in apparent peptide depletion over a 3-day period post-incubation. The extent of change in peptide depletion for DNCB and isoeugenol was relatively small such that the classification

of these two chemicals with respect to skin sensitisation capacity remained unchanged. However, for cinnamaldehyde and methyl salicylate, the marked changes in apparent peptide depletion over a 3-day period post-incubation led to eventual misclassification of the sensitising reactivity of both chemicals.

Similarly for the cysteine heptapeptide incubated with cinnamaldehyde, there was an apparent decrease in peptide depletion over the 3-day period post-incubation such that it was initially categorised as having moderate reactivity but after 3-days it was mis-classified as having low reactivity. The extent of lysine peptide depletion over the 3-day post-incubation assessment period remained unchanged for glutaraldehyde, ethyl acrylate and methyl salicylate but not cinnamaldehyde, for reactions carried out in polypropylene vessels.

Comparison of the extent of lysine heptapeptide depletion by ethyl acrylate for reactions conducted in borosilicate glass and polypropylene materials showed that it was 47.3% and 24.7% at 24 h post-incubation. This difference was not due to adsorptive losses of peptide as the lysine heptapeptide QCs with known concentrations remained unchanged when stored in vessels comprised of either type of material, for up to 3 days. However, in work by others, the extent of lysine depletion by ethyl acrylate reported by others were 2.1% and 93.7% (Gerberick et al., 2007, Troutman et al., 2011), results that would misclassify ethyl acrylate as either a no/minimal sensitiser or a strong sensitiser, respectively. My total lysine depletion results determined using 96-well polypropylene plates are aligned with the classification of ethyl acrylate as weak sensitiser by the LLNA (Gerberick et al., 2005).

Overall, my DPRA data suggest that the stability of the covalent bonds formed between the test chemical and the heptapeptide of interest, appears to be dependent upon both the type of chemical being assessed as well as the heptapeptide utilised. Although the number of test chemicals assessed was small, my data provide novel insight showing that use of the DPRA for screening a large number of chemicals in a single experiment with autosampler storage extending over several days post-chemical/peptide incubation, is unwise. This is because of the potential instability of test chemical-peptide complexes such that the apparent heptapeptide concentration may change markedly, resulting in chemical misclassification.

After successfully converting the DPRA to 96-well plate format and re-optimising the assay parameters in my research described in Chapter 3, I next examined its accuracy for correctly classifying 19 chemicals against their corresponding LLNA skin sensitisation data

(Chapter 4). Overall, my findings were similar to previously published data whereby DNCB reacted with the thiol group of the cysteine and Cor1-C420 peptides but not the amine group of the lysine heptapeptide (Natsch, 2010, Natsch and Gfeller, 2008). Conversely, there was no/minimal peptide reactivity for all three heptapeptides with salicylic acid, a finding in agreement with previously published data (Gerberick et al., 2007, Natsch and Gfeller, 2008). Importantly, there was no/minimal reactivity of the Cor1-C420 heptapeptide with α-hexylcinnamaldehyde (2.3% depletion) in contrast to the 93% of Cor1-C420 depletion reported by Natsch and Gfeller (2008). My DPRA data more accurately reflects the LLNA classification of α-hexylcinnamaldehyde as a weak sensitiser.

Next, I assessed the applicability of my optimised DPRA for assessing skin sensitisation potential of five representative epoxy resin compounds. Specifically, PEGGE and TMPTGE depleted all three heptapeptides. Somewhat surprisingly, DGEBA had no/minimal reactivity for all three tested heptapeptides whereas it was classified by the LLNA as a moderate to strong sensitiser and it is reportedly the most common sensitising chemical based on human data (Aalto-Korte et al., 2015, Gamer et al., 2008). Poor aqueous solubility of compounds such as epoxy resins can pose a major challenge in *in vitro* assays. To address this solubility issue, I evaluated a range of solvents and solvent mixtures regarding their ability to successfully dissolve epoxy resin compounds without adversely affecting DPRA accuracy. Herein, I showed for the first time that a solvent mixture comprising methanol:acetonitrile 1:1 containing 1% *tert*-butanol, was effective in solubilising all five epoxy resin compounds following addition of suitable aliquots to the DPRA reaction buffer. Importantly, this solvent mixture alone had minimal effect on peptide depletion for all three heptapeptides. Using my optimised solvent system for dissolution of all five tested epoxy resin compounds and the Cor1-C420 heptapeptide in the DPRA, the epoxy resin compounds, DGEBA, TMPTGE, PPGE and PEGGE were all classified as having moderate reactivity whereas THETGE was classified as having low reactivity. For DGEBA, my findings were total cysteine depletion of 44.6% and total lysine depletion of 1.7% using methanol:acetonitrile 1:1 containing 1% *tert*-butanol as solvent, in a manner similar to a recent report by Natsch et al. (2013).

Skin sensitisation in humans involves cross-talk between multiple pathways and so this complexity ultimately limits the accuracy of any single *in vitro* test to identify skin allergens. Hence, in the last part of my PhD research, I evaluated the predictive capacity of my optimised h-CLAT and DPRA methods relative to the corresponding data generated using the *in vivo* LLNA test (Chapter 4; Table 4-11). In the spirit of the 3Rs, I performed the ‘gold

standard' mouse LLNA only for the epoxy resin compounds of interest where there were no previously published LLNA data or it was not possible to make accurate predictions using the information in the OECD QSAR skin sensitiser databases. Using the LLNA, I showed for the first time that the skin sensitising potency of PPGE is that of weak sensitiser with an EC3 value of 13.3% whereas PEGGE and THETGE were categorised as non-sensitisers.

Based upon the afore-mentioned LLNA data, my optimised h-CLAT method showed that it accurately classified four out of the five epoxy resin compounds assessed whereas the and DPRA correctly classified three of five epoxy resins compounds relative to the corresponding LLNA data. Despite the small number of epoxy resin compounds assessed, my findings suggest that both the h-CLAT and the DPRA have the potential to be adapted to successfully identify skin sensitisers amongst epoxy resin compounds. At this early stage, it is not possible to identify the ideal strategy for *in vitro* evaluation of the sensitising potential of epoxy resin compounds. Nonetheless, adaptation of the DPRA and h-CLAT methods holds promise in hazard assessment.

In summary, during my doctoral research program, I have successfully adapted and optimised the DPRA and h-CLAT *in vitro* methods for assessing the sensitising capacity of industrial chemicals, primarily epoxy resin compounds, that to date have not been widely assessed using *in vitro* test methods (Wong et al., 2015). Additionally, my findings showed for the first time the use of a solvent mixture comprising acetonitrile:methanol with addition of 1% *tert*-butanol improved the solubility of epoxy resin compounds and the capacity of the DPRA for correctly classifying the sensitising potency of these compounds. Also, my h-CLAT data show that cultured THP-1 cells incubated with epoxy resin compounds induced increased secretion of IL-8 and IL-6 by THP-1 cells into the culture media, raising the possibility that pro-inflammatory cytokines such as IL-8 may have potential as a quantitative endpoint for assessing skin sensitisation potency. My data on the sensitisation potential of the three epoxy resin compounds, PEGGE, THETGE and PPGE as determined by LLNA, are novel and they will extend the LLNA database within the OECD QSAR toobox. It is acknowledged that there are many challenges that remain to be addressed in future work beyond the scope of that encompassed by my PhD research program that was focused on bringing innovation into both the DPRA and h-CLAT methods and then applying these methods to a representative set of five epoxy resin compounds.

5.2. Future directions

- My PhD research findings show that the optimised h-CLAT and DPRA methods that I developed hold considerable promise for assessing the skin sensitisation potential of epoxy resin compounds. However, a large number of epoxy resin compounds need to be assessed in the future before conclusions can be drawn with confidence on the accuracy of these methods for correctly classifying epoxy resin compounds.

- My findings also showed that the stability of the covalent bond formed in the peptide-test chemical complex in the DPRA appears to be dependent upon the chemical class being assessed as well as the heptapeptides utilised. Further investigations are warranted to examine the stability of the covalent bond in peptide-chemical complexes by monitoring adduct formation (and their reversal) between the heptapeptide of interest and a range of test chemicals, using MS/MS. Such information will be very informative on the maximum number of samples that can be analysed in a single assay and the possibility of adapting the DPRA to high-throughput assay.

- Previous work by others have incorporated the use of enzymes such as horseradish peroxidise (HRP) alone and in combination with hydrogen peroxide (HRP/P) (Gerberick et al., 2009), or aroclor-induced rat liver microsomes (S9) (Chipinda et al., 2011b), and skin-like recombinant human cytochrome P450 cocktails (Bergström et al., 2007) for assessing the skin sensitisation potential of chemical compounds that become sensitisers after metabolic activation (i.e. prohaptens and/or prehaptens). This approach improves the identification of potential skin sensitisers. In my DPRA work PPGE gave a false negative result. Hence, it is possible that it may require metabolic activation before becoming a sensitiser. Therefore, this requires future investigation.

- My present findings showed that the pro-inflammatory cytokine, IL-1β, was unstable in cell culture at 37°C for a period of 24 h. Hence, future work aimed at defining the time-dependent kinetic profiles of cytokines released from cultured THP-1 cells incubated with various chemical compounds for 24 h in the h-CLAT, will be an invaluable contribution to the field.

- In addition, the incorporation of a flow through device such as a transwell cell culture system may improve the throughput of cytokine sampling in my h-CLAT. Future work is warranted to optimise and assess the feasibility of using the above device in h-CLAT.

- Recently, Urbisch et al. (2015) used a 'two out of three' prediction model of skin sensitisation whereby the combination of the DPRA, h-CLAT and KeratinoSens™ assays (another *in vitro* method as outlined in Chapter 1 Section 1.8.2.3) increased the accuracy of skin sensitiser identification to 90% (N=101) and 82% when compared with known human and LLNA data respectively. Previous work showed that the KeratinoSens™ assay accurately identified DGEBA as a skin sensitiser (Natsch et al., 2013). Hence, future work aimed at assessing the accuracy of a panel of *in vitro* assays comprising my optimised DPRA and h-CLAT methods as well as the KeratinoSens™ assay, for assessing the skin sensitising potential of epoxy resin compounds, has considerable potential.

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Appendices

frontiers in Pharmacology

REVIEW
published: 05 May 2015
doi: 10.3389/fphar.2015.00094

In vitro methods for hazard assessment of industrial chemicals – opportunities and challenges

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Allergic contact dermatitis (ACD) is a delayed-type hypersensitivity immune reaction mediated by T-lymphocytes as a result of repeated exposure of an allergen primarily on skin. ACD accounts for up to 95% of occupational skin diseases, with epoxy resins implicated as one of the most common causes of ACD. Efficient high-throughput *in vitro* screening for accurate identification of compounds and materials that may pose hazardous risks in the workplace is crucial. At present, the murine local lymph node assay is the 'method of choice' for predicting the sensitizing potency of contact allergens. As the 3Rs principles of reduction, refinement, and replacement in animal testing has gained political and economic momentum, several *in vitro* screening methods have been developed for identifying potential contact allergens. To date, these latter methods have been utilized primarily to assess the skin sensitizing potential of the chemical components of cosmetic products with scant research attention as to the applicability of these methods to industrial chemicals, particularly epoxy resins. Herein we review the currently utilized *in vitro* methods and identify the knowledge gaps with regard to assessing the generalizability of *in vitro* screening methods for assessing the skin sensitizing potential of industrial chemicals.

Keywords: allergic contact dermatitis, epoxy resins, *in vitro* methods, skin sensitization, integrated hazard classification

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Predictive Toxicology, a section of the journal Frontiers in Pharmacology

Received: *19 February 2015*
Accepted: *16 April 2015*
Published: *05 May 2015*

Citation:
Wong CL, Ghassabian S, Smith MT and Lam A-L (2015) In vitro methods for hazard assessment of industrial chemicals – opportunities and challenges. Front. Pharmacol. 6:94. doi: 10.3389/fphar.2015.00094

Introduction

Occupational skin diseases (OSDs) are a significant public health concern both in terms of employee pain and suffering as well as socioeconomic burden. In 2012 for the U.S. alone, the estimated annual direct and indirect costs of OSDs exceeded USD1 billion per annum (Lushniak, 2004; Cashman et al., 2012). Additionally, the cost of dermatological treatments is forecast to reach USD18.5 billion per annum by 2018 (Evers, 2013). These high socioeconomic costs have provided the impetus for development of efficient *in vitro* screening methods for accurately identifying chemicals with high skin sensitization risk so that their industrial use can be avoided, thereby reducing OSDs. One of the most commonly reported OSDs is contact dermatitis, which accounts for up to 95% of occupation-related skin diseases (Lushniak, 2000) in the areas of medicine, beauty products, manufacturing, and the construction industries (Gimenez-Arnau, 2011; Lowney and Bourke, 2011; Sosted, 2011).

Contact dermatitis is an inflammatory skin reaction resulting from direct contact with foreign substances, mainly affecting exposed skin areas such as the hands, arms, legs, and

face (Belsito, 2005). Contact dermatitis can be classified into irritant contact dermatitis and allergic contact dermatitis (ACD). In this review, we address (i) ACD and its associated contact allergens, with particular attention on epoxy resins and their constituents and (ii) *in vitro* methods that may be used for risk assessment of ACD.

Allergic contact dermatitis is a type IV delayed hypersensitivity cutaneous immune reaction that is mediated by T-lymphocytes, and which occurs upon repeated skin exposure to contact allergens (Kimber et al., 2002a). Briefly, ACD develops in two stages, the sensitization phase and the elicitation phase (**Figure 1**; Toebak et al., 2009; Kimber et al., 2011). During the sensitization phase, contact allergens/haptens initially come into contact with the stratum corneum, the outermost layer of the skin and subsequently gain access to the body system through the viable epidermis. The invasion of haptens triggers the local release of proinflammatory molecules which subsequently induce the binding of haptens to skin proteins (Kimber et al., 2002a). The release of proinflammatory molecules also stimulates the disentanglement and subsequent migration of Langerhans cells (LCs) from the surrounding keratinocytes toward the hapten–protein complex (Schwarzenberger and Udey, 1996). The hapten–protein complex binds to the major histocompatibility complex (MHC) on LCs and is then transported into the lymph nodes via the afferent lymphatics (Toebak et al., 2009). During the transitory migration to the lymph nodes, the activated LCs differentiate into mature antigen presenting cells (APCs) resulting in morphological changes such as the loss of endocytic/phagocytic receptors and the upregulation of co-stimulatory molecules and MHC molecules (Toebak et al., 2009). The hapten–protein complex is presented by the APCs to the naïve hapten-responsive T-cells, followed by selective clonal expansion of effector and memory T-cells. The proliferated population of primed antigen-specific T-cells is then disseminated into the blood circulation resulting in the sensitization of an individual (Kimber et al., 2011). Elicitation is triggered when the haptens interact with either the same or a different skin site (Kimber et al., 2011). Upon re-exposure, epidermal cells release a cocktail of proinflammatory cytokines and chemokines which draw the hapten-specific T-cells from the peripheral circulation into the epidermal layer (Kimber et al., 2011). The infiltrating T-cells produce pro-inflammatory cytokines which in turn trigger the secretion of chemokines by keratinocytes, resulting in increased infiltration of T-cells from blood vessels into the epidermis leading to the development of ACD (Basketter and Maxwell, 2007; Toebak et al., 2009).

To date, more than 4000 chemical substances are linked to induction of ACD in humans (Cahill et al., 2012). The 18-year

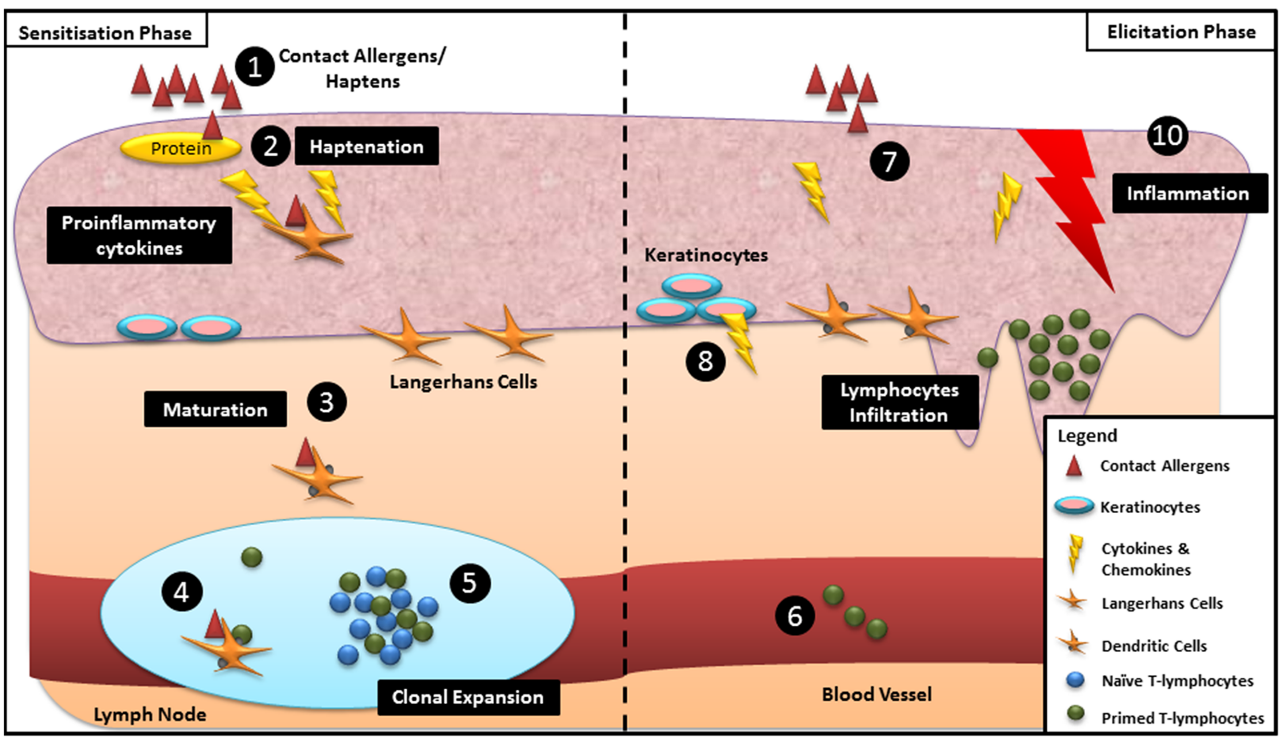

FIGURE 1 | Schematic overview of the mechanisms underpinning skin sensitization during sensitization and elicitation phases: (1) Haptens gain access through the viable epidermis. (2) Binding of haptens and skin proteins. (3) Langerhans cells (LCs) bind to the hapten–protein complex and differentiate into matured dendritic cells (DCs) during migration to the lymph node. (4) LCs present haptenated protein to the naïve T-lymphocytes. (5) Clonal expansion of specific effector and memory T-cells. (6) Proliferated T-lymphocytes disseminate into the blood circulation resulting in sensitization of an individual. (7) Re-exposure of similar haptens to the same individual. (8) Release of proinflammatory cytokines and chemokines by epidermal cells. (9) Infiltration of T-cells from blood vessels into the site of contact. (10) Development of allergic contact dermatitis (ACD).

retrospective analysis of ACD patients identified a number of frequently defined contact allergens, some of which have been summarized in **Table 1** (Cahill et al., 2012). These chemicals have been compiled in several human patch tests series including the North American Series, the European Baseline Series, the International Standard Series and the Thin-layer Rapid Use Epicutaneous Tests (TRUE). In brief, these series identify chemical substances commonly implicated in the population of a given geographical area, to cause ACD (Spiewak, 2008).

Epoxy resin-induced ACD was first reported in the 1950s, a time when there was extensive development of epoxy resin systems (ERSs) in industry (Broughton, 1965). In general, an ERS is comprised of an epoxy resin, hardener, reactive diluent, or other additives such as solvents, modifiers and fillers which together control the chemical and physical properties of the ERS (Geraut et al., 2009; Nixon et al., 2012).

Epoxy resin system components are the third most common allergen types for occupational ACD after chromates and rubber allergens, with ERS the primary source of ACD in the plastics manufacturing industry (Geraut et al., 2009). The prevalence of ERS-induced ACD by country is summarized in **Table 2**.

It was estimated that for individuals with ERS-associated ACD, ∼60–80% were sensitized to diglycidyl ether bisphenol A (DGEBA), an ERS that is widely used in industry (Björkner et al., 2011). This high prevalence resulted in the inclusion of DGEBA in the human patch test series since the 1960s (Geraut et al., 2009). Other epoxy resins including diglycidyl ether bisphenol F (DGEBF) and tetraglycidylmethylenedianiline, are also associated with induction of ACD (Geraut et al., 2009; Nixon et al., 2012).

Apart from epoxy resins, epoxy hardeners, predominantly polyamine compounds such as triethylenetetramine (TETA) and diethylenetriamine (DETA), as well as reactive epoxy diluents (e.g., phenyl glycidyl ether and *p*-tert-butylphenyl glycidyl; Geier et al., 2004), also cause ACD. A retrospective analysis of the records of 182 patients with ACD induced by epoxy resins over a 22-year period showed that 23.6% had developed an allergic response to epoxy hardeners (Jolanki et al., 2001). In a prospective study involving 92 individuals with suspected and/or prior exposure to ERS, patch tests showed that they were responsive to the epoxy diluents, 1,6-hexanediol diglycidyl ether (19.5%), and 1,4-butanediol diglycidyl ether (18.5%; Geier et al., 2004), highlighting cross-reactivity between epoxy compounds for induction of ACD in humans.

Although the high propensity of ERS to induce ACD is known, they are nevertheless used widely in commercial thermosetting products due to their strong adhesive bonding properties between different surfaces while exhibiting excellent resistance in harsh chemical and environmental conditions (Cahill et al., 2012). Worldwide demand for epoxy resins is forecast to reach ∼3 million tons by the end of 2017, with an estimated value of USD9.2 billion per annum (GIA forecasts the global market, 2012; Markets and Markets, 2014). The high global demand for epoxy resins is due to their ever increasing utility in a wide range of industrial applications including automotive coatings, electronic coatings, construction and adhesive products (Dietrich and Mirasol, 2012; GIA forecasts the global market, 2012). At present, research on assessment of the generalizability of *in vitro* tests developed for identifying the skin sensitizing potential of small molecules used in the toiletries and cosmetics industries, to that of epoxy resins and their components, is limited. Hence, this knowledge gap needs to be addressed. Herein, we review recent developments in non-animal tests for screening industrial chemicals for skin sensitization potential, with particular attention to the applicability of these tests to the epoxy resin chemical class.

TABLE 1 | Common allergens and sources of exposure.

Allergens	Source
Epoxy resin system (ERS)	Adhesives, paints
Formaldehyde	Pesticides, home cleansers
Fragrance mix	Toiletries, cosmetics
Neomycin sulfate	Creams, deodorants
Nickel sulfate	Costume jewelry, tools

Contact Allergens Screening Approaches

Development of the first animal models more than 80 years ago, to substitute for human skin patch testing of chemical compounds as potential contact allergens, was a landmark in terms of minimizing if not altogether avoiding the need for human testing (Landsteiner and Jacobs, 1935). About 40 years later, animal

TABLE 2 | Reported prevalence of occupational allergic contact dermatitis (ACD) due to epoxy resin systems (ERS).

Study period	Country	Study population (number of individuals)	Prevalence of ERS-induced ACD (%)	Reference
1993–2002	Australia	1354	3.0	Cahill et al. (2005)
1996–2006	North America	2540	0.9	Amado and Taylor (2008)
1997–2001	Norway	2336	1.0	Romyhr et al. (2006)
1999–2008	Portugal	2440	0.6	Canelas et al. (2010)
2001–2010	Denmark	219	8.2	Mose et al. (2012)
2001–2006	China	1354	8.5	Cheng et al. (2011)
2005–2009	Denmark	20 808	1.3	Bangsgaard et al. (2012)
2006–2008	Lithuania	816	1.5	Beliauskiene et al. (2011)

models were introduced for assessing the sensitizing capacity of ERS (Thorgeirsson and Fregert, 1977; Thorgeirsson, 1978; Gamer et al., 2008; Ponten et al., 2009). At present, the murine local lymph node assay (LLNA) is the 'gold standard' for assessing the skin sensitization potential of contact allergens. However, the use of animals for this type of testing has provoked much ethical debate (Carlson et al., 2004; Basketter, 2009) and provided the impetus over the past decade for the development of *in vitro* methods to replace, reduce, and refine (3Rs) this type of animal testing (Flecknell, 2002). Although several of these non-animal testing methods are at the pre-validation stage, they have been used primarily to assess the skin sensitization potential of small molecules (molecular weights <500 Da) such as those used in the manufacture of cosmetic and toiletry products (Kaplan et al., 2012). However, their applicability for assessment of the skin sensitization potential of ERS is largely unexplored. Hence this knowledge gap needs to be addressed to enable the best method or combination of methods to be identified for the reliable assessment of the skin sensitization potential of epoxy resin compounds. In the following sections we review the current non-animal testing approaches that have been developed based upon key mechanistic events in the process of skin sensitization and address the limitations of these methods for assessing the skin sensitization potential of ERS.

In Chemico Assays: Peptide–Chemical Interactions

Epoxy resins and/or epoxy resin composite materials, in common with other classes of haptens, react with skin proteins. The hapten–protein complex is then internalized and processed by LCs (Aleksic et al., 2007). Protein modification, in a process known as haptenation, is a key step in the initiation of skin sensitization (Chipinda et al., 2011). Majority of contact allergens are electrophilic in nature, consisting of Michael acceptors, S_NAr and S_N2 electrophiles, Schiff base formers, or acylating agents, which underpin their ability to react with the nucleophilic amino acid residues of skin proteins (Chipinda et al., 2011; Lalko et al., 2012). For epoxy resins, the electrophilic epoxide groups react with the nucleophilic moieties of skin proteins via S_N1 or S_N2 type nucleophilic reactions (Obach and Kalgutkar, 2010).

This haptenation process is mimicked *in vitro* by the direct peptide reactivity assay (DPRA; **Figure 2A**) which assesses depletion of small proteins (peptides) secondary to their interaction with potential haptens (Gerberick et al., 2007). Briefly, in this model, synthetic peptides containing nucleophilic residues including cysteine or lysine are incubated with test chemicals at a pre-determined ratio for 24 h to allow the binding of the active side chain of the peptide to the hapten. Based upon the irreversible covalent bond formation that occurs between haptens and amino acid residues in proteins, the DPRA quantifies the amount of unbound (remaining) peptide in the reaction mixture using high performance liquid chromatography (HPLC). Subsequently, the quantification of the bound (depleted) peptides is determined as a measure of reactivity of the test chemical (Gerberick et al., 2004).

At present, the DPRA has been validated by the European Centre for the Validation of Alternative Methods (ECVAMs) for the assessment of contact allergens as a replacement for the *in vivo* LLNA (Troutman et al., 2011). A test guideline has been promulgated by the Organization for Economic Co-operation and Development (OECD) highlighting the generalizability of peptide reactivity with small molecules (OECD, 2015). However, the suitability of the DPRA test system for chemicals such as epoxy resins that contain an epoxide group remains to be assessed.

DPRA: Chemicals Tested to Date

Use of the DPRA to assess the ability of 82 compounds that are mainly used as ingredients in cosmetic and toiletry products, to deplete cysteine-, lysine-, and glutathione-based peptides, indicated a significant correlation between peptide depletion and their sensitizer potency as previously established from *in vivo* LLNA data (Gerberick et al., 2007).

Steps undertaken to improve the accuracy of the DPRA for identification of potential skin sensitizing chemicals have included incorporation of oxidizing agents such as horseradish peroxidase and hydrogen peroxide (HRP/P) as well as cytochrome P450 enzymes to metabolically activate unreactive haptens into their more reactive hapten form, a process that may take place in human skin *in vivo* (Bergström et al., 2007; Troutman et al., 2011). By incorporating HRP/P into the DPRA, 83% of 70 chemicals with known sensitizing potential were identified accurately as compared with the standard DPRA reported previously (89%; Troutman et al., 2011). This apparently reduced accuracy of the HRP/P-added DPRA analysis is misleading, however, as the initial chemical set used to evaluate the previous DPRA prediction model did not include pre-/pro-haptens (Gerberick et al., 2007).

More recent refinements aimed at increasing the robustness of the DPRA to identify skin sensitizing chemicals include using pH conditions that more closely mimic human skin pH and measurement of concomitant chemical-specific mass changes indicative of peptide adduct formation (Dietz et al., 2013). In other work, the rate constant for reactivity of various test chemicals with the DPRA peptide was determined to assess whether quantitative kinetic reactivity data generated by measuring cysteine depletion at multiple test chemical concentrations and at various incubation times, were correlated with their potency as sensitizers (Roberts and Natsch, 2009; Natsch et al., 2014). However, drawbacks of this approach are that chemical reactivity varies markedly between various functional groups and the reaction rate of test chemicals with the DPRA peptide may not be linearly related to their *in vivo* sensitization potency (Roberts and Natsch, 2009).

DPRA: Application to ERS

While cysteine and lysine are the most widely utilized peptides for the *in vitro* DPRA, other modified peptides have been investigated. More recently, the utility of the DPRA for classifying the sensitizing capacity of several epoxies including novel analogs of DGEBF and phenyl glycidyl ether (PGE), has been examined using a synthetic peptide, viz PHCKRM (Pro-His-Cys-Lys-Arg-Met). The extent of peptide (PHCKRM) depletion by six novel epoxy analogs and the parent epoxide, PGE, was correlated with

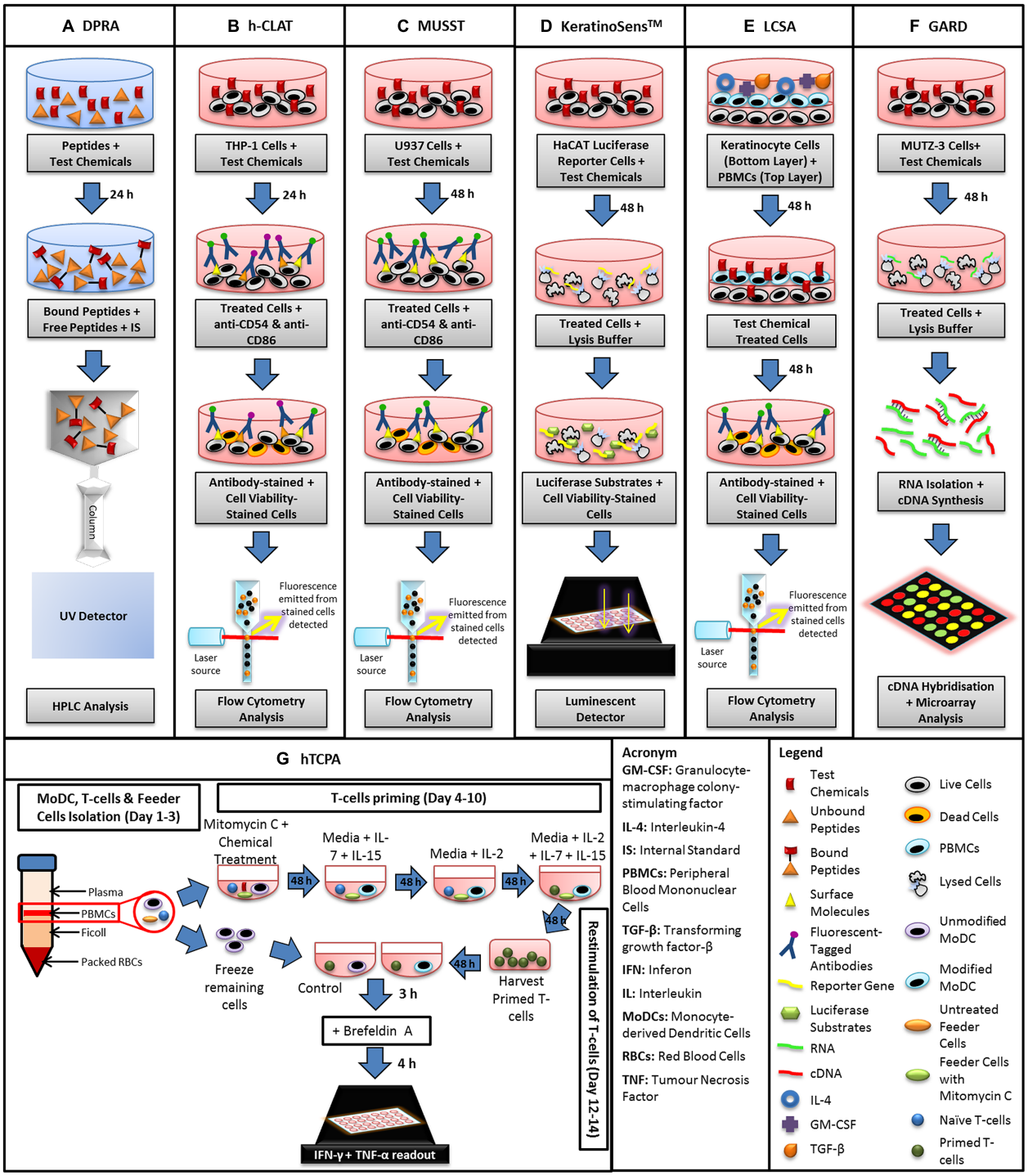

FIGURE 2 | Schematic diagram summarizing the steps involved in the conduct of *in vitro* assays currently available for assessment of skin sensitization potential. (A) Direct peptide reactivity assay (DPRA), **(B)** human cell line activation test (h-CLAT), **(C)** myeloid U937 skin sensitization test (MUSST), **(D)** KeratinoSens™, **(E)** loose-fit coculture-based sensitization assay (LCSA), **(F)** genomic allergen rapid detection (GARD), and **(G)** human T-cell priming assay (hTCPA).

the sensitizing potency of these epoxies determined using *in vivo* LLNA assessment (Niklasson et al., 2009). The strong sensitizer, PGE produced 88% peptide depletion whereas the weak epoxide sensitizers, butyl glycidyl ether, and butenyl glycidyl ether produced 46 and 54% peptide depletion, respectively (Niklasson et al., 2009). In a DPRA evaluation of DGEBF (containing two

epoxide groups) and two variants (Variant A and Variant B) using the same synthetic peptide (PHCKRM), the thiol (cysteine) binding of DGEBF and its variants appeared to be affected by the terminal epoxide groups (O'Boyle et al., 2012). Variant A (DGEBF without terminal epoxide groups) did not react with free thiols whereas variant B (DGEBF with one terminal epoxide group) did react with thiol groups albeit to a slightly lesser extent than the diepoxide DGEBF. Interestingly, the reaction rate for DGEBF that contains two terminal epoxide groups was slightly faster than that of variant B. These findings are aligned with the sensitizing capacity of DGEBF and its variants determined using the LLNA and the KeratinoSens^{TM} assay (O'Boyle et al., 2012).

To date, reports on the applicability of the incorporation of enzymes into the DPRA, as a means of bioactivation for assessing the skin sensitization potential of epoxy resins, are lacking. It is known that the enzyme, epoxide hydrolase, catalyzes the hydrolysis of epoxides to their respective dihydrodiol metabolites which react readily with skin proteins. Conversely, the enzyme, glutathione-*S*-transferase catalyzes the detoxification of epoxides by formation of glutathione conjugates (Obach and Kalgutkar, 2010). Hence, future investigation involving incorporation of epoxide hydrolase and/or glutathione-*S*-transferase into the DPRA for analysis of epoxy resin compounds is warranted, to more closely mimic possible bioactivation and deactivation processes within human skin that produce reactive electrophilic intermediates and detoxified species, respectively.

Issues relating to the poor aqueous solubility of industrial compounds that have high octanol/water partition coefficients, present another obstacle for use of DPRA to assess compounds such as epoxy resins. Although various solvents including dimethylsulfoxide (DMSO), methanol and acetonitrile have been used to dissolve lipophilic compounds, only small volumes of these solutions can be used due to their limited miscibility with an aqueous solution of the peptide to be depleted. To that extent, microemulsion systems have potential to improve miscibility between an organic solution of a lipophilic test compound and that of an aqueous peptide solution; preliminary data suggest that this approach is worthy of further investigation (Merckel et al., 2010).

Unacceptable modulation of the test systems by organic solvents limits the range of solvents that can be used for dissolution of epoxy resins. For example, organic solvents routinely used in laboratories inhibit cytochrome P450-mediated metabolic reactions, and may potentially fail to activate the enzyme-dependent sensitizing chemicals in the test system (Li et al., 2010; Troutman et al., 2011). DMSO is unsuitable for use in the DPRA as its high reactivity means that it may react with assay peptides resulting in false positive results. The use of DMSO in the DPRA would require an additional costly step of purging the reaction system with an inert gas such as argon, to prevent oxidation of DMSO (Niklasson et al., 2009).

In Vitro Assays: Cell-Based Models

Human LCs and dendritic cells (DCs) play key roles in skin sensitization (Coutant et al., 1999). Hence, there has been considerable research attention on development of *in vitro* systems that mimic the roles of LCs and DCs in skin sensitization. Initial *in vitro* assays using LCs/DCs were limited due to the scarcity of available LCs and inter-donor variability of DCs (Yoshida et al., 2003). These factors were compounded by between-laboratory variability in cell isolation and cell culture techniques, which led to assay reproducibility problems (Yoshida et al., 2003). The inter-donor variability was circumvented by the use of human myeloid cell lines, such as KG-1, THP-1, MUTZ-3, and U937 that have the ability to differentiate into cells with DC-like characteristics (Hu et al., 1996; Koss et al., 1996; Yoshida et al., 2003). Several *in vitro* model systems using human cell lines to assess the skin sensitizing potential of contact allergens have been developed. These include the human cell line activation assay (h-CLAT), myeloid U937 skin sensitization test (MUSST), the KeratinoSens^{TM} test (**Figures 2B–D**) and the LuSens which were under ECVAM evaluation (Ade et al., 2006; Ashikaga et al., 2006; Sakaguchi et al., 2006; Python et al., 2007; Emter et al., 2010; Bauch et al., 2012). These methods have been reviewed extensively by others (Mehling et al., 2012; Vocanson et al., 2013), and hence will not be covered in this review.

Loose-Fit Coculture-Based Sensitization Assay (LCSA)

An allergen-sensitive *in vitro* method that combines two layers of cells, termed the loose-fit coculture-based sensitization assay (LCSA), was developed using human primary keratinocytes from healthy donors, and mobile DC-like cells viz peripheral blood mononuclear cells (PBMCs; **Figure 2E**; Schreiner et al., 2008). As keratinocytes are proposed to have a role in haptenation via maturation of DCs, this assay has the advantage of being able to detect prohaptens such as isoeugenol (Schreiner et al., 2008), that are not detected by many *in vitro* model systems. In short, inclusion of keratinocytes in this two-tiered cell-based system facilitated metabolic activation of prohaptens into sensitizing agents akin to that which occurs in the skin *in vivo* (Wanner et al., 2010).

Similarly to MUSST and h-CLAT (as depicted in **Figures 2B,C**), LCSA quantifies the increase in expression of the cell surface marker, CD86 (Schreiner et al., 2007). Additionally, LCSA accuracy and sensitivity for assessing metal allergens such as nickel and cobalt, was improved by measuring accumulation of the proinflammatory cytokine, interleukin-6 (IL-6) and the chemokine macrophage inflammatory protein 1-β (MIP-1β; Schreiner et al., 2008). In a comparative evaluation of the *in vitro* LCSA relative to the *in vivo* LLNA for assessing the skin sensitizing potential of a group of textile disperse dyes, both methods identified 87.5% of these dyes as having skin sensitizing potential. Hence, the LCSA is a promising *in vitro* method for identifying agents with skin sensitizing potential for use in combination with other non-animal testing methods (Sonnenburg et al., 2012). However, the current challenges in using the LCSA include the necessity to obtain keratinocytes and PBMCs from healthy human donors which makes the method susceptible to inter-donor variability. Additionally, the complexity and time required for seeding keratinocytes and PBMCs in this co-culture assay makes it low throughput and so future innovation is required to adapt the LCSA to high throughput format.

Genomic Allergen Rapid Detection (GARD)

Apart from quantification of changes in cell surface expression of molecules of interest, genomic methods may offer an alternative or complementary *in vitro* testing paradigm. For example, genomic allergen rapid detection (GARD) employs the myeloid cell line, MUTZ-3 that resembles skin DCs with respect to transcriptional profiles and the ability to activate specific T-cell populations (**Figure 2F**; Johansson et al., 2013). GARD uses a complete genome expression array approach to measure expression levels of 200 transcripts involved in the activation of various signaling pathways involved in skin sensitization.

Unlike the KeratinoSens^{TM}, MUSST and h-CLAT *in vitro* methods that use specific markers for classifying sensitizers, GARD utilizes 'biomarker signatures' for identifying skin sensitizers, thereby potentially increasing the predictive ability of the method. An added advantage of GARD is that it can distinguish respiratory and skin allergens by their unique biomarker signatures (Johansson et al., 2013). Encouragingly, use of GARD to assess 38 chemicals with known skin sensitization potential in a preliminary study, showed that the accuracy, sensitivity, and specificity of the method was high at 99% (Johansson et al., 2011).

Recently, Albrekt et al. (2014) stressed that chemical reactivity properties were key factors for consideration when developing *in vitro* screening models of chemical sensitizers. Sensitizing chemicals were divided into groups based upon their mechanistic reactivity and assessed against various cell-signaling pathways using the GARD assay. Interestingly, different chemical reactivity groups induced differential changes in various cell signaling pathways, particularly those involved in cell cycling and metabolism. Potency in modulating these pathways appeared to be correlated with skin sensitization potential (Albrekt et al., 2014). However, care is required to avoid over-interpretation of these associations with respect to potential sensitizer classification. More work is clearly required using larger numbers of chemicals with a broad range of functional groups of varying reactivity, as well as a range of concentrations and reaction times. Nevertheless, the GARD assay can provide invaluable information on the various cell signaling pathways underpinning the sensitization process which is invaluable in informing further development of *in vitro* sensitization test methods. Future research is warranted to assess the extent to which the epoxide group in ERS will modulate cell-signaling responses based upon their reactivity domain and/or their sensitizing potency.

T-cell Activation Model

During skin sensitization, specific effector and memory T-cells are activated by DCs triggered by sensitizing agents. While activation and proliferation of T-cells reflect the ultimate step in inducing sensitization, there are very few assays that address this aspect of the sensitization process. At present, only the *in vivo* LLNA is used widely to evaluate the activation and expansion of T-cells. More recently, an *in vitro* assay known as the human T-cell priming assay (hTCPA) was developed to assess T-cell responses initiated by contact allergens (**Figure 2G**; Dietz et al., 2010; Richter et al., 2013). The hTCPA uses naïve T-cells isolated from PBMCs of healthy donors that are depleted in $CD25^{+}$ and $CD45RO^{+}$, a T-cell population responsible for regulating hapten-specific interferon-γ (IFN-γ)-producing T-cells in lymph nodes (Vocanson et al., 2013). The modified T-cells are co-cultured with hapten-treated monocyte-derived DCs at two stages, priming and re-stimulation. After re-stimulation, the increase in T-cell production and the cytokines, IFN-γ and TNF-α (tumor necrosis factor-α), are quantified using an enzyme-linked immunosorbent assay (ELISA) and an intracellular cytokine assay (Richter et al., 2013; Vocanson et al., 2013).

The hTCPA has been used successfully to assess the skin sensitizing potential of the strong sensitizers, 2,4-dinitrochlorobenzene (DNCB), 2,4-dinitrobenzenesulfonic acid (DNBS) 2,4,6-trinitrobezene sulfonic acid (TNBS), and moderate/weak sensitizers, fluorescein isothiocyanate (FITC), and α-hexyl cinnamaldehyde (HCA) as well as the non-sensitizers, methyl salicylate, DMSO, and sodium lauryl sulfate (SLS; Vocanson et al., 2014). Hence, the hTCPA has potential as an *in vitro* method for assessing the sensitizing potential of contact allergens. However, similar to the LSCA, this method is time-consuming and fraught with difficulty in assay reproducibility due to the scarcity of T-cell donors and inter-donor variability. More work is warranted to assess the applicability and generalizability of this cell-based model system using a larger number and a wider range of chemical compound classes. For example, the hydrophobic compound, DNCB that reduced DCs uptake did not stimulate T-cell proliferation (Dietz et al., 2010). While the use of nanoparticle encapsulation of lipophilic compounds significantly increased the ability of DNCB to stimulate T-cell proliferation and thus increase the assay sensitivity (Vocanson et al., 2013), inclusion of this additional step adds another level of complexity and increases the cost of the assay.

Cell-Based Models and ERS

Despite significant progress in the development and optimization of non-animal testing assays, a major limitation in their use for accurately identifying the skin sensitizing capacity of test compounds, is poor water solubility, particularly for aqueous cell-based assays (McKim et al., 2012). To date, few ERS compounds have been assessed using cell-based *in vitro* model systems. While the KeratinoSens^{TM} assay has been used successfully to classify the skin sensitizing potential of DGEBA, DGEBF, and PGE (Delaine et al., 2011; O'Boyle et al., 2012; Natsch et al., 2013) to match the LLNA results, the generalizability of other *in vitro* cell-based methods reviewed herein is a knowledge gap and remains to be determined.

Maintaining a suitable balance between the final solvent composition, test compound solubility and deleterious solvent-related effects within the assay, is pivotal for generating meaningful data on skin sensitization potential. In general, the solvent-related issues associated with *in vitro* assays relate to toxicity and/or solvent-mediated modulation of the assay response, thereby confounding assay readouts resulting in inaccurate assessment of skin sensitization potential. High solvent concentrations in cell-based assays adversely affect cellular integrity, resulting in cell death (Tapani et al., 1996; Galvao et al., 2014). Concentration-related toxic effects of the solvent need be evaluated to identify the maximum 'no effect' levels for each *in vitro*

assay. The balance between acceptable solvent percentage in the aqueous cell-based test system whilst maintaining solubility of high molecular weight and low solubility test compounds, particularly industrial epoxy resins is yet to be adequately addressed. This issue is arguably the most significant obstacle to be overcome in adapting current *in vitro* skin sensitization assays to assessment of epoxy resin hazard risk.

Skin Models

While selection of solvents compatible with *in chemico* assays may improve the ability of the DPRA to identify epoxy resins that have skin sensitizing properties, it is more difficult to attain a suitable balance between epoxy resin solubility and cell viability in aqueous culture-based assays. Moreover, future investigation is required regarding the fact that most test compounds are applied in solution to *in vitro* assays which may not necessarily be reflective of the situation in humans where there may be topical application of the compound in the solid state to the skin. To address this issue, the reconstructed human epidermis (RHE) has considerable potential. The RHE comprises an acellular dermal matrix mimicking the human skin epidermis layer. It has been used together with cytokines and growth factors to better represent the human skin micro-environment (Gibbs et al., 2007). Preliminary data using the RHE system showed that it was responsive to known sensitizers (Uchino et al., 2011).

More recently, EpiSensA, an *in vitro* skin sensitization assay that utilizes a commercially available RHE has become available (Saito et al., 2013). In brief, using this skin model system, skin sensitizing potential of test compounds is assessed based upon changes in the expression of genes related to the cellular stress response. Preliminary data from 16 test compounds were promising (Saito et al., 2013). Despite considerable progress, the challenge remains for a more complete human skin model system to become available that has a high degree of accuracy for correctly identifying and classifying the skin sensitization potential of novel compounds. This challenge is multi-factorial encompassing inter-individual differences at both the cellular and molecular levels such as genotypic variation, differences in epidermal thickness and metabolic activity of the skin, as well as inter-individual differences in rates of skin cell differentiation (Gibbs et al., 2007). Nevertheless, EpiSensA has promise for improving *in vitro* assessment of the skin sensitizing properties of compounds with poor aqueous solubility such as epoxy resins.

Challenges in Assessing Epoxy Resin Compounds Using non-Animal Testing Systems

Apart from use of RHE model systems, the accuracy of *in vitro* methods for skin sensitization assessment of industrial chemicals may be improved by including multiple assay readouts using an 'assay panel' approach (Natsch et al., 2009; Jaworska et al., 2011; Bauch et al., 2012). However, questions on the generalizability of these *in vitro* methods to accurately identify chemicals containing very different functional groups, is as yet unclear. In particular, most *in vitro* methods were developed and evaluated using small molecule chemicals that are widely utilized in the manufacture of cosmetic and toiletry products. This is a significant limitation as it has now been shown that different functional groups with varying chemical reactivity produce differential engagement of cell signaling pathways (Albrekt et al., 2014).

For example, a dataset of 145 chemical compounds assessed using the KeratinoSens^{TM} and MUSST assays, those that were preferentially lysine-reactive resulted in false negatives (Natsch et al., 2013). These findings mirror work by others (Migdal et al., 2013) whereby chemicals with high reactivity toward cysteine, and not lysine, activated the nuclear factor erythroid-derived 2-related factor 2 (Nrf2)-ARE pathway in THP-1 cells, a well-known toxicity pathway activated by skin sensitizers (Natsch, 2010) that underpins the design principles of both the KeratinoSens^{TM} and LuSens tests. ERS compounds such as DGEBA, DGEBF, and PGE react selectively with thiol groups (cysteine; O'Boyle et al., 2012; Natsch et al., 2013). Hence, the KeratinoSens^{TM} and LuSens assays that are based on the aforementioned pathway are worthy of future investigation for their applicability and reliability to assessment of the skin sensitizing potential of epoxy resins.

However, it is important to bear in mind that a single standalone method based upon a single mechanistic pathway to assess novel derivatives of ERS compounds is fraught as the novel derivatives may produce skin sensitization by a different mechanistic pathway. To address this issue, ECVAM recommendations are that the KeratinoSens^{TM} be used as part of an integrated assessment approach that may also include the DPRA (ECVAM, 2014). Hence, future research is required to assess the applicability of current *in vitro* methods to assess the skin sensitizing potential of a broader range of chemical compounds as a means to identify the most appropriate *in vitro* assays and assay readout ranges, for establishing benchmarks to use for classifying the skin sensitization potency of novel compound classes.

Another consideration to this discussion is the inherent accuracy of the LLNA itself with respect to existing human data. The LLNA is widely utilized as the benchmark for evaluating the predictive accuracy of non-animal methods. However, when compared against the human maximization and patch test, the accuracy of the LLNA was 72% (Anderson et al., 2011). More recently, a retrospective comparison of a moderately large dataset (>100) of test compounds revealed an 82% predictive accuracy for LLNA when compared with established human data (Urbisch et al., 2014). In other work, use of an integrated testing strategy-based on data from '2 out of 3 *in vitro* prediction models' resulted in a higher overall accuracy ($\geq$90%) when compared with human data, as opposed to $\leq$83% using the LLNA dataset (Bauch et al., 2012; Urbisch et al., 2014). Factors potentially contributing to the discordance between human and LLNA data include the difference in skin penetration rates between the mouse and human, as well as the application method of the test compounds on the skin (Anderson et al., 2011; Delaine et al., 2011). The volatility and cytotoxicity of compounds such as the components of ERS, could affect potency outcomes given the open nature of substance

application to the mouse ear in the LLNA in contrast with the occluded dressing used in human patch tests (Delaine et al., 2011). Hence, where possible, it is important to compare data produced by various *in vitro* skin sensitization tests with human data where available rather than relying solely on comparisons with LLNA data.

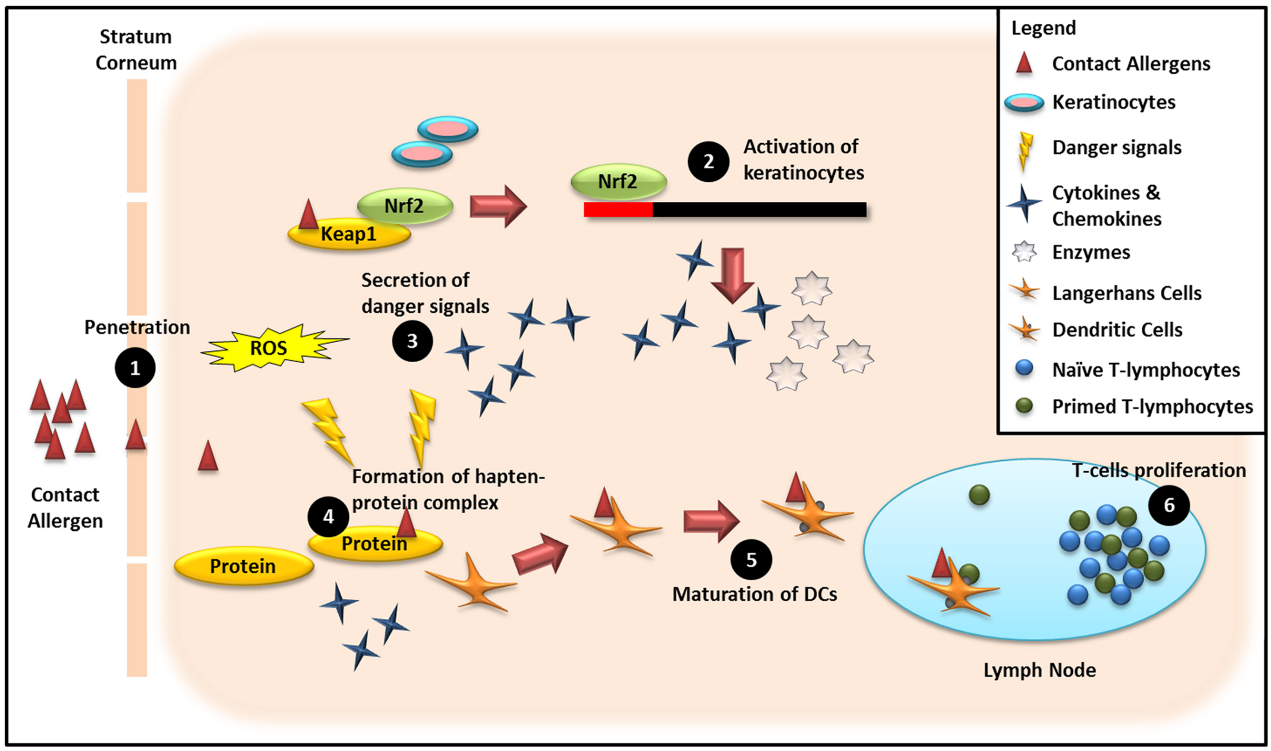

FIGURE 3 | Schematic overview of mechanisms underpinning non-animal methods for assessing the sensitizing potential of chemical compounds. (1) Penetration of haptens through the viable epidermis: Quantitative Structure–Activity Relationship (QSAR). (2) Activation of keratinocytes in the epidermal layer by haptens: KeratinoSens™, LuSens. (3) Secretion of danger signals from the epidermal compartment due to invasion of haptens: ROS production, genomic fingerprints, and proteomics biomarkers. (4) Formation of the hapten–protein complex: DPRA, peroxidase peptide reactivity assay (PPRA) and QSAR, Allergen-peptide/protein interaction assay (APIA). (5) Maturation of DCs when migrating from epidermal compartment to auricular lymph nodes via afferent lymphatics: h-CLAT, MUSST, LCSA. (6) T-cell proliferation in auricular lymph nodes: hTCPA.

TABLE 3 | *In vitro* methods used in combination for classifying and predicting skin sensitization potential of novel chemical compounds.

Combination methods	Description	Accuracy	Reference
(a) Peptide reactivity (b) Cell-based ARE† assay (c) TIMES-SS‡ computer modelling (d) Calculated octanol-water partition coefficient	• Scores of 0–4 for each individual test • A binary system is applied for *in silico* test results	88% (based on LLNA data) (116 test substances)	Natsch et al. (2009)
(a) DPRA (b) LuSens (similar principle with KeratinoSens™ assay) or KerotinoSens™ assays (c) h-CLAT or MUSST	• A sensitizer if DPRA and LuSens yield negative results and MUSST is positive • If contradictory results between DPRA and LuSens, or h-CLAT, then weight of evidence approach is used	94% (based on human data) 83% (based on LLNA data) (54 test substances)	Bauch et al. (2012)
Bayesian network Integrated Testing Strategy (a) TIMES§ (b) DPRA (c) ARE luciferase activity (d) MUSST	• Adaptive testing strategy where the choice and sequence of tests performed are based on available information • Reduces uncertainty of the sensitizing capacity of a test substance before proceeding to the experiment.	–	Jaworska et al. (2011)

†*ARE, antioxidant response element.*
‡*TIMES-SS, tissue metabolism simulator for skin sensitization.*
§ *TIMES, tissue metabolism simulator.*

Integrating Non-Animal Assay Readouts: Classifying Potential Skin Sensitizers

The OECD has proposed that the hazard classifying system for chemicals should consider the potential severity of allergic manifestations from human and animal-based epidemiological data [Organisation for Economic Co-operation and Development (OECD), 1998]. A strong sensitizer is defined as a compound that has a high occurrence of sensitization within an exposed population whereas low to moderate sensitizers produce a low or moderate frequency or severity of sensitization [Organisation for Economic Co-operation and Development (OECD), 1998]. At present, this chemical classification system is based solely on the 'gold standard' LLNA which assesses the potency of skin sensitizers based on the extent to which they induce T-cell proliferation in the auricular lymph nodes of mice (Kimber et al., 2002b). Current and future research aimed at gaining a deeper understanding of the various cellular and immunological mechanisms and their interplay that contribute to the extent of sensitization evoked, is essential. Such new knowledge will be invaluable for informing future research aimed at optimization of *in vitro* methods for hazard identification of industrial chemicals, particularly ERS, as well as enable quantitative risk assessments to be performed (Kimber et al., 2011).

The available non-animal testing methods for assessing the various stages of ACD are summarized in **Figure 3**. This schematic diagram clearly shows that single testing methods are unable to evaluate potential cross-talk between the various phases of the skin sensitization process. Thus, a single *in vitro* test representative of a single event in the human skin response to contact allergens cannot adequately capture the complexity of the human response to a contact allergen, thereby potentially leading to generation of false negative results (Aeby et al., 2010). Thus, a panel of complementary non-animal tests that together mimic the complexity inherent in *in vivo* test methods (e.g., LLNA, human patch test), has considerable potential utility as a screening tool for more accurately classifying novel compounds as extreme, strong, moderate or weak sensitizers.

An integrated hazard classification scheme involving assessment of multiple steps in the skin sensitization process, including bioavailability, structural alerts, formation of hapten–protein conjugates, DC maturation, and T-cell proliferation, has been proposed (Kimber et al., 2003; Jowsey et al., 2006). Using this approach, greater weight is given to *in vitro* tests that produce quantitative data. An index of sensitizing potency is calculated based upon the product of values obtained from each test representing a key step in the skin sensitization process, for comparison of skin sensitization potency with the corresponding mouse LLNA data (Kimber et al., 2003; Jowsey et al., 2006). Various non-animal test combinations proposed for identifying potential skin sensitizers are summarized in **Table 3**.

Conclusion

A strategy encompassing the integration of readouts from multiple *in vitro* tests as a means to improve the accuracy for identification of novel compounds that are contact allergens has merit. However, implementation of such a strategy requires extensive validation and assessment of its generalizability for multiple chemical classes before gaining widespread acceptance. Additionally, use of an integrated panel of *in vitro* methods to screen large numbers of industrial chemicals is likely to be unattractive from a cost and time perspective and so development of a hierarchy of individual high throughput *in vitro* tests is needed.

At present, single *in vitro* assays in high throughput format enable large numbers of compounds to be screened in a short time frame. However, the choice of *in vitro* method for screening purposes, either as part of an integrated or hierarchical strategy, should be informed by knowledge of the chemical class/domain. In conclusion, the choice of *in vitro* methods for inclusion in a panel for assessing skin sensitization potential will be the best balance between predictive power of the selected tests relative to the time and cost of generating the data and its value to the organization that requires the data.

Author Contributions

CW and AL wrote the manuscript. All authors reviewed and commented on the manuscript drafts.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.