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## Toxicology in Vitro

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## Mass spectrometric identification of covalent adducts of the skin allergen 2,4-dinitro-1-chlorobenzene and model skin proteins

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### ARTICLE INFO

#### Article history:

Received 30 November 2007

Accepted 11 March 2008

Available online xxxx

#### Keywords:

Skin sensitisation

Human keratin 14

Human cofilin

Protein adducts

Mass spectrometry

*In vitro*

Hapten

### ABSTRACT

A large proportion of allergic skin reactions are considered to be the result of skin exposure to small organic chemicals that possess the intrinsic ability to covalently modify skin proteins, either directly or following activation. In the absence of information about specific skin protein targets, studies of chemical modifications are limited to the use of model proteins. We have previously demonstrated that selected well known skin sensitizers (2,4-dinitro-1-chlorobenzene and phenyl salicylate) have the ability to covalently modify residues selectively on the model protein, human serum albumin. In the present work, we focus on the differences in covalent binding observed for two additional model proteins, human cytokeratin 14 and human cofilin, both constituent proteins of skin. Using matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) and nano LC-MS and -MS/MS strategies, the amino acid residues targeted by 2,4-dinitro-1-chlorobenzene on the two model proteins have been identified. In contrast, a structurally related non-sensitizer (2,4-dichloro-1-nitrobenzene) and a non-sensitising irritant (benzalkonium chloride) did not covalently modify the model proteins. Detailed examination of the results for the sensitizers indicate that reactive chemicals target nucleophilic amino acids residing in specific microenvironments of the 3D protein structure that are conducive to reactivity. This observation has important implications for the development of hapten-peptide binding assays. It is envisaged that the data from such assays will be integrated with outputs from other *in vitro* assays in the future to give a prediction of the sensitisation potential of novel chemicals.

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### 1. Introduction

Chemical reactivity with skin proteins is a key step in the induction of skin sensitisation (Landsteiner and Jacobs, 1935; Dupuis and Benezra, 1982; Lepoittevin et al., 1998; Divkovic et al., 2005; Divkovic, 2006). Skin sensitizers are either directly protein-reactive or can be activated by interaction with environment or metabolising enzymes in skin (Karlberg et al., 1992; Smith and Hotchkiss, 2001). Knowledge about how sensitising chemicals react with

proteins could contribute towards the development of new non-animal experimental models that can generate data to help in predicting whether new chemicals have the potential to act as skin sensitizers. Today, the murine local lymph node assay (LLNA) (Basketter et al., 2001) is used for the assessment of skin sensitisation potential of chemicals. However, with implementation of the 7th amendment of the EU Cosmetics Directive in 2013 (EU, 2003) this assay can no longer be used for cosmetic ingredients. Development of reliable *in vitro/in chemico/in silico* testing strategies and effective integration of their data outputs to address this endpoint will only be possible through a detailed understanding of the key events in the induction of skin sensitisation (Fentem et al., 2004; Jowsey et al., 2006). Covalent modification of proteins by chemicals is one of the determining events in skin sensitisation and therefore better understanding of the chemical mechanisms and specificity is needed to develop new assays for chemical reactivity for the skin sensitisation induction process. It is envisaged that data from such assays (Gerberick et al., 2004a,b; Aptula et al., 2006; Gerberick et al., 2007) could be used for screening purposes or integrated with data from other related *in vitro* predictive assays (e.g. skin penetration assays, dendritic cell activation assays, T cell

**Abbreviations:** ACD, allergic contact dermatitis; LLNA, local lymph node assay; MALDI-MS, matrix assisted laser desorption/ionisation mass spectrometry; ES-MS, electrospray mass spectrometry; ES-MS/MS, electrospray tandem mass spectrometry; MeCN, acetonitrile; TFA, trifluoroacetic acid; MHC, major histocompatibility complex; HSA, human serum albumin; K14, human cytokeratin 14; DTT, dithiothreitol; DNCB, 2,4-dinitro-1-chlorobenzene; DCNB, 2,4-dichloro-1-nitro benzene; BC, benzalkonium chloride; DNP, dinitrophenol; TPCK, L-(tosylamido-2-phenyl) ethyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid.

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proliferation assays, etc. (Jowsey et al., 2006)) for future estimation of relative skin sensitising potency of chemicals.

Due to the complexity of the skin proteome and the lack of knowledge about the specific protein targets of sensitising chemicals, investigations of reactions between proteins and allergens are largely focused on the use of isolated models, which have previously included model nucleophiles (Alvarez-Sanchez et al., 2003; Franot et al., 1994; Meschkat et al., 2001a,b), peptides (Ahlfors et al., 2003; Alvarez-Sanchez et al., 2004a,b; Hanson et al., 1999; Nilsson et al., 2005) and proteins (Alvarez-Sanchez et al., 2004a,b; Meschkat et al., 2001a,b). To investigate reactions between chemical allergens and a protein, we have previously used human serum albumin (HSA) as a representative protein abundant in skin (Aleksic et al., 2007) and reported high specificity in amino acid residue modification by known sensitisers 2,4-dichloro-1-nitro benzene (DNCB) and phenyl salicylate. However, as a secreted protein, HSA is rich in disulphide bonds and has only one free Cys residue (Peters, 1996). Here we are using two additional skin proteins as models to further explore chemical reactions with a sensitiser, non-sensitiser and an irritant to test the hypotheses that (a) that the microenvironments within different proteins cause covalent modification of specific nucleophilic residues and (b) than non-sensitisers and irritants do not bind to proteins under any conditions.

Human cytokeratin 14 (K14, MW 51663 Da) is type I keratin expressed in the basal layer of epidermis, where it forms filaments with its type II pair, K5. Keratins are the most abundant epidermal proteins, likely to be amongst the first biological macromolecules to encounter the hapten (Elahi et al., 2004). These are major structural proteins in keratinocytes where they form a cytoplasmic network of intermediate filaments. Human cofilin (MW 18503 Da) is a cytoskeletal actin binding protein found in dendritic cells (antigen presenting cells) and keratinocytes. Phosphorylation of Ser at position 3 of cofilin interferes with actin binding in immature dendritic cells (Moriyama et al., 1996) resulting in their activation. A recent study shows that cofilin is potentially involved in signalling events during dendritic cell maturation (Verdijk et al., 2004). Both K14 and cofilin are cytosolic proteins and thus have no disulphide bridges, there-

fore all of their Cys residues (5 Cys residues on K14 (4, 18, 40, 367 and 389) and 4 on cofilin (39, 80, 139 and 147)) are theoretically available for reaction with chemicals. Additionally, both proteins are commercially available at a high level of purity.

In this study we incubated a known skin sensitiser DNCB, a known non-sensitiser 2,4-dichloro-1-nitrobenzene (DCNB) and a known irritant benzalkonium chloride (BC) (Fig. 1A, 1, 2 and 3, respectively) with model proteins to establish their intrinsic ability to modify amino acid residues and to investigate residue specificity. DNCB is a strong sensitiser and it reacts with specific nucleophiles on HSA via nucleophilic substitution reaction on an aromatic ring (Fig. 1B) resulting in a stable dinitrophenol (DNP) adduct (Aleksic et al., 2007). In contrast, DCNB is often used as a non-sensitising structural analogue to DNCB and thus is presumed not to covalently modify proteins (although a weak sensitising potential of DCNB has been demonstrated in the LLNA (Basketter et al., 1996)). The irritant BC was also incubated with model proteins to investigate its binding potential to model proteins. Irritant responses are not due to the chemical ability to covalently modify proteins, but arise from the direct toxic effect of the irritant chemical on the exposed cells.

## 2. Materials and methods

### 2.1. Sample preparation

Test chemicals 2,4-dinitro-1-chlorobenzene (DNCB), 2,4-dichloro-1-nitrobenzene (DCNB) and benzalkonium chloride (BC) were supplied by Sigma as >95% pure and solubilised in acetone. The working solutions of chemicals were further diluted in buffer corresponding with protein solutions they were incubated with. Recombinant human K14 was supplied by Progen Biotechnik (Heidelberg, Germany) as 250 µg lyophilised powder and reconstituted in 175 µl MilliQ water, obtaining a working solution at a concentration of 1 mg/ml in 30 mM TRIS HCl buffer (pH 8.0) containing 9.5 M urea, 2 mM dithiothreitol (DTT), 2 mM EDTA and 10 mM methylammonium chloride. Recombinant human cofilin was supplied from Cytoskeleton Inc. (Denver, USA) as 100 µg lyophilised powder

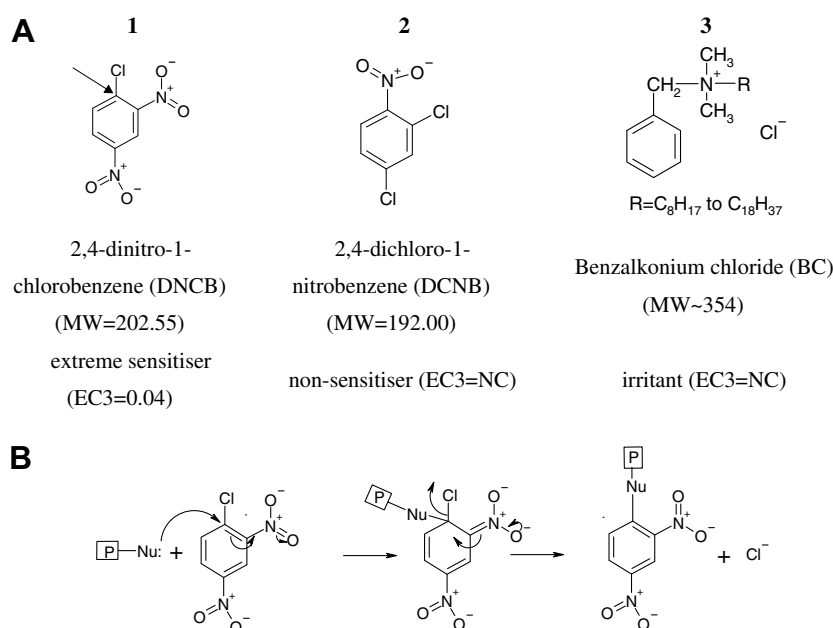


**Fig. 1.** (A) Structures, class and MW of compounds used in protein binding experiments: (electrophilic centre indicated by arrows). (B) Reaction mechanism of DNCB with nucleophile is a nucleophilic substitution on the aromatic ring, where the mass shift expected is 166 Da (Aleksic et al., 2007); (EC3 value reported from (Gerberick et al., 2004) NC not calculated); P – protein; Nu – nucleophile.

and reconstituted in 40  $\mu$ l of MilliQ water obtaining a working solution at a concentration of 5 mg/ml in 20 mM TRIS buffer (pH 7.4) containing 20 mM NaCl and 1 mM DTT. HSA was purchased from Sigma (>99%, fatty acid free) and served as a positive control taking account of previously described DNCB modifications of HSA. HSA solution was prepared at a 1 mg/ml concentration in 30 mM TRIS HCl buffer (pH 8.0) containing 2 mM DTT and 2 mM EDTA. Samples were prepared by mixing solutions of each test chemical and protein at a 1:100 protein:chemical molar ratio. However, the effective nucleophile:electrophile molar ratios for these experiments are 1:1.2 for K14, 1:0.8 for HSA and 1:2.4 for cofilin, respectively, calculated using the number of nucleophilic side chains on each protein. Control samples were prepared for each protein by mixing the protein solutions with an appropriate dilution of acetone in water. Samples were incubated at 37 °C for 7 days. Remaining test chemical was removed by dialysis against 50 mM ammonium bicarbonate through a CelluSep<sup>®</sup> dialysis membrane (Triple Red Ltd. Oxon, UK) (MWCO 12–14 kDa for HSA and K14 or 3.5 kDa for cofilin) for 24 h at 4 °C with one change of buffer solution after 5–6 h. After lyophilisation samples were reduced in a solution of DTT in degassed TRIS buffer (0.6 M, pH 8.5, for 45 min at 37 °C) and derivatised by addition of iodoacetic acid (at room temperature in the dark for 90 min). The reaction was terminated by dialysis, samples were lyophilised and digested by trypsin as previously described (Aleksic et al., 2007) and reconstituted in 0.1% trifluoroacetic acid prior to MALDI-MS and ES-MS/MS analysis.

## 2.2. Mass spectrometric analysis

### 2.2.1. MALDI-MS analyses

MALDI mass spectra were obtained on a Voyager-DE STR ToF mass spectrometer (Applied Biosystems, Foster City, CA) in the reflector mode employing delayed extraction, with an accelerating voltage of 20 kV and a 3 m flight path. The instrument is equipped with a nitrogen laser operating at 337 nm. Following lyophilisation the digested peptides were co-crystallised with  $\alpha$ -cyano-4-hydroxycinnamic acid ( $\alpha$ -CHCA), a saturated solution of which was prepared by dissolving ~10 mg of  $\alpha$ -CHCA in 1 mL of 50% acetonitrile (MeCN) in 0.3% trifluoroacetic acid (TFA). Peptide samples (~1  $\mu$ l) were deposited on a stainless steel target plate and an equivalent volume of matrix solution was added and gently mixed. Co-crystallisation was achieved by drying the mixture under vacuum. Data were acquired and processed using the Voyager Data Explorer software (Applied Biosystems, Foster City, CA). The ToF data were externally calibrated using leucine enkephalin, bradykinin, adrenocorticotrophic hormone (ACTH) fragment 1–7, ACTH fragment 7–38, ACTH fragment 1–39 and bovine insulin as standards.

### 2.2.2. Online-nano LC-ES MS and MS/MS analyses

Tryptic digests were analysed by nano-LC-MS/MS using a reverse-phase nano-HPLC system (Dionex, Sunnyvale) connected to a quadrupole ToF mass spectrometer (Q-STAR Pulsar-i, MDS Sciex, Canada). The peptides were separated by a binary nano-HPLC gradient generated by an Ultimate pump fitted with a Famos autosampler and a Switchos micro column switching module (LC Packings, Amsterdam, Netherlands). An analytical C<sub>18</sub> nanocapillary (75 mm i.d.  $\times$  15 cm, PepMap) and a micro pre-column C<sub>18</sub> cartridge were employed for on-line peptide separation. The mixture was first loaded onto the pre-column and eluted with 0.1% formic acid in water for 4 min. The eluent was then transferred onto an analytical C<sub>18</sub> nanocapillary HPLC column and eluted at a flow rate of 150 nl/min using the following gradient: 0–5 min 99% A, 5–10 min 99–90% A, 10–70 min 90–60% A, 70–71 min 60–50% A, 71–75 min 50–5% A, 75–85 min 5% A, 85–86 min 5–95% A, and

86–90 min 95% A. Solvent A = 0.05% (v/v) formic acid in 95:5 (v/v) water: MeCN; solvent B = 0.04% formic acid in 95:5 (v/v) MeCN:–water. Data acquisition was performed using Analyst QS software with an automatic information dependent acquisition (IDA) function.

All interpretations of MS and MS/MS spectra were carried out without the aid of automated sequencing programs.

## 3. Results

### 3.1. Peptide mapping

Initial peptide mapping MALDI MS analyses of control samples from both model proteins showed that all of the observed signals could be identified as native K14 or cofilin peptides, derived from tryptic digestion or assigned to peptides produced by trypsin auto-digestion. These analyses also revealed that on average more than 90% of the each protein sequence was observed in any one MALDI-ToF spectrum (Fig. 2A and B). Therefore, reliable detection of covalent adducts could be confirmed by appearance of completely new signals with mass shifts corresponding to hapten adducts. As previously observed with HSA analyses (Aleksic et al., 2007), the relative signal intensities were variable depending on the position of the laser beam and laser intensity at the time of MALDI-MS data acquisition.

### 3.2. Reactions of chemicals with model proteins

The incubation conditions used in these experiments allowed significant levels of protein modification and are not intended to reflect *in vivo* conditions. All samples remained clear in appearance throughout the incubation time indicating that no bacterial growth or protein aggregation occurred, however some denaturation is likely to have occurred in K14 samples due to a high urea concentration.

Using K14 and cofilin as model proteins, comparison of the DNCB sample spectra (Fig. 3A and B) to the control spectra (Fig. 2A and B) has revealed new signals in the DNCB sample spectra which could not be assigned to native K14, cofilin or trypsin autodigestion peptides. New signals (circled in Fig. 3) are accompanied by previously reported satellite signals representing the loss of 16 amu, thought to be an artefact of the MALDI ionisation process. Concomitantly, some strong signals observed in Fig. 2 appear significantly reduced in intensity in Fig. 3, indicating that DNCB may have modified the peptides represented by them and/or affected the tryptic digestion (for example, for K14 sample  $m/z$  767,  $m/z$  1107 and  $m/z$  1425 (Fig. 3A), for cofilin sample  $m/z$  659 (Fig. 3B)).

BC is classed as an irritant (Basketter et al., 2004) and DNCB is classed as a non-sensitiser although it has been reported to have a weak sensitising potential in the LLNA (Basketter et al., 1996). MALDI-MS analyses of DNCB and BC samples did not show any new signals in comparison to the respective control sample spectra, which could not be assigned to either native K14 or cofilin peptides or products of the trypsin autodigestion (data not shown).

### 3.3. Identification of DNCB-protein adducts

To further confirm these observations, and to determine the exact localisation of the modifications, samples were analysed using nanoES-MS/MS. As previously observed with HSA, DNCB covalently modified select nucleophilic HSA residues via a nucleophilic substitution on an aromatic ring. The reaction involved a loss of chlorine and resulted in an observable mass shift of 166 Da corresponding to dinitrophenol (DNP) adduct. When incubated with K14, DNCB showed the highest preference for tyrosine, with five residues (of

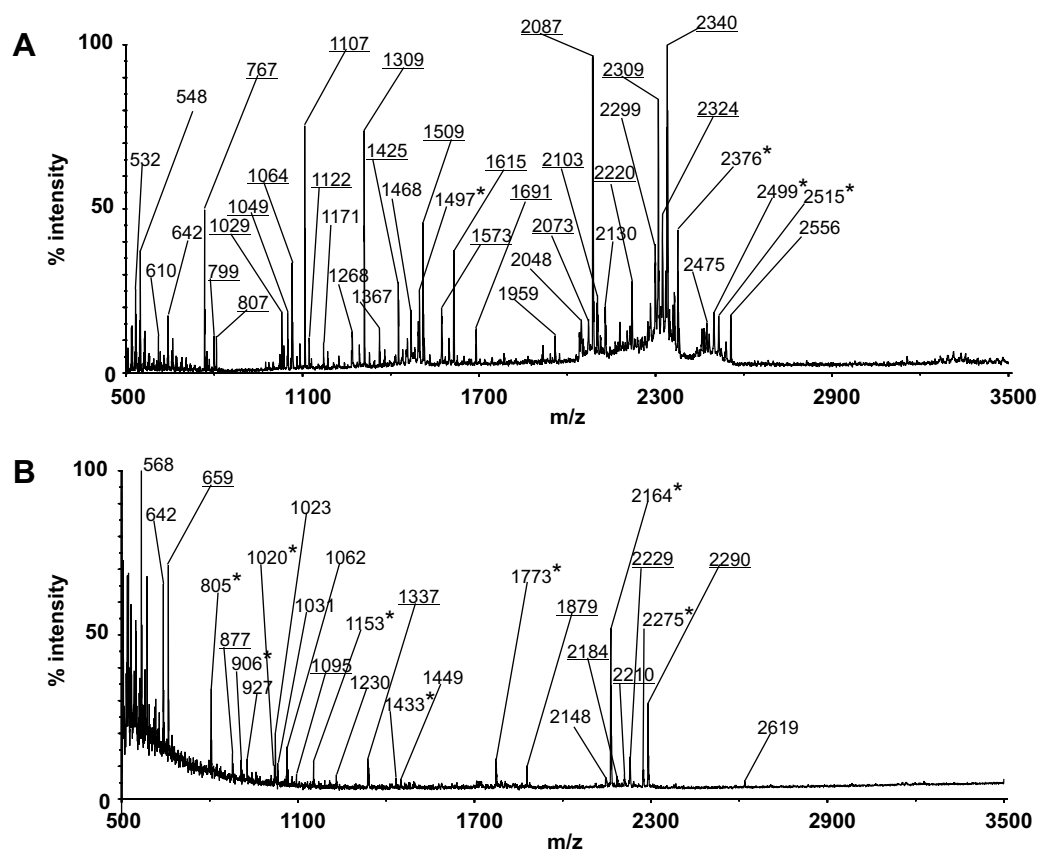


**Fig. 2.** Model protein peptide maps following tryptic digestion: (A) human cytokeratin 14 (K14) and (B) human cofilin; underlined are K14 and cofilin peptides mapped using the MALDI-TOF instrument; \* peptides resulting from trypsin autodigestion.

14) found modified (Tyr 46, 129, 150, 204 and 415). Also modified were Lys 132, 203 and 363 (of 22) as well as a single Cys residue (Cys 40, of 5) (Table 1). No His or N-terminal modifications were located in the K14-DNCB sample. When incubated with cofilin, DNCB modified three (of 4) cysteines (Cys 39, 80 and 147), two (of 25) lysines (Lys 19 and 78) and a single Tyr residue (Tyr 82, of 6) (Table 1).

DNCB showed a differential preference for Lys and Tyr residues. Interestingly, two Tyr residues, Tyr 129 and 204 were found modified by DNCB only when the neighbouring Lys residues (Lys 132 and 203, respectively) were unexpectedly unavailable during the incubation. The observed low level of Lys modification with the net mass shift of 43 Da is probably carbamylation of Lys side chains resulting from urea in the incubation medium. K14 is commercially supplied lyophilised with high urea content acting as a chaotropic agent. This rendered some Lys residues unavailable for reaction with DNCB, thus the closest Tyr residues were modified. Tyr 129 and 204 were not found modified if respective Lys residues (Lys 132 and 204) were free to react with DNCB. This is evident from the online nano-LC-MS/MS analysis of doubly charged signals at  $m/z$  717 and  $m/z$  738. These signals correspond to the singly charged signals at  $m/z$  1432 and  $m/z$  1475, respectively, observed in the MALDI-MS spectrum. The signal at  $m/z$  1432 represents the K14 peptide TKYETELNLR (residues 202–211) modified at Lys 203 and the signal at  $m/z$  1475 represents the same peptide modified at Tyr 204 when the side chain of Lys 203 is carbamylated. Fig. 4 shows the CAD MS/MS spectra resulting from the fragmentation of the doubly charged  $m/z$  738.3 ion. The spectra produced allowed assignment of the initial  $b$  ions ( $b_1$  and  $b_2$ ), with the corresponding  $a$  ion series and ions representing loss of ammonia ( $-17$  amu). Clearly, the  $b_2$  ion appears modified by

43 amu (Fig. 5C). The  $a_3$  ion indicates that Tyr is the site of modification and this is further confirmed by the presence of the  $y''_8$  ion which is clearly 166 amu higher than its counterparts in the theoretical fragmentation pattern and in case of adjacent Lys modification (Fig. 5A and B, respectively). These analyses confirmed that the site of the DNCB modification is Tyr 204 when Lys 203 is carbamylated.

Comparable results were obtained when the doubly charged signals at  $m/z$  615 and  $m/z$  638 were analysed. The signal at  $m/z$  615 represents the K14 peptide LASYLDKVR (residues 126–134) modified by DNCB at Lys 132 and the signal at  $m/z$  638 represents the same peptide carbamylated at Lys 132 and modified by DNCB at Tyr 129.

Additionally, K14, cofilin and HSA protein samples incubated with DNCB and BC have been subjected to nanoLC-ES-MS/MS analyses and have failed to produce any evidence of covalent modifications of the model proteins by DNCB or BC. It was therefore concluded that neither DNCB nor BC covalently modify the three model proteins in this test system.

#### 4. Discussion

In this study, the nature and specificity of covalent modification of proteins by chemicals has been interrogated by expanding the range of model proteins examined from the single secreted protein HSA (Aleksic et al., 2007) to encompass two representative cytoplasmic proteins. For this purpose, a highly abundant skin protein from the intermediate filament family (cytokeratin 14) and an intracellular signalling protein involved in maturation of Dendritic cells (cofilin) were selected. Through this work, in both additional skin proteins, we have obtained evidence of the promiscuous nat-

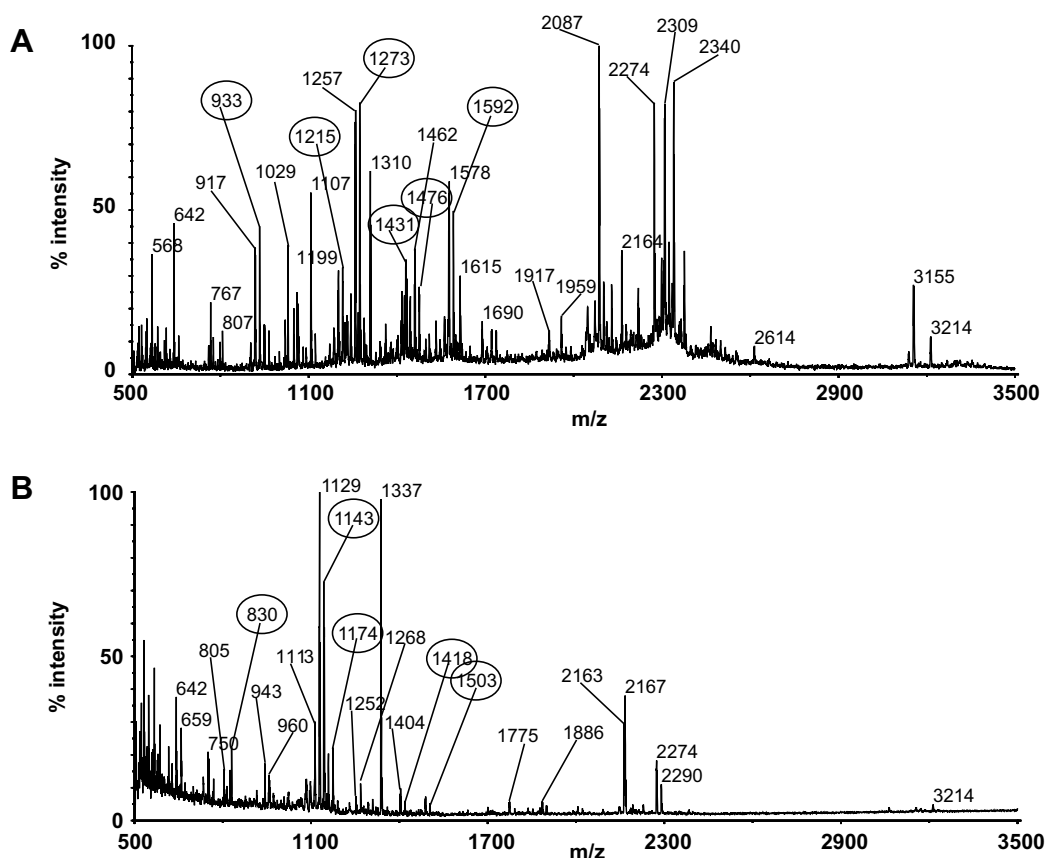


**Fig. 3.** Partial MALDI-MS spectra of the model protein samples incubated with DNCB: (A) human cytochrome 14 (K14) and (B) human coflin; signals not previously observed in the control sample spectra (Fig. 2A and B) are circled and represent peptides modified by DNCB mapped using the MALDI-ToF instrument (mass shift of 166 Da); the circled signals are preceded by a satellite signals representing the loss of 16 amu (e.g. circled signals in spectrum A at  $m/z$  933 and 1273 and their respective satellite signals at  $m/z$  917 and 1257).

**Table 1**

Summary of signals observed in MALDI-MS and ES-MS/MS experiments for DNCB samples incubated with K14 and coflin

| K14-DNCB sample           |                          | Corresponding control K14 signal ( $m/z$ )    |                          | K14 sequence – modified residue in bold (residue position) ( $K_{ca}$ – carbamylated Lys) |
|---------------------------|--------------------------|-----------------------------------------------|--------------------------|-------------------------------------------------------------------------------------------|
| Modified signal ( $m/z$ ) |                          |                                               |                          |                                                                                           |
| ( $M+H$ ) <sup>+</sup>    | ( $M+2H$ ) <sup>2+</sup> | ( $M+H$ ) <sup>+</sup>                        | ( $M+2H$ ) <sup>2+</sup> |                                                                                           |
| 933                       | 467                      | 766                                           | 383.5                    | DWYQR (150)                                                                               |
| 1215                      | 608                      | 1049                                          | 525                      | ISSVLGGSCR (40)                                                                           |
| 1230                      | 615.5                    | 1064                                          | 532.5                    | LASYLDKVR (132)                                                                           |
| 1273                      | 637                      | 1107                                          | 554                      | LASYLK <sub>ca</sub> VR (129)                                                             |
| 1288                      | 644.5                    | 1122                                          | 561.5                    | LEQEIATYR (415)                                                                           |
| 1431                      | 716                      | 1265                                          | 633                      | TKYETELNLR (203)                                                                          |
| 1476                      | 738.5                    | 1309                                          | 655                      | TK <sub>ca</sub> YETELNLR (204)                                                           |
| 1592                      | 796.5                    | 1426                                          | 713.5                    | APNTYGGGLSVSSSR (46)                                                                      |
| 1599                      | 800                      | 1433                                          | 717                      | ASLENSLEETKGR (363)                                                                       |
| Coflin-DNCB sample        |                          | Corresponding control coflin signal ( $m/z$ ) |                          | Coflin sequence – modified residue in bold (residue position)                             |
| Modified signal ( $m/z$ ) |                          |                                               |                          |                                                                                           |
| ( $M+H$ ) <sup>+</sup>    | ( $M+2H$ ) <sup>2+</sup> | ( $M+H$ ) <sup>+</sup>                        | ( $M+2H$ ) <sup>2+</sup> |                                                                                           |
| 830                       | 415.5                    | 664                                           | 332.5                    | CTLAEK (147)                                                                              |
| 1143                      | 572                      | 977                                           | 489                      | MLPDKDCR (80)                                                                             |
| 1174                      | 587.5                    | 1008                                          | 504.5                    | VFNDMKVR (19)                                                                             |
| 1309                      | 655                      | 977                                           | 489                      | MLPDKDCR (78 and 80)                                                                      |
| 1418                      | 709.5                    | 1252                                          | 626.5                    | AVLFCLSEDKK (39)                                                                          |
| 1503                      | 752                      | 1337                                          | 669                      | YALYDATYETK (82)                                                                          |

ure of DNCB covalent modification of amino acid side chains, a result consistent with our earlier observations with HSA. Overall, DNCB was found to be reactive with Lys, Cys and Tyr residues on K14 and coflin. However, previously observed His and N-terminus modifications by DNCB when HSA was employed as the model pro-

tein (Aleksic et al., 2007) were not identified using K14 and coflin. This could be explained by the inaccessibility of His side chains and N-termini, lower affinity of DNCB towards His residues compared to Cys, Lys and Tyr residues, fewer His residues in K14 and coflin (3 in K14 and only 1 in coflin), as well as the relatively high pH of

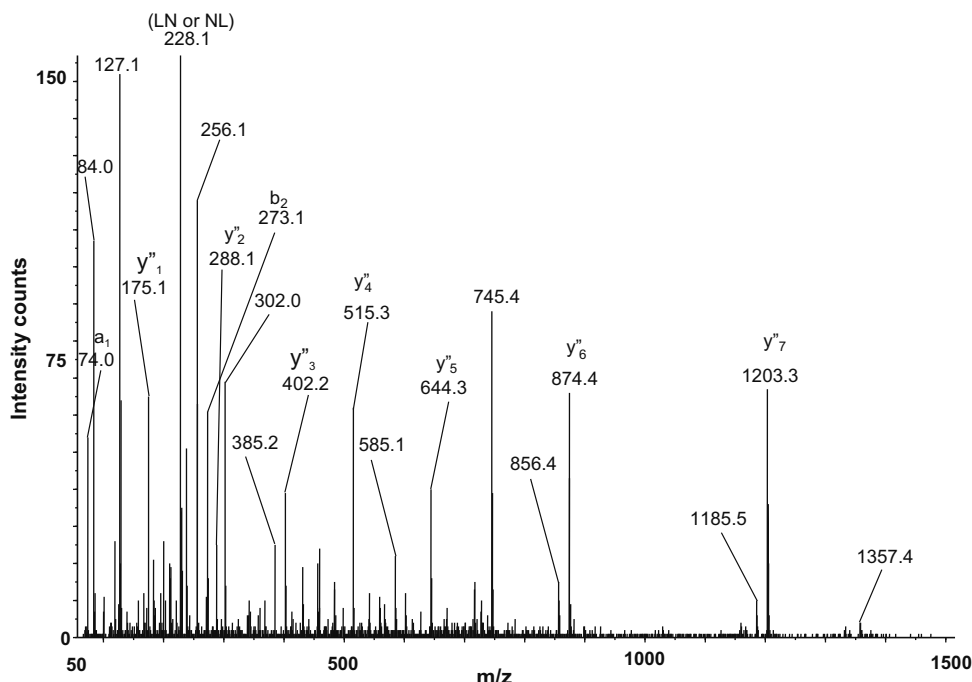


Fig. 4. CAD MS/MS spectra of the  $[M+2H]^{2+}$  738.3 ion, derived from a tryptic digest of the K14 incubated with DNCB.

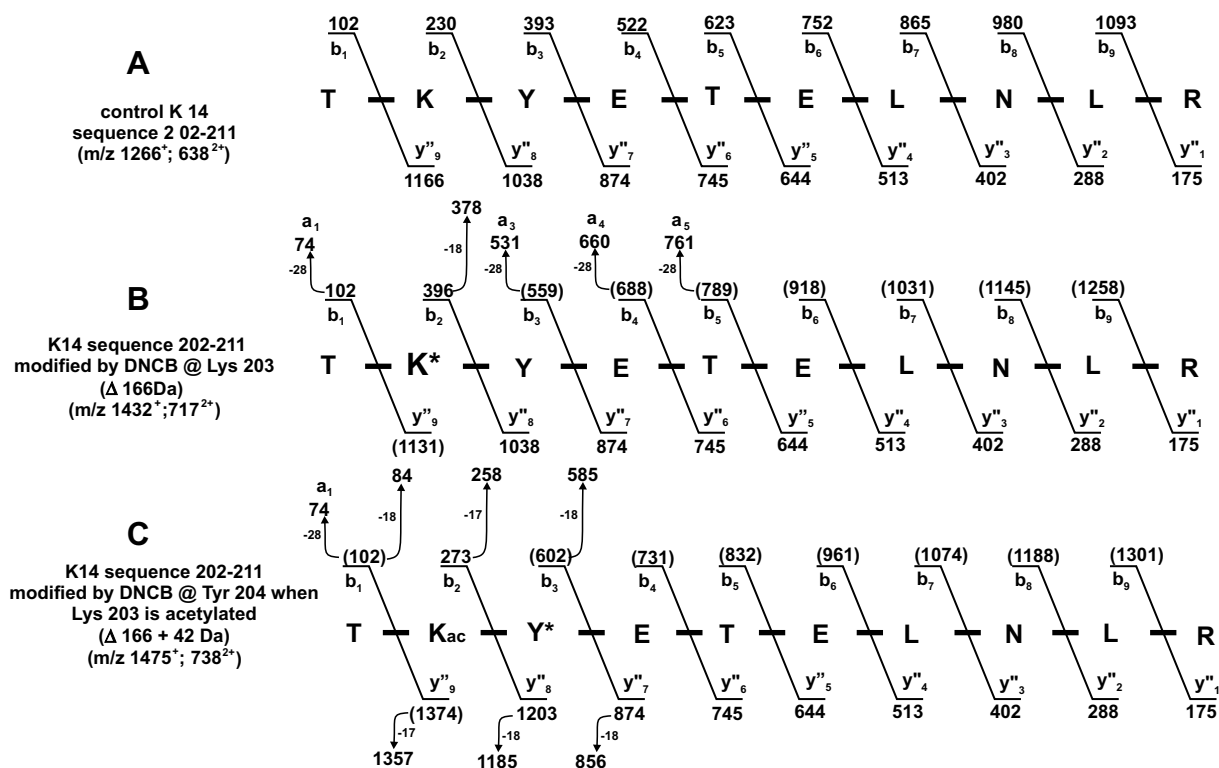


Fig. 5. (A) CAD MS/MS derived data from  $[M+2H]^{2+}$  638, (B) 717 and (C) 738.3 (Fig. 4 shows the MS/MS spectrum of  $m/z$  738). (\*) modified residues). Fragment ion masses in brackets are not observed in the CAD MS/MS spectrum. Differential preference of DNCB for Lys and Tyr residues during incubation with K14; DNCB modification of Tyr 204 occurs when Lys 203 is carbamylated.

the incubation medium being unfavourable for optimal His reactivity.

Of particular interest in these studies are the findings of high affinity towards Tyr and Lys in K14. DNCB overall modified more Tyr than Lys residues in K14 (5 of 14 Tyr and 3 of 22 Lys). However,

the preference for a particular microenvironment within the 3D structure of this intermediate filament protein appears more significant than the preference for a specific amino acid residue. This is exemplified by the modification of Tyr 129 and 204 when the neighbouring Lys 132 and 203, respectively are unavailable

(through carbamylation under the particular experimental conditions). The K14 sequence possesses several similar regions where Tyr and Lys residues are in close proximity and consequently can be assumed to be residing in the same 3D microenvironment. Three other Tyr residues found modified by DNCB (Tyr 46, 151 and 415) are situated in regions of the protein which are relatively free of Lys.

DNCB showed a high affinity for Cys residues of cofilin, with 3 (of 4) Cys residues being found to be modified. We have previously observed affinity of DNCB and phenyl salicylate for adjacent Lys of residues on HSA (Aleksic et al., 2007). Cofilin is particularly rich in Lys residues and has several instances of adjacent Lys residues in its sequence, or lysines residing in close proximity of one another. The two lysines (of 25) found modified by DNCB on cofilin (Lys 19 and 78) were not part of this pattern, but were surrounded by other basic amino acids (Arg, Tyr, Lys).

Previous observations regarding the importance of the 3D structure of the model protein molecule for the location of covalent modifications (Aleksic et al., 2007; Alvarez-Sanchez et al., 2004a,b) are reinforced by the present findings but the modifications observed with such model proteins cannot be directly related to induction of skin sensitisation *in vivo*. The proteins we studied were isolated from their natural *in vivo* environment and exposed to laboratory conditions, such as high urea concentration for K14, which would inevitably lead to some loss of native protein conformation. However, the low aqueous solubility of isolated skin keratins necessitates the deployment of chaotropic agents as a prerequisite of *in vitro* studies such as those described here.

It is evident that despite the presence of numerous nucleophilic residues on these model proteins, modification are identified for only a few specific residues, presumably due to the conditions of the protein microenvironments being conducive to reactivity. Additionally, in this study the irritant and the non-sensitising chemicals did not form adducts.

The results reported here show that the very potent sensitiser DNCB, which is capable of covalently modifying a wide variety of nucleophilic amino acids, targets specific Cys, Lys and Tyr residues on two structurally and functionally distinct skin proteins. Furthermore, the data suggest that specificity of these reactions is largely dependant on not only the 3D structure of model protein and localisation of target amino acid side chains within protein microenvironments conducive to reactivity, but also the general amino acid content of the model protein(s). The reactivity patterns of other sensitising chemicals with equal or lesser sensitising potency (as defined by the EC3 value in the LLNA) such as MCI and phenyl salicylate have been shown to be very different when HSA was used as a model protein.

Gerberick and colleagues have recently reported a development of a peptide reactivity assay for screening skin sensitising chemicals (Gerberick et al., 2004a,b; Gerberick et al., 2007). This very promising development enables assessment of direct *in chemico* reactivity of chemicals with two key protein nucleophiles (Cys and Lys). It is likely that this assay would detect majority of chemical sensitisers, however the full spectrum and nature of the chemical reactivity would not be obvious from the data. Moreover, as the assay relies solely on measurement of the depletion of the parent peptide, the obtained depletion value could sometimes be attributable to oxidation (for Cys peptide) rather than covalent modification by the sensitising chemical. Attempts to design physiologically relevant peptides (and thus to increase the sensitivity in chemical reactivity studies) have demonstrated that alternative Cys containing peptide sequences could be used to assess chemical reactivity (Natsch et al., 2007). Their study reports the use of heptapeptides derived from actual nuclear and cytoplasmic target proteins modified by biotin tagged thiol reactive chemicals in HEK293 cell line (Dennehy et al., 2006). The peptide portfolio includes

amongst others a Cys containing segment of cofilin sequence (residues 136–142, Cys 139 was not modified by DNCB in the present study). The increased reactivity of chemicals with these peptides could only be attributed to the presence of additional nucleophilic side chains (Lys, Tyr and Arg), which could influence the reactivity of thiol, but are also likely to be modified themselves.

Whilst it may represent a possible route to novel predictive assay, screening for peptide reactivity with selected nucleophiles does not reveal the mode of *in vivo* haptenation or the immunogenic relevance of detected modifications. It is therefore necessary to identify the human skin protein targets of sensitising chemicals and investigate their immunogenic potential if progress is to be made in the more fundamental area of knowledge of pathophysiological relevant covalent protein modification related to the induction of skin sensitisation.

## Acknowledgements

The authors thank the BBSRC and Unilever Research for the BBSRC CASE studentship for Maja Aleksic and BBSRC and Wellcome Trust for funding. A.D. is a BBSRC Professorial Research Fellow.

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