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THE ROLE OF NON-COVALENT PROTEIN BINDING IN SKIN SENSITISATION POTENCY OF CHEMICALS

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Skin sensitisation is a delayed hypersensitivity reaction caused by repeated exposure to common natural and synthetic chemical allergens. It is thought that small chemical sensitizers (haptens) are required to form a strong irreversible bond with a self protein/peptide and generate an immunogenic hapten–protein complex in order to be recognised by the immune system and stimulate T cell proliferation. The sensitizers are usually electrophilic chemicals that are directly reactive with proteins or reactive intermediates (metabolites) of chemically inert compounds (prohaptens). Sensitising chemicals are also capable of weak, non-covalent association with proteins and there is an ongoing debate about the role of weak interactions of chemicals and proteins in the chemistry of allergy. The non-covalent interactions are reversible and thus have a major impact on skin/epidermal bioavailability of chemical/reactive metabolites. We investigated the relationship between the relative level of non-covalent association to a model protein and their relative potencies as determined by the EC₃ values in the murine local lymph node assay (LLNA) for a number of chemicals. Using human serum albumin as a model protein, we determined that no observable relationship exists between the two parameters for the chemicals tested. Therefore, at least for this model protein, non-covalent interactions appear not to be a key determinant of allergen potency.

Keywords: Human serum albumin; LLNA; Non-covalent binding; Skin sensitisation

INTRODUCTION

Skin exposure to low molecular weight (<800 g/mol) sensitising chemicals can result in the occurrence of allergic contact dermatitis, which is a two phase process: induction of sensitisation then elicitation. Evidence to date suggests that the induction of sensitisation is dependent on an intrinsic ability of a chemical (or its metabolite) to covalently modify self skin proteins (1). These modified proteins are processed into antigenic peptides by Langerhans cells (resident antigen presenting cells in the skin). The Langerhans cells mature and migrate through the skin into the lymphatic circulation, carrying antigens. Recognition of the modified (haptenated) peptide antigens by the immune system is achieved by presentation in association with major histocompatibility molecules (MHC) (human leukocyte

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antigen (HLA) in man) on the surface of the antigen presenting cells. The MHC:peptide:hapten complexes are presented to T cell receptors of naïve T cells in the lymph node resulting in clonal expansion of hapten-specific T cells. The clinical manifestation (erythema, oedema, and itching) occurs as a result of an elicitation phase, where at the site of a subsequent exposure to the same, or a cross-reactive chemical (resulting in the formation of an identical adduct) inflammation is effected through the release of a number of inflammatory mediators and infiltration of the hapten-specific T cells, which migrate to the site of exposure.

The skin sensitisation potential of chemicals is currently assessed using the murine local lymph node assay (LLNA), which is used for hazard identification as well as to estimate relative sensitising potency of a chemical (EC3% value, i.e. the chemical concentration which causes a three-fold increase in T cell proliferation in the LLNA) (2).

Both the induction and elicitation phases of skin sensitisation are considered to be thresholded dose-dependent processes (3) and depend upon delivery of toxic (reactive) species to the epidermis to effect a response directly in the skin. Small molecule contact allergens (either as parent molecule or skin-derived metabolites) are typically reactive species (e.g. electrophiles) that are able to form strong high energy (200–420 kJ/mol) irreversible covalent bonds with proteins (4). Formation of covalent protein modifications is thought to be a key step in the induction of contact sensitisation to small chemicals (5,6). Recent studies have confirmed the ability of a range of skin allergens to form covalent bonds with peptides/proteins *in vitro* (1,7–13). However, it is also likely that sensitising chemicals can form weak (~50 kJ) non-covalent interactions with biomolecules in the skin. Non-covalent bonds are not thought to play the key role in determining the allergenic potential of a chemical (4). However, there is an ongoing debate about the role of weak interactions between chemicals and proteins in contact allergy. Pichler and colleagues have demonstrated that some chemicals are able to stimulate chemical-specific T cell proliferation without processing by antigen presenting cells or indeed formation of a covalent bond with the MHC-peptide complex (14). Additionally, Burkhart and colleagues have demonstrated that the inert chemical may associate non-covalently with a multitude of HLA-bound peptides, or even directly to HLA and successfully stimulate the specific T cell clone (15). The stimulation of naïve T cells by the non-covalently bound sulphamethaxazole and its covalently bound nitroso metabolite was recently demonstrated, albeit with different frequency of responses (16).

It is possible that reversible association of a chemical to a protein can increase the likelihood of a irreversible (covalent) modification of that protein by delivery of the chemical to specific microenvironments within a protein with optimal conditions for that reaction to occur. Additionally, the non-covalent reversible association with carrier proteins could be a way of delivery of sensitising chemicals to the reaction site. This is proposed to be the case in nickel allergy, where it has been demonstrated that HSA and apo-transferrin can act as carrier proteins, delivering nickel to the interface between peptide bound to MHC of antigen presenting cell and T cell receptor (17,18). It is typically seen that higher doses of chemical are required for induction of sensitisation than those required to elicit a response (19), thus induction and elicitation of contact allergy may have different requirements for delivery of chemical to cells and nature of chemical association with protein.

In order to meet the requirements of the 7th Amendment of the EU cosmetics legislation (20), which will ban the use of *in vivo* animal assays to predict skin sensitisation potential of chemicals, there is a need to better understand this process on a molecular level. It is envisaged that future consumer safety decisions will be supported by the risk assessment based on new paradigms, with data derived from specifically developed *in vitro* tests integrated using powerful informatics and statistical analysis tools (21). For example, the need for understanding of the epidermal disposition (tissue bioavailability) of chemicals in skin sensitisation in relation to their ability to exert their local toxic effect was highlighted at a recent workshop in European Centre for the Validation of Alternative Methods (ECVAM) (22). Epidermal bioavailability is the concentration of the freely available chemical at target sites (e.g. keratinocytes and Langerhans cells *in situ*), and is closely related to the rate of penetration (which is in turn linked to physicochemical properties, such as molecular mass, hydrophobicity, ionisation state, and physical factors such as exposure site, thickness, damage, temperature, and occlusion). Understanding of this parameter will be crucial for development of integrated alternative approaches (23). Whilst the attempts have already begun to model epidermal bioavailability *in silico* using pharmacokinetic modelling principles (e.g. 24,25), actual *in vivo* determination of human epidermal bioavailability of skin sensitisers is very challenging experimentally and there is no such data available to date. It is envisaged that one of the key parameters to influence epidermal bioavailability will be the ability of a chemical to bind reversibly to tissue proteins. Therefore it is important to investigate the nature and level of both reversible and irreversible interactions of sensitising chemicals with proteins.

One of the most abundant serum proteins, human serum albumin (HSA) is often used as a model protein to estimate the level of non-covalent binding of chemicals in serum. HSA is abundant in the skin (around 40% of extravascular albumin (26)), and we explore here whether and to what extent skin sensitising chemicals can bind in a reversible way to this model protein. We utilised the principles of high performance affinity chromatography (HPAC) to investigate the relative amount of non-covalent association of skin sensitising chemicals to human serum albumin (27). Recently described methodology for determination of the amount of non-covalent association of chemicals to HSA (28) was adapted for this purpose. A chemical dataset of 50 chemicals based on that published by Gerberick and colleagues (29) was used for this investigation to assess the variable of reversible protein binding. The dataset contains a range of sensitisation potencies as determined by the LLNA derivation of EC3 values (2): five extreme sensitisers (EC3 0.003–0.04%), seven strong sensitisers (EC3 0.1–0.7%), fifteen moderate sensitisers (EC3 1.4–5.8%), eleven weak sensitisers (EC3 10.6–46.4%) and thirteen compounds that were non-sensitising up to the maximum concentration tested in LLNA. The validity of LLNA assessments of potency was recently supported by an EU Expert Group (19). An attempt was made to explore whether any direct correlation existed between the sensitising potency of chemicals with their relative extent of non-covalent binding to model protein. We also explored the effects of an additional parameter, $\log K_{o/w}$ (octanol/water partition coefficient, used as a descriptor of hydrophobicity of the chemical) on this relationship.

MATERIALS AND METHODS

Materials

Ammonium acetate, 2-propanol, extreme sensitizers (diphenylcyclopropenone, oxazolone, 1,4-benzoquinone, 1-chloro-2,4-dinitrobenzene), strong sensitizers (hydroquinone, 1,4-phenylenediamine, phthalic anhydride, CD3 (N⁴-Ethyl-N⁴-(2-methanesulfanomid-oethyl)-2-methyl-1,4-phenylenediamine (sesquisulfate, monohydrate)), moderate sensitizers (2-hydroxyethyl acrylate, 2-methyl-4-isothiazolin-3-one hydrochloride, 3-dimethylaminopropylamine, 1,2-benzisothiazolin-3-one, cinnamic aldehyde, 3-aminophenol, isoeugenol, benzylidene acetone, β -propylidenephthalide, squaric acid, 1-bromopentadecane, citral, 3,4-dihydrocoumarin, diethyl maleate), weak sensitizers (α -amyl cinnamaldehyde, α -hexylcinnamaldehyde, eugenol, benzyl benzoate, lilial, hydroxycitronellal, linalool, ethyleneglycol dimethacrylate, penicillin G salt) and non-sensitizers (glycerol, hexane, diethyl phthalate, octanoic acid, 2-hydroxypropyl methacrylate, 1-butanol, 4-hydroxybenzoic acid, 6-methylcoumarin, methyl salicylate, chlorobenzene, 1-bromobutane, 2,4-dichloro-1-nitrobenzene) were all purchased as >95% pure from Sigma-Aldrich (Poole, UK). Additionally, extreme sensitizer 5-chloro-2-methyl-4-isothiazolin-3-one (1.2% solution in H₂O), strong sensitizers glutaraldehyde (50% solution in H₂O) and formaldehyde (36% solution in H₂O), moderate sensitizer phenylacetaldehyde (>90%), weak sensitizers abietic acid (75%) and cyclamen aldehyde (>90%) and non-sensitizer lactic acid (90%) were also sourced from Sigma Aldrich (Poole, UK).

Methods

HPAC separation. All chemicals were dissolved individually in 2-propanol: 0.05 M pH 7.4 ammonium acetate 50:50 (v/v) to give a concentration of approximately 0.5 mg/mL. Chromatograms were obtained on an Agilent HP1100 HPLC instrument (Agilent, Stockport). 5 μ L aliquots of chemical samples were injected onto a Chromtech Immobilized HSA column, 3 μ , 50 $\times$ 3 mm (Chromtech, Congleton) and eluted at a flow rate of 1.8 ml/min at a column temperature of 30°C. Mobile phase A was 0.05 M ammonium acetate pH 7.4 solution and mobile phase B was 2-propanol. A linear gradient from 0 to 30% 2-propanol was applied from 0 to 7 minutes. From 7 to 10 minutes the mobile phase composition was held at 30% 2-propanol. From 10 to 10.5 minutes, mobile phase B was reduced to 0% and remained that way until the end of the run. Each run was stopped after 15 minutes. Chromatograms were collected at 230 and 254 nm by a diode array UV detector and retention times of tested chemicals were recorded. Data were acquired and processed using Agilent Chemstation software (Agilent, Stockport).

Statistical analysis. To investigate the possible correlation between sensitization potency and non-covalent protein binding, Minitab (MINITAB[®] for Windows, release 12.1, Copyright 1998, Minitab Inc.) and Microsoft Excel (Excel, Microsoft[®] Corporation, Copyright 2000) were used to graphically examine the data and to generate regression equations. The average retention time (tR, n = 2) was used as descriptor of the non-covalent protein binding (relative affinity of the chemicals for the immobilized HSA). The chromatographic data were not corrected for the system

void volume as the experimental variation in the solvent front was minimal throughout the course of the analyses. For investigation of the effect of physicochemical parameters on the relationship between non-covalent protein binding and relative sensitizing potency (EC3%), an additional descriptor, $\log K_{o/w}$ (a descriptor of hydrophobicity) was calculated using the KowWin software from the US EPA (Copyright, 2000, US EPA vs.1.67). For the statistical analysis the chemical sensitizing potency data was treated as the dependent (y) variable and the chemical descriptors as the independent (x) variables. Linear regression analysis and multiple linear regression analysis were performed to assess the possible correlations. Regression equations were assessed based primarily on the value of r^2 (r = correlation coefficient, as calculated by either Microsoft Excel or Minitab software).

RESULTS AND DISCUSSION

In this study, we used the HPAC methodology to determine the relative affinity of 50 chemicals to immobilised HSA and determined the extent to which selected sensitising and non-sensitising chemicals bind reversibly to this model protein. Table 1 shows the average $\log t_R$ (derived from the measured retention times of chemicals, $n = 2$) as a measure of their relative affinity (non-covalent association) for immobilised HSA.

A number of tested chemicals were not detected in the chromatograms. For example, structures lacking a good chromophore are not likely to be detected by UV (5-chloro-2-methyl-4-isothiazolin-3-one, formaldehyde, 1-bromopentadecane, hydroxycitronellal, linalool, octanoic acid, glycerol, hexane, 1-butanol, lactic acid, and 1-bromobutane). Mass spectrometry could be used as a detection method to overcome this problem. However, some chemicals may not associate non-covalently with HSA and are thus not sufficiently retained on the column. This could be the likely explanation for compounds that possess a good chromophore, such as cyclamen aldehyde, lilial, and chlorobenzene.

An attempt was made to directly correlate the parameter of reversible protein binding to relative sensitising potency as determined by the LLNA molar EC3 values (2) and to $\log K_{o/w}$. The relative sensitising potency ($\log(1/EC3)$ (molar)), as derived from the LLNA) as well as $\log K_{o/w}$ are also given in Table 1. No direct correlation was found between the observed affinity for binding towards HSA and the relative sensitising potency of tested chemicals ($r^2 < 0.1$) (Fig. 1). No significant improvement of the regressions was noted on inclusion of $\log K_{o/w}$ as a second descriptor ($r^2 < 0.1$, regression equation not shown). Therefore, at least for this model protein, non-covalent interactions appear not to be a key determinant of allergen potency.

It is however possible that the level of non-covalent association with relevant target proteins within the epidermis may be responsible for driving covalent modifications in the specific microenvironments of the protein molecules. Further research is possible in the area of molecular modelling to elucidate the regions/domains of proteins involved in non-covalent associations with chemicals. This may highlight examples of non-covalent interactions facilitating covalent modification of protein within the same domain.

Another possible application of the type of data derived in these experiments could be to supplement the wealth of data generated from covalent reactivity studies

Table 1 Relative affinity of test chemicals to immobilised HSA (average tR and logtR), their relative sensitisation potency (EC3% and log (1/EC3) molar) and estimated octanol/water partition coefficient (logK_{o/w}, used as a descriptor of hydrophobicity). E – extreme, S – strong, M – moderate, W – weak, NS – non-sensitiser

Test chemical	Average tR (min ± SD) (n = 2)	logtR (mins)	EC3%	log (1/EC3) (molar)	log K _{o/w}	Potency Category
Diphenylcyclopropanone	3.8 ± 0.021	0.58	0.0030	4.8	3.3	E
Oxazolone	0.25 ± 0.0007	– 0.60	0.0030	4.9	1.5	E
1,4-benzoquinone	0.25 ± 0.0	– 0.60	0.010	4.0	0.25	E
1-chloro-2,4-dinitrobenzene	2.0 ± 0.0092	0.30	0.040	3.7	2.3	E
Hydroquinone	0.32 ± 0.0007	– 0.49	0.10	3.0	1.0	S
Glutaraldehyde	0.30 ± 0.0046	– 0.52	0.10	3.0	– 0.18	S
1,4-phenylenediamine	0.24 ± 0.0007	– 0.62	0.10	3.0	– 0.39	S
Phthalic anhydride	0.22 ± 0.0007	– 0.66	0.16	3.0	2.1	S
CD3 (N4-Ethyl-N4(2-methano-sulfanomid-oethyl)-2-methyl-1,4-phenylenediamine (sesquisulfate, monohydrate)	0.35 ± 0.0	– 0.46	0.60	3.1	–	S
2-hydroxyethyl acrylate	0.22 ± 0.0021	– 0.66	1.4	1.9	– 0.25	M
2-methyl-4-isothiazolin-3-one hydrochloride	0.22 ± 0.0	– 0.66	1.9	1.8	– 0.83	M
3-dimethylaminopropylamine	1.5 ± 0.0071	0.17	2.2	1.7	– 0.45	M
1,2-benzisothiazolin-3-one	2.9 ± 0.030	0.46	2.3	1.8	0.64	M
Phenylacetaldehyde	0.62 ± 0.0021	– 0.21	3.0	1.6	1.5	M
Cinnamic aldehyde	0.99 ± 0.0014	– 0.0044	3.1	1.6	1.8	M
3-aminophenol	0.28 ± 0.0	– 0.55	3.2	1.5	0.24	M
Isoeugenol	2.2 ± 0.024	0.34	3.5	1.7	2.7	M
Benzylidene acetone	1.7 ± 0.011	0.22	3.7	1.6	2.0	M
β-propylidenephthalide	2.6 ± 0.017	0.42	3.7	1.7	2.0	M
Squaric acid	0.23 ± 0.0	– 0.64	4.3	1.4	– 0.44	M
Citral	1.3 ± 0.0057	0.12	5.1	1.5	3.5	M
3,4-dihydrocoumarin	1.4 ± 0.0021	0.14	5.6	1.4	0.97	M
Diethyl maleate	0.31 ± 0.00070	– 0.51	5.8	1.5	2.2	M
α-amyl cinnamaldehyde	6.5 ± 0.015	0.81	10.6	1.3	4.3	W
α-hexylcinnamaldehyde	6.5 ± 0.013	0.81	11.7	1.3	4.3	W
Eugenol	2.7 ± 0.0060	0.44	12.9	1.1	2.7	W
Abietic acid	0.21 ± 0.00070	– 0.68	14.7	1.3	6.5	W
Benzyl benzoate	6.0 ± 0.014	0.78	16.7	1.1	3.5	W
Ethyleneglycol dimethacrylate	2.0 ± 0.0035	0.29	36.5	0.74	2.2	W
Penicillin G salt	0.86 ± 0.0	– 0.066	46.4	0.89	– 3.0	W
Diethyl phthalate	3.0 ± 0.0004	0.47	–	–	1.9	NS
2-hydroxypropyl methacrylate	0.13 ± 0.0014	– 0.89	–	–	0.72	NS
4-hydroxybenzoic acid	0.36 ± 0.0014	– 0.44	–	–	1.4	NS
6-methylcoumarin	2.5 ± 0.013	0.39	–	–	2.1	NS
Methyl salicylate	2.8 ± 0.0032	0.44	–	–	2.6	NS
2,4-dichloro-1-nitrobenzene	3.9 ± 0.012	0.60	–	–	3.1	NS

with model peptides. It has already been demonstrated that the level of reactivity resulting in covalent modification of model peptides generally correlates well with sensitisation potency (30). However, it is well established that this is not the only factor to influence sensitising potency of chemicals. Therefore, outliers from the

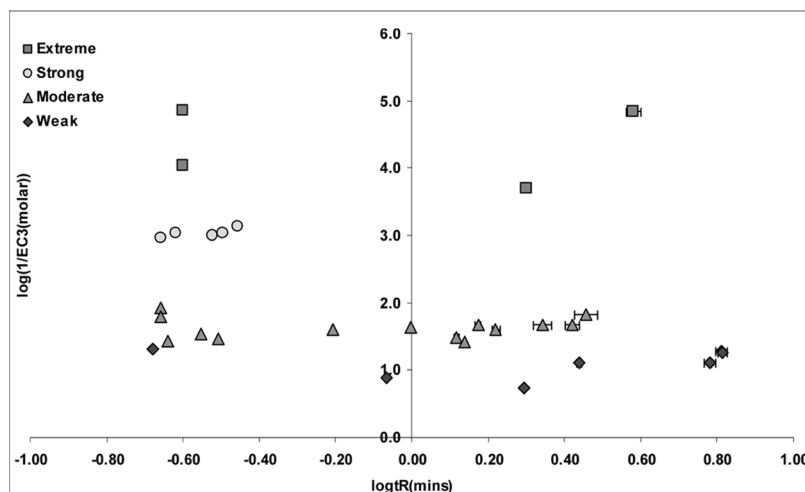


Figure 1 The plot of relative sensitizing potency values of tested chemicals and logtR. No observable correlation was found between the two parameters, indicating that no relationship exists between the level of non-covalent binding to proteins and the relative sensitizing potency of chemicals.

correlations between sensitisation potency and covalent reactivity from peptide assays could be explained by considering these additional interactions.

Whilst no direct correlation between non-covalent binding to a model protein and relative sensitising potency of chemicals can be demonstrated, data on reversible protein binding could be integrated with other factors relevant to epidermal bioavailability (e.g., penetration, metabolism, and clearance), for correct interpretation in the context of predicting skin sensitisation. Accurate characterisation of skin sensitisation potential of chemicals and an estimation of sensitising potency without the use of animals is likely to be achieved by effective integration of a number of related parameters (23). To date, appropriate data on the key parameters for skin sensitisers does not exist, further emphasising the need for better understanding of induction and elicitation of skin sensitisation on a molecular and kinetic level.

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