**Proteomic Analysis of Haptenation by Skin Sensitisers: Diphencyprone and Ethyl Acrylate**

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**Running title**: Protein haptenation of HSA and HaCaT lysates by DPCP and EA.

**Abstract**

The potential risk of skin sensitisation, associated with the development of allergic contact dermatitis (ACD), is a consideration in the safety assessment of new ingredients for use in personal care products. Protein haptenation in skin by sensitising chemicals is the molecular initiating event causative of skin sensitisation. Current methods for monitoring skin sensitisation rely on limited reactivity assays, motivating interest in the development of proteomic approaches to characterise the skin haptenome. Increasing our mechanistic understanding of skin sensitisation and ACD using proteomics presents an opportunity to develop non-animal predictive methods and/or risk assessment approaches. Previously, we have used a novel stable isotope labelling approach combined with data independent mass spectrometry (HDMSE) to characterise the haptenome for a number of well-known sensitisers. We have now extended this work by characterising the haptenome of the sensitisers Diphenylcyclopropenone (DPCP) and Ethyl Acrylate (EA) with the model protein Human Serum Albumin (HSA) and the complex lysates of the skin keratinocyte, HaCaT cell line. We show that haptenation in complex nucleophilic models is not random, but a specific, low level and reproducible event. Proteomic analysis extends our understanding of sensitiser reactivity beyond simple reactivity assays and offers a route to monitoring haptenation in living cells.

**Keywords:** allergic contact dermatitis, Diphencyprone, Ethyl Acrylate, HSA, MSE, proteomics, sensitization, skin

**Abbreviations:** Diphenylcyclopropenone (Diphencyprone, DPCP), ethyl acrylate (EA),

**Introduction**

Contact allergy is one of the most common forms of immunotoxicity in humans (Thyssen et al. 2007). Chemical allergens are small electrophilic molecules (haptens), capable of forming covalent bonds with skin protein nucleophiles. This chemical reaction (haptenation) is a molecular initiating event (MIE) and first in the sequence of events leading to skin sensitisation (Landsteiner and Jacobs 1935). Subsequent events are described and expanded on by several authors (e.g. (Basketter et al. 1995; Lepoittevin 2006) and used to form an adverse outcome pathway (AOP) for skin sensitisation (OECD 2014). This AOP is a framework for our current knowledge and a basis for expanding the mechanistic understanding of the chemical effects on a molecular and sub-cellular level. Specifically, further characterisation of the protein haptenation facilitates the development and refinement of non-animal predictive methods and/or risk assessment approaches (Burden et al. 2015; MacKay et al. 2013; Maxwell et al. 2014).

To date, numerous studies have examined electrophilicity of a variety of haptens in detail and consequently, there is a considerable amount of knowledge about electrophilicity and relative reactivity of allergens. These insights have been distilled into novel methods for routine determination of aspects of chemical reactivity and confident inference of skin sensitisation hazard (Aleksic et al. 2009; Gerberick et al. 2004; Gerberick et al. 2007; Natsch and Gfeller 2008; Roberts and Natsch 2009; Sanderson et al. 2016). However, the knowledge of the nature and identity of the skin protein nucleophilic targets haptenated during sensitisation is sparse. In reactivity studies, protein nucleophile is usually represented by models (amino acids or small molecules representing the side chains of nucleophilic amino acids, small peptides (native or artificial) and model proteins). Detailed studies in complex protein mixtures are relatively rare and often explore methodologies available to investigate a complex low level changes in proteomes (Codreanu et al. 2009; Conrad et al. 2001; Hong et al. 2005; Jacobs and Marnett 2010; Mello et al. 2007; Shearn et al. 2016; Simonsson et al. 2011; Spiess et al. 2011). From these we conclude that, besides electrophilicity of the chemical, nucleophilicity of the protein target and steric constraints, further factors, such as types of proteins present, expression levels, levels of glutathione and other ‘defence’ molecules, difference in local pH and microbioavailability, are all determinants of the type and level of haptenation. The optimum conditions for haptenation are unclear, but evidence shows that the epidermis and dermis are the skin sites where these modifications become available to the immune system (Kimber et al. 2011; Kimber and Dearman 2003; Pickard et al. 2009). Furthermore, we understand that the generic nucleophilic ‘make up’ of skin matches the rest of the human proteome, with basic, amine nucleophiles by far the most abundant (Parkinson et al. 2014b).

Due to lack of highly sensitive methods for detection and investigation of haptenation, studies identifying haptenated proteins *in vitro* and *ex vivo* are limited. We have previously demonstrated the increased sensitivity for detection of haptenated peptides using HSA (Parkinson et al. 2014a). We further showed the utility of this approach to define the haptenome of HaCaT cell lysates and human skin treated with dinitrochlorobenzene (DNCB), 5-chloro-2-methylisothiazol-3-one (MCI), cinnamaldehyde (CA) and 6-methyl coumarin (6-MC) (Parkinson et al. 2018) by combining stable isotope labelling with data-independent acquisition (DIA) mass spectrometry. We have shown that the haptenation of proteins *in chemico* is not driven solely by their abundance and that the preference for specific amino acid modifications remains comparable to the other *in chemico* reactivity studies (e.g. peptide reactivity). Here, the dataset is extended to include protein haptenation by diphenylcyclopropenone (diphencyprone, DPCP) and ethyl acrylate (EA).

DPCP and EA are both known skin sensitisers, electrophiles which react to protein nucleophiles (Roberts and Natsch 2009). DPCP is an extreme skin sensitiser (ECETOC-DocNo46 2008; Ryan et al. 2000) which has found its uses in a variety of clinical settings ranging from treatment of alopecia aerata and recalcitrant warts to benign dermatoses and atopic dermatitis (Choe et al. 2018; Park et al. 2018; Zerbinati et al. 2018). EA is a weak sensitiser (Dearman et al. 2007; Gerberick et al. 2005) used in the production of polymers including resins, plastics, rubber, acrylic nails and denture materials (Spencer et al. 2016).

Whilst qualitative and quantitative differences in reactivity of DPCP and EA have been observed in major *in chemico* studies (summarised in Table 1), it is evident that both DPCP and EA react to more than one nucleophile and often react rapidly forming stable adducts. However, the similarities in *in chemico* reactivity do not translate directly into understanding the outcome of sensitisation by these two chemicals. Here we investigate whether their overall reactivity remains comparable when more complex protein models are used.

Table 1

Figure 1

For comparison with previous work, we utilise HSA and HaCaT cell lysates. The unreliable supply of quality viable human skin tissue prompted the need to seek a suitable surrogate which is easier to obtain and maintain. We have previously used HaCaT cells successfully and have shown that their proteome is reflective of human skin proteome for these purposes.

**Materials and Methods**

**Test chemicals**

Diphenylcyclopropenone (DPCP) (98% purity; MW 206.24 Da) and Ethyl Acrylate (99% purity, MW 100.12 Da) were obtained from Sigma-Aldrich (Poole, UK). DPCP-D10 (97.1% purity; MW 211.27 Da) was custom synthesised by Quotient Bioresearch (Rushden, UK). Ethyl-d5 Acrylate (99% purity, MW 105.15 Da) was obtained from CDN Isotopes (Quebec, Canada). Stock solutions of each chemical were made by dissolving a 50:50 mix, by molar concentration, of deuterated and unlabelled chemical in ethanol to a final concentration of 750 mM. Exact locations of the stable isotopes for each chemical are shown in Table 2.

Table 2

**Generation of HaCaT cell lysates**

The adherent keratinocyte cell line (HaCaT) was cultured in Dulbecco’s Modified Eagle Medium (DMEM), high glucose, (supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100U/ml Penicillin and 100ug/ml Streptomycin), at 37°C and 5% CO2. Cells were harvested once they had reached 70% confluency by scraping and washing with PBS. To the cell pellets, 500 µl of lysis buffer (0.1% SDS in 0.1M TEAB) was added and the cells transferred to a reinforced 1.5ml tube containing ceramic beads (Matrix D – QBioGene, Cambridge, UK). The cells were lysed via a FastPrep macerator (MP Biomedical, UK) for 5 cycles of 45 seconds; speed setting 6, chilling on ice for one minute between cycles. Insoluble material was pelleted by centrifugation at 9000 *g* for 5 minutes and discarded.

**Sensitiser modification of HSA and preparation for MS analysis**

To study the effect of DPCP and EA haptenation over time, HSA, in either PBS or 0.1M TEAB, at a concentration of 1mg/ml was modified with 1.5 mM of the test chemical (0.2% ethanol) at 37°C for 24 hours, 1 week, 2 weeks or 4 weeks. Modified HSA and control samples (20-40 ug) were prepared for GeLC-MS/MS as described previously (Parkinson et al. 2014a). Briefly, the samples were denatured in final sample buffer containing 10 mM DTT at 70°C for 10 minutes, before resolving for 45 minutes through a NuPage 4-12% Bis-Tris gel (Life Technologies, Paisley, UK) at 200V. Bands at approximately 66 kDa were excised and subjected to *in* *situ* trypsin digestion using a published method (Shevchenko et al. 1996). The resulting peptides were extracted, dried down under vacuum and stored at -20 °C.

**Sensitiser modification of HaCaT cell lysates and preparation for MS analysis**

Lysates were modified and prepared for MS analysis as previously described (Parkinson et al. 2018). Briefly, cell line lysates were dissolved in 0.1M TEAB to a final concentration of 1mg/ml and modified with solutions of deuterated and unlabelled mixes of either DPCP or EA (as described above) at 1:100 molar excess of sensitiser to protein at 37°C for 4 weeks. To 100 µg of sensitiser modified cell line lysate, 4 volumes of methanol was added, the sample vortexed, to this 1 volume of chloroform was added and the sample vortexed again before finally adding 3 volumes of water and vortexing once more. The sample was centrifuged at 20,000 g for 1 minute, focusing the proteins between the organic and inorganic phases. The aqueous phase was removed and 4 volumes of ethanol was added, followed by a short vortex. The precipitate was pelleted by centrifuging at 20,000 g for 2 minutes, the ethanol was removed and the pellet was air-dried.

The pellet was resolubilised in buffer containing 6M urea, 2M thiourea and 10 mM Hepes, pH 7.5. Proteins were reduced with DTT for 1 hour at room temperature, alkylated with 5.5 mM IA for 45 min in the dark at room temperature, and then digested for 4 hrs with Lys-C (1/50 w/w). Peptides were then diluted four times with 20 mM ammonium bicarbonate and then digested with sequencing grade modified trypsin (1/50 w/w) overnight at room temperature. After digestion, the peptides were fractionated along Immobiline IPG strips, pH 3-10 using the Agilent 3100 OFFGEL Fractionator. Peptides (100ug) were made up to a final volume of 1.4 mL with a 1:50 solution of IPG buffer, pH 3-10 (GE Life Sciences, Buckinghamshire, UK) diluted in 5% Glycerol; then focussed for 20 kVh at a maximum current of 50 µA and a maximum power of 200 milliwatt. The collected fractions were and acidified with TFA to pH <3.0 and peptides bound to a C18 SPE 96 well plate (3M, Minnesota). Contaminants were washed off 0.5% acetic acid, and peptides were eluted with 80% acetonitrile + 0.5% acetic acid. The fractions were dried down and stored at -20 °C.

**Mass spectrometry**

Samples were resuspended in 0.1% Formic Acid and approximately 1 - 2 µg of sample loaded onto a reverse phase trap column (Symmetry C18, 5 µm, 180 µm x 20mm, Waters), at a trapping rate of 5 µL/min and washed for 10 minutes with buffer A prior to the analytical nano-LC separation using a C18 Reverse phase column (HSS T3, 1.8 µm, 200mm x 75µm, Waters Corporation, Milford, Massachusetts). Peptides were fractionated over a continuous gradient from 3-40% acetonitrile at a flow rate of 300 nL/min for 30 minutes, for the HSA samples, or 90 minutes, for the HaCaT fractions. Eluted samples were sprayed directly into a Synapt G2-S mass spectrometer (Waters Corporation, Wilmslow, UK) operating in MSE mode. Data was acquired from 50 to 2000 *m/z* using alternate low and high collision energy (CE) scans. Low CE was 5V and elevated CE ramp from 15 to 40V. Ion mobility was implemented prior to fragmentation using a wave height of 650 m/s and wave velocity of 40V. The lock mass Glu-fibrinopeptide, (M+2H)+2, *m/z* = 785.8426) was infused at a concentration of 100 fmol/µl with a flow rate of 250 nl/min and acquired every 60 seconds.

**Database searches**

The raw mass spectra were processed using ProteinLynx Global Server Ver 3.0.2 (Waters Corporation, Wilmslow, UK), and the data processed to generate reduced charge state and deisotoped precursor and associated product ion peak lists. These peak lists were searched against the human UniProt sequence database (obtained from UniProt 02/2015). A maximum of two missed cleavages were allowed for tryptic digestion and the variable modifications were set to contain oxidation of methionine, carboxyamidomethylation of cysteine and 3 possible DPCP modifications of +206, +222 and +224 amu of cysteine, lysine, tyrosine, histidine and arginine or 2 possible EA modifications of +100 and +200 amu. Precursor ion and product ion mass tolerances were calculated automatically during data processing and the false discovery rate was set at 4% and subsequently filtered to 1%.

**Data filtering, protein abundance and nucleophile concentration calculations**

Following database searching the data was filtered, and protein abundance and nucleophile concentrations were calculated as previously described (Parkinson et al. 2018).

**Results**

The current data shows low levels of modified protein residues on HSA and low level of haptenated proteins overall in HaCaT cell lysates, which is similar to our previous observations using both HSA and relevant cell/tissue lysates as models (Parkinson et al. 2018; Parkinson et al. 2014a).

To maximise the ability to detect and identify low abundance haptenated peptides as well as reduce the bias towards highly abundant peptide species, the data independent mode of acquisition, MSE (Geromanos et al. 2009; Michalski et al. 2011) was used together with a previously described dual labelling approach and high molar ratio of hapten to protein. Unambiguous identification of modified residues could be achieved in most cases.

As before, raw MS data were processed using PLGS and searched against the UniProt *Homo sapiens* sequence database. Modified peptides were confirmed only where a peptide signature consisting of two peptide isotope clusters of fixed Δ *m/z* were observed within a single MS spectrum. The product ion spectrum of the modified precursor ion was subsequently manually inspected to determine if the exact site of haptenation could be confirmed. Confirmation of the amino acid site of modification was determined by the presence of high quality fragment ions around the modification site.

In some instances, haptenated peptides were detected for which the exact sites of haptenation could not be unambiguously identified since the fragmentation spectra were of insufficient quality. Occasionally, the exact location of such modifications could be implied if a single nucleophile is present in the haptenated peptide sequence. If more than one nucleophile is present and fragmentation spectra are of insufficient quality to confirm which nucleophile is modified, we consider that any present nucleophile could be the target. Nevertheless, these modifications are demonstrated by the presence of the unique isotopic signature achieved by dual labelling method.

**Modification of HSA by DPCP and EA**

A total of 49 HSA residues were found haptenated by DPCP and 27 by EA (**Supplementary Data 1**). Of those, 28 and 14, respectively, could be unambiguously assigned to an exact nucleophile location. This level of HSA haptenation is comparable to previously tested chemicals (Parkinson et al. 2014a). Differences in reactivity of DPCP and EA observed in peptide reactivity assays are still evident with HSA as a model, with somewhat lower level of haptenation now more obvious for EA, possibly due to evaporative loss over long incubation with HSA.

DPCP initially forms single adducts (+206 amu), mainly with Lys, Arg, Cys and His, which further react either via oxidation (+222 amu) or ring opening (+224 amu) of the original adduct. Oxidative and ring opening changes were not reserved only for Cys residues (as observed in peptide reactivity assays). In fact, the only confirmed modifications of HSA Cys 34 (the only Cys not involved in S-S bridges on HSA) was a DPCP adduct with an opened ring (+224 amu). Majority of DPCP modifications were in fact found on amine-based nucleophiles (27 Lys, 10 Arg and 6 His modifications), whereas no Tyr modifications were detected. Interestingly, similar to DNCB and MCI, previously tested sensitisers of extreme potency, DPCP shows a high preference for HSA Lys residues. However, DPCP also modifies high levels of Arg residues, similar to CA (moderate sensitiser).

For EA, confirmed modifications were found on Cys 34, Cys 53 and Cys 514 (despite the two latter residues reported unavailable due to S-S bridging), as well as single adducts with Lys (6), His (4), Tyr (3) and Arg (6). Modification of Cys residues, involved in S-S bridge formation, was previously observed with DNCB and was not thought to be a result of a reducing capacity of the test chemical itself (Parkinson et al. 2014a). Overall, EA did not show a particular preference for any specific nucleophile type.

In comparison to the sensitisers investigated previously, it is evident that whilst the extent of modification is not the same for all, the target HSA residues are mostly common amongst all (Figure 3). Notable exceptions include EA haptenation of His 510 and Cys 514 and DPCP haptenation of His 288, neither of which were observed as targets with previous test chemicals. His 288 has been previously shown as a target for trans-4-hydroxy nonenal, endogenous electrophile, generated as an end product of lipid peroxidation (Liu et al. 2012).

The DPCP modifications were observed throughout the time course, some as early as 24h, however, more confirmed modifications occur at later time points (Figure 2A). Contrastingly, EA modifications are more prominent at 24h and, whilst few of those remain, new modifications were seen at later time points (Figure 2B).

Figure 2

Figure 3

**Modification of proteins in HaCaT cell lysates by DPCP and EA**

In total, 110 peptides related to 84 proteins were modified by DPCP and 8 peptides related to 8 proteins by EA (1.2% and 0.1%, respectively, of the keratinocyte cell proteome). In comparison to DNCB, MCI and CA which showed a low level of haptenation in HaCaT cell lysates (2.5, 2.5 and 1.1%, respectively, (Parkinson et al. 2018)), the level of haptenation with DPCP and EA in these lysates was lower still (Figure 4). We note that this is evident despite the fact that experimental conditions are all favourable for haptenation (high concentration of DPCP and EA, prolonged incubation time and cell lysis ahead of incubation). The detected haptenation by DPCP and EA is of varying abundance (data not shown), again indicating that protein abundance is not the only factor driving protein haptenation.

Across the identified haptenome a total of 107 amino acid residues in HaCaT cell lysates were haptenated by DPCP, 60 of which could be assigned to the exact location as confirmed by the presence of high quality fragmentation around the proposed modification site, and only 8 by EA (the exact location of which could not be confirmed from inspection of the fragmentation spectra; shown in grey in Figure 4). The percentage of haptenated residues observed as a proportion of the total number of modifiable nucleophilic residues within the HaCaT proteome is therefore very low (Figure 4). DPCP haptenation of HaCaT lysate proteins is comparable to previously obtained data for other sensitisers (Figure 4), whereas EA haptenation was found to be considerably lower. Indeed, no observed modifications could be fully assigned to an exact nucleophile by fragmentation spectra inspection. DPCP modifications were mostly located on Lys and His residues, whereas Cys, Arg and Tyr were sparsely modified. Interestingly, Tyr modifications by DPCP were not previously reported in peptide reactivity assays nor were any Tyr residues found modified on HSA by DPCP. In HaCaT lysate, DPCP modified Tyr residues on Q93009 (Ubiquitin carboxyl-terminal hydrolase 7), as well as P15311 (Ezrin) and P15924 (Desmoplakin), amongst others. Likewise, EA was not reported to modify Tyr in peptide reactivity assays, but 3 HSA Tyr residues were found modified (Tyr 30, 341 and 497) and one in the HaCaT lysates (in Nad(P)H-hydrate epimerase, Q8NCW5).

As previously reported, the total theoretical nucleophile concentrations within the HaCaT cell is 19.0%, based upon the total number of modifiable nucleophilic residues (Lys, Arg, Cys, Tyr and His)(Parkinson et al. 2018). Mapping of DPCP and EA modified proteins to a ranked list of proteins according to their nucleophile concentration (high to low) shows a correlation between protein modification and protein nucleophile concentration within the HaCaT cell lysates (Figure 5).

P19338 (Nucleolin) and P59998 (Actin related protein 2/3 complex subunit 4) were found to modified by both DPCP and EA. Interestingly, P14618 (Pyruvate Kinase) was found to be modified in 5 separate locations by DPCP, EA modifications were not observed on this protein. We have previously reported that DNCB, MCI and CA all modified this protein (Parkinson et al. 2018). In addition, P63261 (Actin cytoplasmic 2 protein) was found to be modified in separate locations by DPCP and Q5VTE0 (Putative elongation factor 1) in 3 locations. A summary of all HaCaT cell lysate modified proteins can be found in the Supplementary Table 2.

Figure 4

Figure 5

**Discussion**

The study of DPCP and EA reactivity with single model protein (HSA) and (skin) relevant mixture of proteins (HaCaT cell lysate) is a useful extension of our understanding of the reactivity of previously investigated model sensitisers (DNCB, MCI and CA) and model protein systems (HaCaT cell lysates and *ex vivo* human skin lysates). The comparison here extends to two further sensitisers, DPCP and EA, both known to cause skin sensitisation in humans and with relatively well understood chemical mechanisms of haptenation. This study is a part of method development of the stable isotope labelling approach and whilst it still represents effectively an *in chemico* reactivity study, it helps us further understand haptenation and determine whether the sensitivity of this approach is sufficient for the investigation of haptenation in living cells and tissue models.

The main questions asked from this study were of a comparative nature. Initially, we compared the reactivity of DPCP and EA with a single protein (HSA) and subsequently in a complex mixture of relevant proteins. Whilst still essentially an *in chemico* approach, this enables us to contrast the ‘single nucleophile’ reactivity data with a richer data set from multiple nucleophile mixture and assess whether equivalent information is obtained.

Both DPCP and EA behave similarly in simple reactivity assays showing comparable levels of haptenation with a variety of nucleophiles despite their differing sensitising potency (Table 1).

HSA haptenation data is showing a difference in haptenation by the two chemicals. This is, perhaps, not surprising given that the main factor thought to be responsible for weak sensitising potency of EA is its volatility (Roberts and Natsch 2009). Peptide reactivity assays are performed in closed tubes, largely preventing evaporative loss, which could explain high reactivity of EA observed in these assays. Importantly, similar conditions are used in reactions with HSA, however, the exposure duration is considerably longer. The length of incubation in these experiments poses issues with volatile compounds: our conclusion is that the low level of detected modifications with EA is largely due to experimental evaporative losses over time.

The DPCP and EA haptenation in HaCaT cell lysate is comparable with haptenation of other previously tested sensitisers. We identified 84 proteins haptenated by DPCP and 8 by EA in HaCaT lysates, accounting for only 1.2% and 0.1% of the total protein content in HaCaT cell lysates, respectively (**Supplementary Data 2**). This overall low proportion of protein haptenation was observed previously and further confirms the need to understand the dynamic nature of haptenation during induction/elicitation of sensitisation as well as specificity.

The main differences between simple and complex nucleophile models were related to preference for certain nucleophile types. Both DPCP and EA show some reactivity to Lys and other amine nucleophiles (Arg and His) in peptide reactivity assays. When HSA and cell lysates are used as models, reactivity to amine-based nucleophiles (Lys, Arg and His) is enhanced. Interestingly, Tyr modifications were new targets for both DPCP and EA in complex models and not previously observed in peptide reactivity studies. Skin nucleophilic ‘make up’, reflected in the cell lysate used here as surrogate and to an extent in HSA, has considerably higher levels of amine type nucleophiles compared to thiols (Parkinson et al. 2014b). On further inspection, DPCP favours amine-based nucleophiles to similar extent as DNCB and MCI observed previously, whereas EA appears to show a more even level of haptenation.

Whilst not all nucleophiles are accessible or available for haptenation, lengthy incubation and high amine-based nucleophile concentration result in higher level of haptenation of those residues. Despite this, overall haptenation is low-level, non-stoichiometric and specific event.

Once again, we have observed differences in haptenation between early and late time points of incubation. It is likely that the modifications observed at later time points are occurring due to altered 3D structure of proteins during initial haptenation events. However, the loss of the initially observed modifications raises a question of overall stability of haptenation. Whilst it is possible that further oxidation or methylation may have occurred and resulted in obscuring these peptides from database searches, this phenomenon warrants further investigation in more physiologically relevant models.

Physico-chemicals characteristics, such as EA volatility, or possibility of free radical polymerisation of EA are likely to account for difference in potency observed in the LLNA (Roberts and Natsch 2009). However, further insights are gained from investigating reactivity using more complex nucleophile models. In reactivity assays most often only one type of nucleophile is present. It is therefore not possible to speculate about the preference of the electrophile for any particular nucleophile when multiple nucleophiles are present and their microbioavailability varies.

The question remains how the reactivity information obtained from ‘single nucleophile’ reactivity assays can be adequately interpreted to provide information for risk assessment purposes. Direct conclusions on sensitising potency of chemicals are unlikely to be drawn solely from *in chemico* reactivity assays. To improve our ability to interpret this data and inform the risk assessments for skin sensitisation, it will be important to consider the mechanistic relevance of *in chemico* measurements of chemical reactivity to model nucleophiles. Quantitative understanding of the dynamics of cellular events relevant to electophile reaction mechanism(s) may play a considerable role in the final level of protein haptenation.

For the majority of reactive chemicals, their inherent reactivity is also their main detoxification route, often enzymatically facilitated. For example, dinitrohalobenzenes bind and deplete GSH (Jacquoilleot et al. 2015) and activate cell defence mechanisms (Spriggs et al. 2016). The latter phenomenon has been utilised to develop one of the novel *in vitro* assays for skin sensitisation (Emter et al. 2010). Whilst there is at least one notable exception to this rule (MCI, which, in its interaction with GSH generates further reactive species rather than being detoxified (Alvarez-Sanchez et al. 2004)) there is solid evidence for GSH conjugation of some electrophiles in exposed cells and tissues. These concomitant reactions are likely to impact the level of haptenation and antigen generation. It may be necessary to adapt the interpretation of the reactivity measurements obtained from *in chemico* approaches in the context of multiple reactivity related events, and consider the dynamics of the cellular response to improve our ability to predict sensitisation thresholds.

Additional reactivity-related events in cells upon treatment with reactive chemicals have been considered by several groups. Pickard and colleagues investigated the molecular events associated with affecting the ‘outer epidermal redox barrier’, a thiol rich layer in the stratum corneum, which appeared to effectively reduce the level of penetration into viable epidermis of DNCB but not DNTB, when both chemicals showed measurable *in chemico* reactivity with GSH (Pickard et al. 2009). The generic reactivity with thiols was also implicated in altering the level of cell surface thiols and inducing the p38 MAPK in human monocytic cell line (THP-1) for a number of skin sensitisers (Suzuki et al. 2009). In this study, unlike most tested sensitisers, DPCP was shown to increase the cell surface thiol content of THP-1 cells. Contrary to what would be expected with a chemical showing high thiol reactivity in peptide reactivity assay, DPCP does not appear to affect GSH/GSSG ratio (Hirota et al. 2010). This may be a consequence of non-toxic DPCP dose and a short (2h) treatment in this study, resulting in rapid cell defence signalling and subsequent increase in thiol content on the cell surface. A clinical study demonstrated that systemic stress-induced modulation (by exercise, known to affect glutathione antioxidant system (Elokda and Nielsen 2007)) impairs both induction and elicitation of DPCP sensitisation in human (Harper Smith et al. 2011). How DPCP interacts with many thiol- and amine-rich skin components and how and at what point do these events result in reduced ability of the tissue to overcome the electrophilic insult remains a subject of investigation. Our dataset here extends the understanding of DPCP reactivity beyond simple reactivity assays, demonstrating selectivity in haptenation by DPCP similar and comparable to that of previously studied sensitisers.

We note that this comparative study of haptenation using nucleophilic models (protein and cell lysate) represents *de facto* study of ‘*in chemico* reactivity in solution’, albeit using complex models. Therefore, full conclusions on all qualitative and quantitative aspects of haptenation would be premature at this stage. Whilst not directly representative of the extent and specificity of reactivity-related events upon human skin exposure to these chemicals, these and previously published data represent baseline studies and physiologically relevant haptenation is likely to be a subset of modifications observed here.

Factors such as reaction mechanisms (including complex reactivity shown here with DPCP and previously with MCI (Alvarez-Sanchez et al. 2004; Parkinson et al. 2018; Parkinson et al. 2014a)), electrophile concentration and role of detoxification, physico-chemical properties as well as nucleophile type and concentration are all influential in overall level of haptenation. Understanding how these factors collide together to determine the amount of antigen that is generated upon exposure of cells to electrophilic chemical remains a challenge. It is likely that the extent to which the skin proteome is haptenated correlates with the number and diversity of T cell epitopes and is therefore strongly indicative of sensitiser potency (Esser et al. 2014) and therefore worthy of detailed investigation. Improved sensitivity, resolution and modes of acquisition of modern mass spectrometry together with the stable isotope labelling approach have established this approach as the ‘method of choice’ for attempting to study haptenation *in vitro*. Further studies on haptenation in living cells or 3D models, relevant to human skin exposure are now plausible. Parameters such as relative nucleophilic ‘make up’ and concentration Vs the electrophile concentration, type of preferred nucleophile(s), mechanisms of haptenation, the dynamic cellular defence response and similar will be necessary for development of mathematical models for prediction of sensitisation. Furthermore, the approach described is applicable to studying reactivity related events beyond skin allergy research.

**Supplementary data description**

**Supplementary Data 1**

Excel file summarising the identification of HSA modified amino acids after exposure to either DPCP or EA, at 24h, 2 week and 4 week time points. Yellow cells denote confirmed sites of modification after inspection of fragmentation spectra; blue cells denote sites with an observed haptenation (by detection of a unique isotopic signature in the MS spectrum), however the exact nucleophilic amino acid is not confirmed due to insufficient quality of fragmentation spectra.

**Supplementary Data 2**

Excel file summarising the identification of modified amino acids in proteins of HaCaT lysates after exposure to either DPCP or EA. Amino acids within peptide sequences highlighted in red show sites of fragmentation. Rows in bold highlight peptides where amino acid site of modification can be confirmed, either by the presence of spectra with sufficient quality of fragmentation around the proposed site of modification or as the suggested amino acid is the only nucleophile present within the modified peptide.

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**Figure Legends**

**Figure 1** - Reaction mechanisms, previously observed in peptide reactivity assays or obtained from literature – (A) single DPCP adduct to thiol (Δ mass 206 Da) (\*isobaric with α,β-diphenylacrylamide adducts reported previously (Eicher 1975; Roberts and Natsch 2009), resulting from a crosslink to additional thiol, not observed here); thiol oxidation of the original adduct results in a separate single adduct (Δ mass 222 Da); further single adduct (Δ mass 224 Da) consistent with ring opening of the original adduct by water addition to carbonyl. Observed adducts to amine-based nucleophiles (Lys, His or Arg) were single adducts (Δ mass 206 Da) – (B) single EA adduct to thiol via Michael addition. Observed adducts to Lys and His were also single Michael adducts (Δ mass 100 Da) as well as double Michael adducts to Lys (Δ mass 200 Da).

**Figure 2** - Schematic drawing of the secondary structure of a single molecule of HSA (downloaded from PDB, 1AO6), front and back, modified by DPCP (A) and EA (B). Nucleophilic residues are highlighted on the structure and colour coded as follows: residues modified at 24h only – orange; residues modified at 1w only – green; residues modified at 24 and 1 w – maroon; residues modified at 2w only – blue; residues modified at 1w and 2w – pink; residues modified at 1w, 2w and 4w – yellow (2 and 4 w for EA); residues modified at 4w only – red; residues modified at all time points – black. The remaining structure of the HSA is represented in grey ribbon. This figure was generated using PyMol.

**Figure 3** – A comparison of previously published HSA haptenation data (DNCB, MCI and CA (Parkinson et al. 2014a)) to current dataset (DPCP and EA). Circular representation of the sequence of HSA showing which nucleophiles were haptenated after exposure with the test chemicals DNCB, MCI, CA (cinnamaldehyde), DPCP and EA. The 2 residues (His 510 and Cys 514) haptenated by EA and His 288, haptenated by DPCP, not observed previously as target residues, outlined in red circles.

**Figure 4** – Percentage of modifiable nucleophilic residues haptenated by DNCB, MCI, DPCP and EA in HaCaT lysates (coloured bars represent modifications that were assigned to an exact nucleophile location, grey bars represent modifications observed by unique isotopic signature in the MS spectrum, the exact localisation of which is ambiguous within the observed modified peptide due to low intensity of the ions in fragmentation spectra; DNCB, MCI and CA previously published (Parkinson et al. 2018))

**Figure 5** – comparison of observed modifications by DNCB, MCI, CA, DPCP and EA in HaCaT cell lysates (DNCB, MCI and CA previously published (Parkinson et al. 2018)) based on decreasing nucleophile concentration

**Tables**

**Table 1**. Comparison of reactivity data for DPCP and EA from studies using small peptide nucleophiles

|  |  |  |  |
| --- | --- | --- | --- |
| **DPCP** | **EA** | **Model peptide(s)** | **References** |
| Cys (98.8 and 100%)a  Lys (0.3 and -0.7%)a  His (-0.1%)b  GSH (22%)a | Cys (96.4 and 97.6%)a  Lys (24 and 93%)a  His – no data reported  GSH (89.8%)a | GSH, AcRFAAXAA (X=C, K or H) | (Gerberick et al. 2004; Gerberick et al. 2007) |
| Single adduct to Cys, His, Lys, Arg and N-terminus (Δmass 206 Dac)  Two further adducts to Cys (Δmass 222 Da and 224 Da, respectivelyd)  High His peptide depletion (70%)  No adducts with Tyr | Single adducts to Cys, Lys, His and N-terminus (Δmass 100 Da)  Double adduct to Lys (Δmass 200 Da)  Low His peptide depletion (5%)  No adducts with Tyr or Arg | AcFAAXAA (X=C, K, H, R or Y)  NH2FAAAAA  1:100 molar ratio of peptide to chemical | (Aleksic et al. 2009) |
| Single and double adducts (Δmass 206 Dac and 412 Da, respectively)  Peptide depletion 97.9%  Thiol depletion 100% | Single adduct (Δmass 100 Da)  Peptide depletion 98%  Thiol depletion 100% | AcNKKCDLF  1:10 molar ratio of peptide to chemical | (Natsch and Gfeller 2008) |
| Single adduct (Δmass 206 Dac)  Two further adducts (one Δmass 224 Dad) | Single adduct (Δmass 100 Da) | AcRFAACAA | (Roberts and Natsch 2009) |

a peptide depletion values reported from Gerberick et al 2007 (Gerberick et al. 2007) for 1:10 and 1:50 molar ratio of peptide to chemical, respectively and 1:100 molar ration of GSH to chemical

b peptide depletion values reported from Gerberick et al 2004 (Gerberick et al. 2004) for 1:50 molar ratio of peptide to chemical

c single DPCP adducts (Δmass 206 Da) observed here are isobaric with α,β-diphenylacrylamide adducts reported previously (Eicher 1975; Roberts and Natsch 2009), however, a suggested further Michael addition to the second Cys peptide (a crosslink)(Roberts and Natsch 2009)) has not been observed (DPCP peptide reactivity data not previously published).

d single DPCP adduct to Cys (Δmass 222 Da) consistent with thiol oxidation of the original adduct; further single adduct to Cys (Δmass 224 Da) consistent with ring opening of the original adduct by water addition to carbonyl. (DPCP peptide reactivity data not previously published).

**Table 2.** Structures, position of stable isotope (deuterium), potency category (including EC3 value, as derived from the local lymph node assay), Δ mass (Da) expected following haptenation, reactivity domain, variable modifications of sensitisers for database searching of MS data within PLGS.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Chemical  (MW (Da)) | Structure and position of stable isotope labels (\*) | Potency category (% EC3) | Δ mass (Da) expected for unlabelled and (deuterated) adduct | Expected adduct type |
| Diphencyprone  (206) |  | Extreme (Ryan et al. 2000) (0.0009) | +206.1 (+216.1) | single adduct |
| +222.1 (+232.1) | oxidation of single adduct |
| +224.1 (+234.1) | ring opening of single adduct |
| Ethyl acrylate  (100) |  | Weak (Gerberick et al. 2005) (28) | +100.1 (+105) | single Michael adduct |
| +200.1 (+210) | double Michael adduct |

**References**

Aleksic M, Thain E, Roger D, Saib O, Davies M, Li J, Aptula A, Zazzeroni R. 2009. Reactivity profiling: Covalent modification of single nucleophile peptides for skin sensitization risk assessment. Toxicological sciences : an official journal of the Society of Toxicology. 108(2):401-411.

Alvarez-Sanchez R, Divkovic M, Basketter D, Pease C, Panico M, Dell A, Morris H, Lepoittevin JP. 2004. Effect of glutathione on the covalent binding of the 13c-labeled skin sensitizer 5-chloro-2-methylisothiazol-3-one to human serum albumin: Identification of adducts by nuclear magnetic resonance, matrix-assisted laser desorption/ionization mass spectrometry, and nanoelectrospray tandem mass spectrometry. Chemical research in toxicology. 17(9):1280-1288.

Basketter D, Dooms-Goossens A, Karlberg AT, Lepoittevin JP. 1995. The chemistry of contact allergy: Why is a molecule allergenic? Contact dermatitis. 32(2):65-73.

Burden N, Sewell F, Andersen ME, Boobis A, Chipman JK, Cronin MT, Hutchinson TH, Kimber I, Whelan M. 2015. Adverse outcome pathways can drive non-animal approaches for safety assessment. Journal of applied toxicology : JAT. 35(9):971-975.

Choe SJ, Lee S, Lee H, Choi J, Lee WS. 2018. Efficacy of topical diphenylcyclopropenone maintenance treatment for patients with alopecia areata: A retrospective study. Journal of the American Academy of Dermatology. 78(1):205-207.e201.

Codreanu SG, Zhang B, Sobecki SM, Billheimer DD, Liebler DC. 2009. Global analysis of protein damage by the lipid electrophile 4-hydroxy-2-nonenal. Molecular & cellular proteomics : MCP. 8(4):670-680.

Conrad CC, Choi J, Malakowsky CA, Talent JM, Dai R, Marshall P, Gracy RW. 2001. Identification of protein carbonyls after two-dimensional electrophoresis. Proteomics. 1(7):829-834.

Dearman RJ, Betts CJ, Farr C, McLaughlin J, Berdasco N, Wiench K, Kimber I. 2007. Comparative analysis of skin sensitization potency of acrylates (methyl acrylate, ethyl acrylate, butyl acrylate, and ethylhexyl acrylate) using the local lymph node assay. Contact dermatitis. 57(4):242-247.

ECETOC-DocNo46. 2008. Potency values from the local lymph node assay: Application to classification, labelling and risk assessment

Eicher TW, J.L. 1975. Structure and reactivity of cyclopropenones and triafulvenes. Cyclic compounds. . Topics in Current Chemistry. 57:1109.

Elokda AS, Nielsen DH. 2007. Effects of exercise training on the glutathione antioxidant system. European journal of cardiovascular prevention and rehabilitation : official journal of the European Society of Cardiology, Working Groups on Epidemiology & Prevention and Cardiac Rehabilitation and Exercise Physiology. 14(5):630-637.

Emter R, Ellis G, Natsch A. 2010. Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers in vitro. Toxicology and applied pharmacology. 245(3):281-290.

Esser PR, Kimber I, Martin SF. 2014. Correlation of contact sensitizer potency with t cell frequency and tcr repertoire diversity. Experientia supplementum (2012). 104:101-114.

Gerberick GF, Ryan CA, Kern PS, Schlatter H, Dearman RJ, Kimber I, Patlewicz GY, Basketter DA. 2005. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. Dermatitis : contact, atopic, occupational, drug. 16(4):157-202.

Gerberick GF, Vassallo JD, Bailey RE, Chaney JG, Morrall SW, Lepoittevin JP. 2004. Development of a peptide reactivity assay for screening contact allergens. Toxicological sciences : an official journal of the Society of Toxicology. 81(2):332-343.

Gerberick GF, Vassallo JD, Foertsch LM, Price BB, Chaney JG, Lepoittevin JP. 2007. Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach. Toxicological sciences : an official journal of the Society of Toxicology. 97(2):417-427.

Geromanos SJ, Vissers JP, Silva JC, Dorschel CA, Li GZ, Gorenstein MV, Bateman RH, Langridge JI. 2009. The detection, correlation, and comparison of peptide precursor and product ions from data independent lc-ms with data dependant lc-ms/ms. Proteomics. 9(6):1683-1695.

Harper Smith AD, Coakley SL, Ward MD, Macfarlane AW, Friedmann PS, Walsh NP. 2011. Exercise-induced stress inhibits both the induction and elicitation phases of in vivo t-cell-mediated immune responses in humans. Brain, behavior, and immunity. 25(6):1136-1142.

Hirota M, Motoyama A, Suzuki M, Yanagi M, Kitagaki M, Kouzuki H, Hagino S, Itagaki H, Sasa H, Kagatani S et al. 2010. Changes of cell-surface thiols and intracellular signaling in human monocytic cell line thp-1 treated with diphenylcyclopropenone. The Journal of toxicological sciences. 35(6):871-879.

Hong F, Sekhar KR, Freeman ML, Liebler DC. 2005. Specific patterns of electrophile adduction trigger keap1 ubiquitination and nrf2 activation. The Journal of biological chemistry. 280(36):31768-31775.

Jacobs AT, Marnett LJ. 2010. Systems analysis of protein modification and cellular responses induced by electrophile stress. Accounts of chemical research. 43(5):673-683.

Jacquoilleot S, Sheffield D, Olayanju A, Sison-Young R, Kitteringham NR, Naisbitt DJ, Aleksic M. 2015. Glutathione metabolism in the hacat cell line as a model for the detoxification of the model sensitisers 2,4-dinitrohalobenzenes in human skin. Toxicology letters. 237(1):11-20.

Kimber I, Basketter DA, Gerberick GF, Ryan CA, Dearman RJ. 2011. Chemical allergy: Translating biology into hazard characterization. Toxicological sciences : an official journal of the Society of Toxicology. 120 Suppl 1:S238-268.

Kimber I, Dearman RJ. 2003. What makes a chemical an allergen? Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology. 90(5 Suppl 2):28-31.

Landsteiner K, Jacobs J. 1935. Studies on the sensitization of animals with simple chemical compounds. The Journal of experimental medicine. 61(5):643-656.

Lepoittevin JP. 2006. The chemistry of skin allergy. Altex. 23 Suppl:234-238.

Liu Q, Simpson DC, Gronert S. 2012. The reactivity of human serum albumin toward trans-4-hydroxy-2-nonenal. Journal of mass spectrometry : JMS. 47(4):411-424.

MacKay C, Davies M, Summerfield V, Maxwell G. 2013. From pathways to people: Applying the adverse outcome pathway (aop) for skin sensitization to risk assessment. Altex. 30(4):473-486.

Maxwell G, MacKay C, Cubberley R, Davies M, Gellatly N, Glavin S, Gouin T, Jacquoilleot S, Moore C, Pendlington R et al. 2014. Applying the skin sensitisation adverse outcome pathway (aop) to quantitative risk assessment. Toxicology in vitro : an international journal published in association with BIBRA. 28(1):8-12.

Mello CF, Sultana R, Piroddi M, Cai J, Pierce WM, Klein JB, Butterfield DA. 2007. Acrolein induces selective protein carbonylation in synaptosomes. Neuroscience. 147(3):674-679.

Michalski A, Cox J, Mann M. 2011. More than 100,000 detectable peptide species elute in single shotgun proteomics runs but the majority is inaccessible to data-dependent lc-ms/ms. Journal of proteome research. 10(4):1785-1793.

Natsch A, Gfeller H. 2008. Lc-ms-based characterization of the peptide reactivity of chemicals to improve the in vitro prediction of the skin sensitization potential. Toxicological sciences : an official journal of the Society of Toxicology. 106(2):464-478.

OECD. 2014. The adverse outcome pathway for skin sensitisation initiated by covalent binding to proteins.

Park JY, Park BW, Cho EB, Park EJ, Kim KH, Kim KJ. 2018. Clinical efficacy of diphenylcyclopropenone immunotherapy as monotherapy for multiple viral warts. Journal of cutaneous medicine and surgery.1203475418764106.

Parkinson E, Aleksic M, Cubberley R, Kaur-Atwal G, Vissers JPC, Skipp P. 2018. Determination of protein haptenation by chemical sensitisers within the complexity of the human skin proteome. Toxicological sciences : an official journal of the Society of Toxicology.

Parkinson E, Boyd P, Aleksic M, Cubberley R, O'Connor D, Skipp P. 2014a. Stable isotope labeling method for the investigation of protein haptenation by electrophilic skin sensitizers. Toxicological sciences : an official journal of the Society of Toxicology. 142(1):239-249.

Parkinson E, Skipp P, Aleksic M, Garrow A, Dadd T, Hughes M, Clough G, O'Connor CD. 2014b. Proteomic analysis of the human skin proteome after in vivo treatment with sodium dodecyl sulphate. PloS one. 9(5):e97772.

Pickard C, Louafi F, McGuire C, Lowings K, Kumar P, Cooper H, Dearman RJ, Cumberbatch M, Kimber I, Healy E et al. 2009. The cutaneous biochemical redox barrier: A component of the innate immune defenses against sensitization by highly reactive environmental xenobiotics. Journal of immunology (Baltimore, Md : 1950). 183(11):7576-7584.

Roberts DW, Natsch A. 2009. High throughput kinetic profiling approach for covalent binding to peptides: Application to skin sensitization potency of michael acceptor electrophiles. Chemical research in toxicology. 22(3):592-603.

Ryan CA, Gerberick GF, Cruse LW, Basketter DA, Lea L, Blaikie L, Dearman RJ, Warbrick EV, Kimber I. 2000. Activity of human contact allergens in the murine local lymph node assay. Contact dermatitis. 43(2):95-102.

Sanderson PN, Simpson W, Cubberley R, Aleksic M, Gutsell S, Russell PJ. 2016. Mechanistic understanding of molecular initiating events (mies) using nmr spectroscopy. Toxicology Research. 5(1):34-44.

Shearn CT, Fritz KS, Shearn AH, Saba LM, Mercer KE, Engi B, Galligan JJ, Zimniak P, Orlicky DJ, Ronis MJ et al. 2016. Deletion of gsta4-4 results in increased mitochondrial post-translational modification of proteins by reactive aldehydes following chronic ethanol consumption in mice. Redox biology. 7:68-77.

Shevchenko A, Jensen ON, Podtelejnikov AV, Sagliocco F, Wilm M, Vorm O, Mortensen P, Shevchenko A, Boucherie H, Mann M. 1996. Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two dimensional gels. Proc Natl Acad Sci U S A. 93(25):14440-14445.

Simonsson C, Andersson SI, Stenfeldt AL, Bergstrom J, Bauer B, Jonsson CA, Ericson MB, Broo KS. 2011. Caged fluorescent haptens reveal the generation of cryptic epitopes in allergic contact dermatitis. The Journal of investigative dermatology. 131(7):1486-1493.

Spencer A, Gazzani P, Thompson DA. 2016. Acrylate and methacrylate contact allergy and allergic contact disease: A 13-year review. Contact dermatitis. 75(3):157-164.

Spiess PC, Deng B, Hondal RJ, Matthews DE, van der Vliet A. 2011. Proteomic profiling of acrolein adducts in human lung epithelial cells. Journal of proteomics. 74(11):2380-2394.

Spriggs S, Sheffield D, Olayanju A, Kitteringham NR, Naisbitt DJ, Aleksic M. 2016. Effect of repeated daily dosing with 2,4-dinitrochlorobenzene on glutathione biosynthesis and nrf2 activation in reconstructed human epidermis. Toxicological sciences : an official journal of the Society of Toxicology. 154(1):5-15.

Suzuki M, Hirota M, Hagino S, Itagaki H, Aiba S. 2009. Evaluation of changes of cell-surface thiols as a new biomarker for in vitro sensitization test. Toxicology in vitro : an international journal published in association with BIBRA. 23(4):687-696.

Thyssen JP, Linneberg A, Menne T, Johansen JD. 2007. The epidemiology of contact allergy in the general population--prevalence and main findings. Contact dermatitis. 57(5):287-299.

Zerbinati N, Esposito C, D'Este E, Calligaro A, Valsecchi R. 2018. Topical immunotherapy of alopecia areata: A large retrospective study. Dermatology and therapy. 8(1):101-110.