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Article

Characterizing Adduct Formation of Electrophilic Skin Allergens with Human Serum Albumin and Hemoglobin

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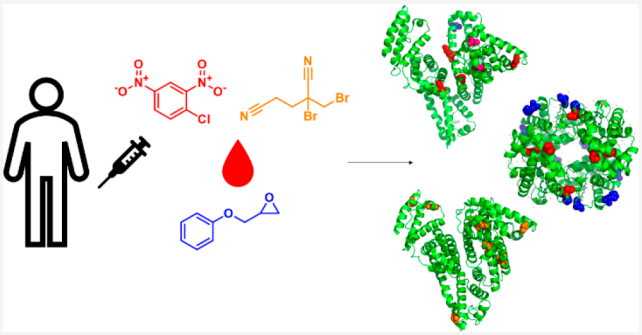
ABSTRACT: Skin (contact) allergy, the most predominant form of immunotoxicity in humans, is caused by small electrophilic compounds (haptens) that modify endogenous proteins. Approximately 20% of the general population in the Western world is affected by contact allergy. Although the importance of the hapten−protein conjugates is well established in the initiation of the immunological reaction, not much progress has been made regarding identification of these conjugates *in vivo* or exploration of their potential as diagnostic tools. In this study, the human serum albumin (HSA) and human hemoglobin (Hb) adductome for three representative contact allergens with different chemical properties, 1-chloro-2,4-dinitrobenzene (DNCB), 1,2-epoxy-3-phenoxypropane (PGE), and 2-bromo-2-(bromomethyl)glutaronitrile (MDBGN), were studied. Plasma and red blood cell lysate were used as a source for HSA and Hb, respectively. The Direct Peptide Reactivity Assay was used to investigate adduct formation of MDBGN with nucleophilic moieties and revealed that MDGBN is converted to 2-methylenepentanedinitrile in the presence of sulfhydryl groups prior to adduct formation. Following incubation of HSA and Hb with haptens, an Orbitrap Q Exactive high-resolution mass spectrometer was used to perform an initial untargeted analysis to screen for adduct formation, followed by confirmation by targeted Parallel Reaction Monitoring analysis. Although a subset of adducted sites was confirmed by targeted analysis, only some of the adducted peptides showed an increase in the relative amount of the adducted peptide with an increased concentration of hapten. In total, seven adduct sites for HSA and eight for Hb were confirmed for DNCB and PGE. These sites are believed to be the most reactive. Further, three of the HSA sites (Cys_{34}, Cys_{62}, and Lys_{190}) and six of the Hb sites (subunit α: Val_1, His_{45}, His_{72}; subunit β: Cys_{93}, His_{97}, and Cys_{112}) were haptenated already at the lowest level of hapten to protein molar ratio (0.1:1), indicating that these sites are the most likely to be modified *in vivo*. To the best of our knowledge, this is the first time that the adductome of Hb has been studied in the context of contact allergens. Identification of the most reactive sites of abundant proteins, such as HSA and Hb, is the first step toward identification of contact allergy biomarkers that can be used for biomonitoring and to develop better diagnostic tools based on a blood sample.



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■ INTRODUCTION

Contact Allergy. Skin (contact) allergy is the most prevalent form of immunotoxicity in humans, accounting for approximately 20% of skin diseases.[1] Contact allergy is caused by a wide range of small electrophilic compounds (haptens) after prolonged and/or repeated exposure. Although too small to trigger the immune system alone, haptens have the potential to react with nucleophilic sites of cellular biomacromolecules, predominantly proteins, forming hapten−protein conjugates. It is these hapten−protein conjugates that have the potential to activate the immune system and thereby trigger an allergic response.[2]

Contact allergy is divided into two phases: sensitization and elicitation. During sensitization, the hapten covalently modifies (haptenates) endogenous proteins. The hapten−protein conjugates are taken up and processed by cutaneous dendritic cells (DCs). DCs migrate from the skin to the local lymph nodes, where the antigens are presented to naíve T cells. When naíve T cells recognize an antigen, they start to proliferate and differentiate into antigen-specific memory and effector T cells that circulate in the blood and the lymphatic system. Sensitization usually does not lead to visible symptoms. However, during elicitation, i.e., re-exposure to the same hapten, the circulating memory T cells are activated and trigger the inflammatory response that results in the clinical manifestation of contact allergy (skin inflammation) at the exposure site, known as Allergic Contact Dermatitis (ACD).[3,4]

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***In Vivo* and *In Vitro* Assays for Prediction of Sensitizing Capacity.** Assessment of the sensitizing capacity of different chemicals is crucial to avoid exposure that could lead to ACD outbreaks in human populations. This assessment was initially performed using the guinea pig maximization test (GPMT)[5] and later replaced by the murine local lymph node assay (LLNA).[6] The LLNA is the most widely used assay for potency assessment of contact allergens, accepted by both the U.S. Food and Drug administration (FDA) and the Organization for Economic Cooperation and Development (OECD, Guideline for the Testing of Chemicals 429. Skin Sensitization: Local Lymph Node Assay).[2] This assay correlates the sensitization capacity of the test compounds with an increase in cell proliferation in the local draining lymph nodes following topical application. Chemicals causing a stimulation index (SI) of 3 or higher are considered to be positive in the LLNA, and their EC3 (estimated concentration to cause an SI of 3) values are used to compare and classify the potency of different chemicals.[7] The LLNA, as well as any other *in vivo* testing of cosmetics for skin-sensitizing properties, has been banned in Europe since 2013. Four key events have been identified for the development of nonanimal based methods for assessment of skin sensitization: key event one, molecular initiation−covalent binding of contact allergens to skin proteins; key event two, inflammatory responses and gene expressions linked to specific signaling pathways that take place inside keratinocytes; key event three, activation of dendritic cells; and key event four, T cell proliferation. One nonanimal based method for skin sensitization assessment employed in this work, although somewhat modified, is the direct peptide reactivity assay (DPRA)[8] which addresses key event one. The DPRA measures the reactivity of possible skin allergens toward peptides with reactive cysteine and lysine moieties.[9,10] Other nonanimal based *in vitro* methods recommended by OECD for predictive testing of skin sensitizers include KeratinoSens[11] (addressing key event two) and h-CLAT[12] (addressing key event three). Although there are a few OECD approved nonanimal based methods, none of them are considered a stand-alone method for replacement of LLNA, and the current approach used by most companies is a combination of two or three nonanimal based methods. Thus, there is still a need for more robust and better mimicking of the *in vivo* conditions in nonanimal based models.

Protein Adducts in Disease Monitoring − The Role of HSA and Hb. Protein adducts are more sensitive biomarkers of exposure to reactive species as compared to DNA adducts partly due to the high abundance and longevity of the proteins, as well as the lack of repair mechanisms of the adducts, enabling an integration of chronic exposures. Thus, adducts of the proteins human serum albumin (HSA) and hemoglobin (Hb) have been extensively studied.[13,14]

HSA is the most abundant protein in blood plasma, with a concentration ranging from 30 to 50 g/L and a half-life of approximately 20 days. The focus of many studies of HSA adducts has been Cys_{34},[15,16] due to its unusually low pK_a that makes it a very strong nucleophilic site.[17] HSA is found in high concentrations in most tissues including skin, and due to its functions as a carrier protein, HSA is the most commonly used protein in studies of reactivity of contact allergens.[18−21]

Hb is found in erythrocytes with a concentration in blood ranging from 120 to 160 g/L. Hb has an estimated lifetime of approximately 120 days, same as that of erythrocytes.[22] Hb adducts have been extensively studied and used for biomonitoring of environmental and occupational exposure.[13,23,24] A big part of the analytical work of Hb adducts is directed toward analysis of adducts formed at the N-terminus Val, mainly due to the possibility of facile detachment of the substituted N-terminus Val from the rest of the protein using modified Edman degradation procedures.[13,25,26] However, the formation and role of Hb adducts with contact allergens are yet to be explored.

Haptens of Interest and Their Sensitizing Capacity. In this study, three different haptens, reacting via different mechanisms, have been used to identify HSA and Hb reaction sites and investigate differences in reactivity. These haptens are 1-chloro-2,4-dinitrobenzene (DNCB), 1,2-epoxy-3-phenoxypropane (PGE), and 2-bromo-2-(bromomethyl)glutaronitrile (MDBGN). The structures of the studied haptens, the published data on the *in vivo* assessment of sensitizing potency based on the LLNA, and the added mass from each hapten after haptenation are shown in Table 1.

Table 1. Name, Structure, Potency Category (EC3 Value Derived from the Local Lymph Node Assay), and Δ Mass (Da) Expected Following Haptenation

Compound name	Structure	EC3 % [w/v]	Category	Δ Mass (Da)
1-Chloro-2,4-dinitrobenzene (DNCB)		0.08	Extreme [28]	166.0015
1,2-Epoxy-3-phenoxypropane (PGE)		0.46	Strong [33]	150.0681
2-Bromo-2-(bromomethyl) glutaronitrile (MDBGN)		5.2	Moderate [36]	183.9636/ 106.0609

Although DNCB can be used to treat warts,[27] it is not a compound that one is easily exposed to, and thus, it is not a common contact allergen from a clinical point of view. However, it is one of the most commonly used haptens for *in vivo* and *in vitro* studies of contact allergy, and a number of studies can be found in the literature where adducts of DNCB with HSA have been identified. We therefore choose to include DNCB in the current work to be able to compare our findings to studies conducted by other research groups. DNCB, which is classified in the LLNA as an extreme hapten,[28] reacts with nucleophilic sites via an aromatic nucleophilic substitution (S_NAr) mechanism, resulting in an added mass of 166.0015 Da, Figure S1(a).

PGE is a model of the most commonly used epoxy resin monomer (ERM), diglycidyl ether of bisphenol A (DGEBA). ERMs are used to produce epoxy resin systems, which have a wide range of uses such as in adhesives, metal coatings, industrial flooring, electrical insulators, and relining of old pipes. Unfortunately, ERS and DGEBA are one the most common causes of occupational ACD.[29−32] LLNA has classified PGE as a strong sensitizer, and it has been shown to react via a second order nucleophilic substitution (S_N2) mechanism.[33] Position 1 of PGE is most prone to nucleophilic attack, but a minor constitutional isomeric adduct is also formed as a consequence of nucleophilic attack on position 2, Figure S1(b). Both of the PGE isomeric adducts add a mass of 150.0681 Da to the nucleophilic site of reaction.

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MDBGN was introduced in the 1980s for use in industrial and cosmetic products but was banned in the EU from leave-on products in 2003 and rinse-off products in 2007, due to an increased incidence of contact allergy.[34] Its sensitizing capacity is well established with a number of clinical studies available.[35] Nowadays, sensitization to MDBGN is still present due to occupational exposure or topical medications.[34] MDBGN is classified as a moderate sensitizer[36] and has been suggested to react via a typical S_N2 mechanism, resulting in an added mass of 183.9636 Da. Sulfhydryl-mediated biotransformation of MDBGN results in the debrominated metabolite 2-MGN.[37,38] Adduction of nucleophilic amino acids by 2-MGN results in an added mass of 106.0609, Figure S2.

Aim of the Study. Contact allergy is the most frequent manifestation of adverse health effects caused by the interaction of chemicals in the environment with our immune system. The public's exposure to various chemicals has increased substantially in recent years; it is, therefore, likely that contact allergy will increase as a health risk in the future unless exposure to these hazardous compounds is limited. Hence, the aim of the current study was to identify sites of HSA and Hb that are likely to become haptenated when an individual is exposed to a contact allergen in order to find potential adduct biomarkers that can be used for biomonitoring purposes and to develop diagnostic methods based on a blood sample.

■ EXPERIMENTAL SECTION

Materials. Human blood with added potassium EDTA was purchased from Biochemed Services (Winchester, VA). Trypsin and chymotrypsin were purchased from Promega Corporation (Madison, WI). Formic acid (FA) was purchased from Honeywell Fluka (Mexico City, MX). Acetonitrile (ACN), LC-MS grade water, phosphate-buffered saline (PBS), sodium chloride, calcium chloride, Coomasie PlusTM Protein Assay Reagent, and PierceTM C18 spin columns were obtained from Thermo Fisher Scientific (Waltham, MA). Sep-Pak C18 1 cc Vac cartridges (50 mg) were purchased from Waters (Milford, MA). Trifluoroacetic acid, potassium chloride, and Amicon ultracentrifugal filters (3K) were purchased from Millipore Sigma (Burlington, MA). DNCB, PGE, MDBGN, 2-methylenepentanedinitrile (2-MGN), reduced L-glutathione (GSH), DL-dithiothreitol, iodoacetamide, and F-moc-lysine were obtained from Sigma-Aldrich (Saint Louis, MO). The peptide Ac-PHCKRM was purchased from Peptide 2.0 (Chantilly, VA).

Human Blood. Upon arrival, the commercial blood was centrifuged at 800*g* for 10 min at 4 °C to separate the erythrocytes from the plasma. The erythrocytes were further washed with an equivalent volume of cold Ringer's solution (250 mM NaCl, 10 mM KCl, 3 mM $CaCl_2$, pH = 7.4). To obtain Hb, erythrocyte lysis was performed by resuspension in an equal volume of distilled water and subjection to 5 min of sonication. The concentration of Hb in the supernatant was estimated using the Pierce Coomassie Plus Assay Reagent and measuring the absorbance at 595 nm. Total protein content of the plasma was assessed using the NanoDrop UV−vis spectrophotometer with the direct A280 application. The amount of HSA was estimated to be 50% of the total plasma protein content.

Direct Peptide Reactivity Assay (DPRA). Adduct formation of MDBGN and its potential metabolite 2-MGN was initially studied using the DPRA. The assay was performed based on the 'OECD Test Guideline No. 442C, In Chemico Skin Sensitization: Direct Peptide Reactivity Assay (DPRA)',[8] with the major difference being that the synthetic N-terminal acetylated cysteine and lysine containing peptide Ac-PHCKRM was used instead of the Ac-RFAACAA-COOH and Ac-RFAAKAA-COOH peptides suggested in the guideline, due to high dimerization rates of the Ac-RFAACAA-COOH peptide (data not shown).

The potential haptens, MDBGN and 2-MGN, were dissolved in methanol (MeOH), with the final reaction conditions used being 25% MeOH in phosphate buffer (pH 7.4). Stock solutions of peptide (Ac-PHCKRM, 1 mM) and reduced GSH (4 mM) were prepared in phosphate buffer (100 mM, pH 7.4). Stock solutions (2 mM) of MDBGN and 2-MGN were prepared in MeOH. The peptide was incubated under three conditions: a) peptide/MDBGN at a molar ratio of 1:10, b) peptide/MDBGN/reduced GSH at a molar ratio of 1:10:20, respectively, and c) peptide/2-MGN at a molar ratio of 1:10. The final volume of all three reaction mixtures was 500 μL. The reaction mixtures were prepared by combining 125 μL of hapten stock solution and 100 μL of peptide stock solution with 275 μL of phosphate buffer for (a) and (c) and 150 μL of phosphate buffer and 125 reduced GSH stock solution for case (b). All incubations were directly performed in amber HPLC vials. The vials were capped, vortexed, and kept in the autosampler at room temperature for the entire analysis time.

A DPRA analysis was performed with the same peptide and 2-MGN, in order to estimate the sensitizing potential of 2-MGN. The depletion of the initial peptide and the formation of the adduct were followed for 24 h. Control samples, as well as reaction mixtures analyzed only after 24 h, were included in the sequence. Quantification was based on a calibration curve in the range of 10−200 μM.

The depletion of the initial peptide and formation of the adduct was monitored using a high-resolution Q Exactive Orbitrap mass spectrometer (MS). Separation was achieved using a reversed-phase Acquity UPLC Protein BEH C4 column eluted at a flow rate of 300 μL/min using buffers A (0.1% FA in water) and B (0.1% FA in ACN). Samples were run on a 15 min gradient with 5% buffer B for 4 min, followed by 5−60% B over 6 min, a 95% buffer B wash for 3 min, and finally a 90−5% decrease in buffer B followed by a 2-min equilibration. The mass spectrometer was operated in positive ion mode using a Top10 Full MS/dd-MS^2 experiment with an expected chromatographic peak fwhm of 15 s. In the full scan mode, resolution was set to 70,000 with an AGC target of 1e6, a maximum IT of 30 ms, and a scan range of 200 to 2000 *m/z*. Tandem mass spectra were captured at 17,500 resolution, AGC target of 5e4, maximum IT of 50 ms, an isolation window of 2.0 *m/z*, and a normalized collision energy of 30. Data was collected in centroid mode.

General Procedures for Studies of Reactions of Proteins with Haptens. *Protein Incubations.* HSA and Hb were diluted with PBS buffer (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4). Stock solutions of PGE and MDBGN were prepared in ethanol, and DNCB stock solution was prepared in dimethyl sulfoxide (DMSO). HSA (diluted plasma with an estimated HSA concentation of 15 μM was mixed with increasing molar ratios of hapten with a final volume of 100 μL. Molar ratios of hapten to estimated HSA concentration at 0.1-, 0.5-, 1-, and 5-fold were used for incubations of HSA with DNCB and PGE, while 0.1-, 1-, 10-, and 100-fold molar ratios were used for incubation of HSA with MDBGN. Hb (15.7 μM) was mixed with increasing molar ratios of hapten with a final volume of 100 μL. Hb was incubated with 0.1-, 0.5-, 1-, and 5-fold molar ratios of DNCB and PGE. The organic solvent of the hapten stock solution constituted 1−5% of the total incubation volume. Incubations of both proteins with each hapten were performed for 24 h at 37 °C. After incubation, the excess of unreacted hapten was removed using 3K cutoff centrifugal filters and centrifuged at 14000*g* for 15 min. Samples were then transferred to new tubes. HSA samples were reduced with dithiothreitol at a final concentration of 5 mM for 40 min at room temperature. Following reduction, samples were alkylated with iodoacetamide at a final concentration of 7 mM, for 30 min, in the dark. Hb samples were only alkylated following the same procedure as above. Each sample was then subjected to enzymatic digestion. HSA samples were digested using a mixture of Trypsin 1:50 w/w and Chymotrypsin 1:100 w/w, while Hb samples were digested only with Trypsin 1:50 w/w. Digestion was carried out for 24 h at 37 °C. After digestion, samples were cleaned up using C18 SepPack cartridges and evaporated to dryness in a speed vac. For both

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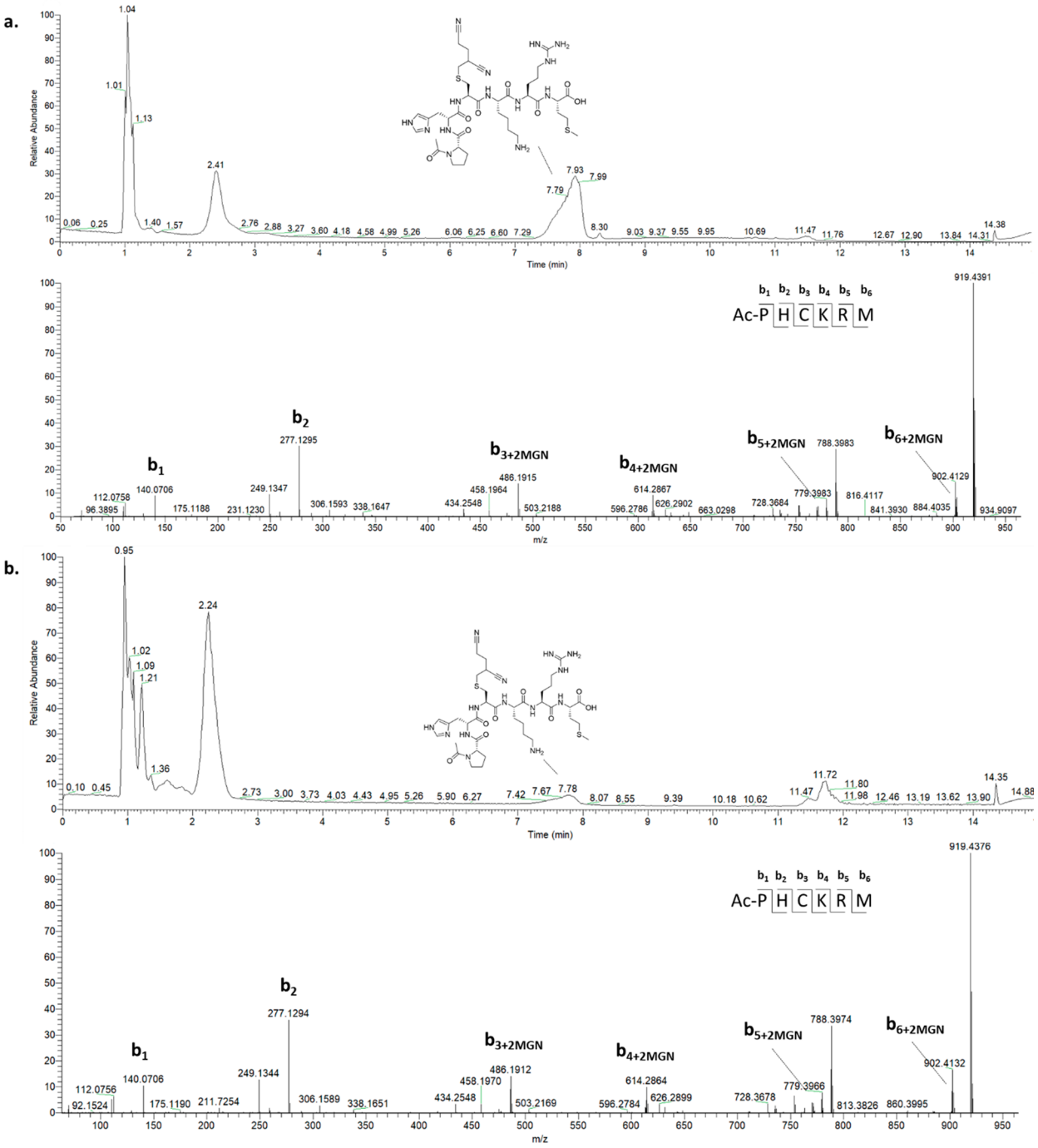

Figure 1. Total Ion Chromatogram (TIC) of the reaction mixture of the peptide Ac-PHCKRM with standard 2-MGN after 24 h, showing adduct formation at 7.5 min retention time and the corresponding MS/MS spectra confirming the added mass (106.06) and the site localization of the adduct (a) and the equivalent TIC and MS/MS spectra for the reaction mixture of the peptide Ac-PHCKRM with MDBGN and reduced GSH after 24 h (b).

proteins, control samples were included and subjected to the same procedure without the presence of haptens.

Liquid Chromatography (LC) Method. Digested HSA and Hb samples were reconstituted in buffer A (0.1% FA in water containing 5% ACN) and analyzed on a high-resolution Q Exactive Orbitrap mass spectrometer (ThermoFisher Scientific, Waltham, MA) with a reverse-phase self-packed Luna C18 nanoLC column (New Objective, Woburn, MA) at a flow rate of 300 nL/min. Samples were run on a 90 min gradient with 5−22% buffer B (0.1% FA in acetonitrile) over 71 min, followed by 22−33% over 5 min, 33−90% over 5 min, a 90% buffer B wash for 4 min, and finally a 90−4% decrease in buffer B over 2 min followed by a 3-min equilibration.

Untargeted Analysis of Protein Digests. Peptides were analyzed in positive ion mode using a Top12 Full MS/dd-MS^2 experiment with an expected chromatographic peak fwhm of 15 s. In the full MS, resolution was set to 70,000 with an AGC target of 1e6, a maximum IT of 30 ms, and a scan range of 300 to 2000 m/z. Tandem mass spectra were captured at 17,500 resolution, AGC target of 5e4, maximum IT of 50 ms, an isolation window of 2.0 m/z, and a normalized collision energy of 30. Data was collected in centroid mode. Control HSA and Hb samples were used to create an exclusion list of unadducted peptides. Untargeted analysis was based on one replicate analysis.

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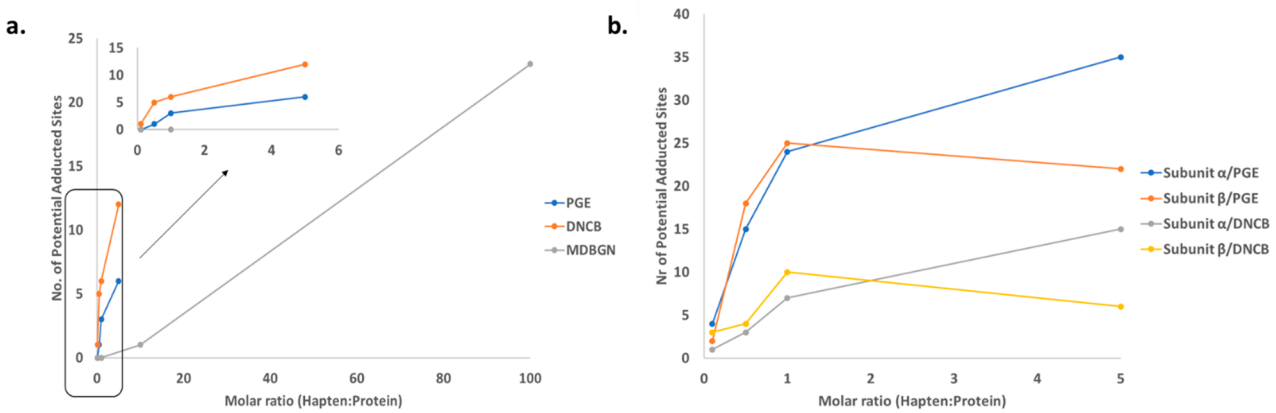

Figure 2. Number of potential adducted sites of HSA incubated with increasing molar ratios of DNCB and PGE (0.1, 0.5, 1, and 5) and MDBGN (0.1, 1, 10, 100) after 24 h of incubation (a) and number of potential adducted sites of Hb incubated with increasing molar ratios of DNCB and PGE (0.1, 0.5, 1, and 5) after 24 h of incubation (b).

Raw mass spectrometry data were processed with Proteome Discoverer 2.2 and analyzed using a modified PWF Fusion Basic Sequest HT processing step. In this modified step, the data was searched against the SwissProt human proteome version 2017-10-25 with variable modifications of oxidation at methionine, carbamidomethyl modification of cysteine, and the added masses for each hapten indicated in Table 1. Modification sites at Cys, His, Lys, Arg, Ser, Thr, Tyr, and N-termini of peptides were included. In addition, a percolator step was added with a strict target FDR of 0.01, a relaxed target FDR of 0.05, and a validation based on the q-value. The files were then processed using a CWF Basic consensus step.

Targeted Analysis of Protein Digests. The peptides identified in the untargeted proteomics study were used to create an inclusion list containing high-confidence peptides modified by each hapten and its corresponding unmodified peptide. This inclusion list was used to analyze digested samples in a targeted analysis using joint Parallel Reaction Monitoring (PRM) and Full MS-SIM experiments. Targeted experiments were run in positive mode with a chromatographic peak width fwhm of 15 s. The PRM experiments were run with a resolution of 70,000, an AGC target of 2e5, a maximum IT of 250 ms, an isolation window of 1.0 m/z, and a normalized collision energy of 25. The accompanying Full MS-SIM experiments were run at a resolution of 70,000, an AGC target of 3e6, a maximum IT of 200 ms, and a scan range of 300 to 2000 m/z. Targeted analyses were performed in triplicate.

Relative Estimation of Modified Peptides. The results from the targeted analysis were processed using Skyline,[39] where the potentially adducted peptides were used to build a targeted list. The original results from the untargeted analysis of the 5-fold molar excess of hapten were used to create a spectral library. Peak areas of the five most intense ions from all the confirmed adducted peptides and their equivalent unmodified peptides were used to estimate the relative percentage of modified peptide compared to unmodified.

Incubation and Targeted Analysis of F-moc-Lys-OH Hydrochloride with MDBGN. Equimolar amounts of F-moc-Lys-OH hydrochloride, reduced GSH, and MDBGN were incubated in ethanol. pH was adjusted to 8, and incubation was allowed to proceed overnight.

An inclusion list containing the mass of the ions corresponding to the starting material, F-moc-Lys-OH, and the expected product ion from the reaction with MDBGN after activation by reduced GSH was used in this case. Otherwise, the same settings as for the targeted analysis of protein digests described above were used for this analysis.

■ RESULTS

In this study, the hapten-dependent adductome of HSA and Hb was characterized by site specific identification of adduct formation. The structures of the studied haptens, their sensitizing potency based on the LLNA, and the added mass from each hapten after haptenation are shown in Table 1.

Direct Peptide Reactivity Assay (DPRA) for MDBGN. To determine the identity of adducts formed by MDBGN activity, experiments with the protected amino acid lysine and the peptide Ac-PHCKRM were performed. The synthetic N-terminal acetylated, cysteine and lysine containing peptide Ac-PHCKRM (observed m/z 813.3857) was incubated under three separate conditions: a) MDBGN at a molar ratio of 1:10, b) MDBGN and reduced GSH at a molar ratio of 1:10:20, respectively, and c) 2-MGN at a molar ratio of 1:10. Incubation of the peptide with only MDBGN led to no adduct formation at any point during the 24-h incubation. Instead, almost instant dimerization of the initial peptide was observed. The doubly charged dimer at m/z 812.3774 was observed at the first time point (approximately at 2 to 3 min), while the nondimerized peptide could not be observed at all, Figure S3. The same phenomenon, also observed by Natsch and Emter,[40] could be explained by the intermediate step in the mechanism suggested by Bao et al.[37] In this mechanism, MDBGN is converted to 2-MGN in the presence of free sulfhydryl groups, usually provided by reduced GSH, simultaneously leading to the formation of oxidized glutathione (GSSG). The initial reaction condition with MDBGN also supported this sulfhydryl-mediated mechanism, where the free sulfhydryl groups presented by the peptide's cysteine residue led to the formation of disulfide bonds and resulted in peptide dimerization. Coincubation of the peptide with MDBGN and reduced GSH at molar ratios of 1:10:20 led to adduct formation of a mass corresponding to the adduct of the peptide with 2-MGN, m/z 919.4394. This 2-MGN adduct formation was observed to increase over time during the 24-h incubation, Figure S4. Importantly, incubation of the peptide directly with 2-MGN resulted in the formation of the same adduct as formed by MDBGN in the presence of reduced GSH. The site of localization of the adduct at the cysteine residue was confirmed in both cases by the MS/MS spectra, Figure 1(a) and Figure 1(b), respectively. These observations strengthen the case for biotransformation of MDGBN by free sulfhydryl groups to 2-MGN that binds to the proteins in the initiation event of contact allergy.

The sensitizing potential of 2-MGN was assessed by measuring the depletion of the initial peptide over the course of 24 h. During this time, the peptide molarity decreased,

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Figure S5, and at the same time the 2-MGN adducted peptide product increased as measured by LC-MS acquired integrated peak area, Figure S6. The peptide depletion was observed to be 69% after 24 h, categorizing 2-MGN as a moderate sensitizer. This sensitization category is in agreement with that of MDBGN when assessed *in vivo* by the LLNA. This further supports the hypothesis that 2-MGN reacts with skin proteins after biotransformation of MDBGN.

Additional confirmation of the reaction mechanism was provided by incubation of equimolar amounts of protected Lys in the form of F-moc-Lys-OH hydrochloride, reduced GSH, and MDBGN. The expected mass addition of 106.0609 results in an F-moc-Lys-MGN product with an expected mass of 474.2267 Da, Figure S7. MS^2 spectra from the PRM analysis of the initial starting material F-moc-Lys (a) and the reaction mixture (b) are shown in Figure S8. The expected product could be detected, with the spectra from the F-moc-Lys (m/z 369.1804, [M + H]) and the final reaction product (m/z 475.2333, [M + H]) sharing common fragments (m/z 179.0853, 130.0862), Figure S8. Fragments at m/z 253.1655 and 297.1553 are unique for the final product with the former confirming modification of the reactive amine of the lysine, Figure S8(b).

Untargeted Proteomic Results from the Incubations of HSA and Hb. To determine whether MDBGN itself or its product after reaction with sulfhydryl groups, 2-MGN, reacts with intact protein nucleophilic side chains, HSA samples were treated with MDBGN and analyzed in Proteome Discoverer both for the mass addition from the metabolite 2-MGN (106.0609 Da) and the mass addition of the direct S_N2 reaction of MDBGN (183.9636 Da). As expected, no potential adducted sites corresponding to the modification of 183.9636 Da could be detected. Analysis of the same HSA samples using the added mass expected as a modification after transformation in the presence of sulfhydryl groups resulted in more than 20 potentially modified sites when incubated with a 100-fold excess of MDBGN, Figure 2(a).

The addition in mass for DNCB and PGE, 166.0015 and 150.0681 Da, respectively, was used in the analysis of the MS^2 data obtained from the untargeted analysis of HSA and Hb in Proteome Discoverer. In order to explore haptenation at different nucleophilic sites, modification of His, Lys, Cys, Ser, Thr, Met, Trp, and Tyr was screened for in the analysis for both HSA and Hb. Modification of the N-terminal Val was also included for Hb. As a result, a large number of potentially modified side chains were identified. Mass spectra of all peptides containing the modification sites were manually evaluated. Sites with spectra containing the b- and y-ions for confident identification and site-localization of the adduct were considered to be valid. In general, treatment of both proteins with increasing molar ratios of haptens showed increasing numbers of potentially adducted sites in the untargeted analysis for both haptens, Figure 2(a) and Figure 2(b). For DNCB, treatment of Hb with the 5-fold molar excess ratio of the hapten did not further increase the number of adducted sites of subunit β in the untargeted analysis but led to identification of other adduct modification sites within the same peptides.

Targeted Proteomic Results from the Incubation of HSA and Hb. Based on the results from untargeted analysis, an inclusion list of the m/z values of the modified peptide samples of HSA and Hb incubated with DNCB and PGE (molar ratios of hapten to protein: 0.1-, 0.5-, 1-, and 5-fold), as well as HSA incubated with MDBGN (molar ratios of MDBGN to HSA: 0.1-, 1-, 10-, and 100-fold), was analyzed in the PRM mode. Skyline was used to process the targeted analysis data using the previously acquired global MS^2 spectra as a database. Each confirmed modified peptide spectra required adduct localization via flanking b- and y-ions.

For HSA, the targeted analysis enabled confirmation of 11 adducted sites after treatment with DNCB and five adducted sites after treatment with PGE at a 5-fold molar excess of hapten, as well as 11 adducted sites after treatment with MDBGN at a 100-fold molar excess of hapten, with the majority of modified sites being cysteines and lysines, Table S1. In total, DNCB was confirmed to modify six cysteine residues (Cys_{34}, Cys_{62}, Cys_{75}, Cys_{90}, Cys_{91}, Cys_{477}), four lysine residues (Lys_{190}, Lys_{199}, Lys_{414}, Lys_{432}), and one serine residue (Ser_{419}). PGE was confirmed to modify three cysteine residues, Cys_{34}, Cys_{90}, and Cys_{177}, and two lysine residues, Lys_{64} and Lys_{190}. Treatment of HSA with a 100-fold molar excess of MDBGN resulted in modification of only cysteine residues, Cys_{62}, Cys_{90}, Cys_{91}, Cys_{177}, Cys_{245}, Cy_{265}, Cys_{279}, Cys_{289}, Cys_{369}, Cys_{514}, and Cys_{567}. Only Cys_{90} was modified by all three haptens, while several sites, Cys_{34}, Cys_{62}, Cys_{91}, and Lys_{190}, were modified by two of the three haptens.

The Hb targeted analysis confirmed a total of 17 reactive sites, Table S2; five of them were modified by DNCB, Tyr_{24}/α, Val_1/β, Cys_{93}/β, Lys_{95}/β, and Cys_{112}/β, and 12 were modified by PGE, Val_1/α, His_{20}/α, Tyr_{24}/α, His_{45}/α, His_{50}/α, His_{72}/α, Ser_{84}/α, His_{77}/β, Ser_{89}/β, His_{97}/β, His_{116}/β, His_{143}/β, and Lys_{144}/β. Only Tyr_{24}/α was a common reactive site for the two haptens.

Relative Quantitative Estimation of Modified Peptides. Although a number of adducted sites in HSA and Hb were confirmed by the targeted PRM analysis, only some of the adducted peptides showed an increase in the relative amount of the adducted peptide with increased concentration of hapten, Tables 2 and 3. This type of estimation exhibits large standard deviations due to experimental variations, including samples from different blood donors; however, the data indicates that these sites are the most reactive toward DNCB and PGE. We would like to stress that the aim of the current study was to identify the sites of HSA and Hb that are most likely to be modified *in vivo*. Hence, it was considered more important to have incubation conditions that represent the actual conditions *in vivo*, rather than using commercial HSA or purifying the HSA from plasma, something that would indeed have resulted in more accurate quantitative data but at the expense of altering the protein conformation and thereby affecting the reactivity of the different sites. The relative quantifications performed should be seen as further support for the reactivity of the haptenated sites rather than an accurate quantification of the level of protein modification.

For HSA, seven of the confirmed sites show a correlation between an increase in hapten concentration and an increase in the relative amount of the adducted peptide, Table 2 and Figures S9−S15, Cys_{34}, Cys_{62}, Cys_{90}, Cys_{177}, Cys_{477}, Lys_{190}, and Lys_{199}. Cys_{34}, the only free thiol group of HSA, and Cys_{90} are modified by both haptens. An increase in the relative amount of adducted HSA is also seen for PGE-modification of Cys_{177} and DNCB-modification of Cys_{62} and Cys_{477}. Adduction of cysteines other than Cys_{34} is worth noting and further exploring, as this observation indicates that some haptens may disrupt the molecular structure of HSA by interfering with the disulfide bonds. The case of two lysine residues, Lys_{190} and

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Table 2. Peptide Sequence and Confirmed Adducted Site Showing a Correlation between Increased Hapten Concentration and an Increase in the Relative Amount of the Adducted Peptide, Hapten Responsible for the Modification, Amino Acid Modified, as well as Theoretical, Observed *m/z* and Charge State of Each Peptide in HSA[a]

Peptide Sequence	Hapten	Adducted Amino acid	Theoretical m/z	Observed m/z	Charge State
LQQ**C**PFEDHVK	DNCB / PGE	Cys_{34}	503.88581 / 498.57468	503.88518 / 498.57481	+3
TC(+IAA)VADESAEN**C**DK	DNCB	Cys_{62}	804.28290	804.28220	+2
ETYGEMAD**C**C(+IAA)AK/ ETYGEMAD**C**CAK	DNCB / PGE	Cys_{90}	772.26041 / 735.78298	772.25980 / 735.78236	+2
AA**C**LLPK	PGE	Cys_{177}	433.24629	433.24606	+2
DEG**K**ASSAK	DNCB	Lys_{190}	529.72296	529.72360	+2
L**K**C(+IAA)ASLQKFGER	DNCB	Lys_{199}	534.92801	534.92790	+3
C(+IAA)**C**TESLVNR	DNCB	Cys_{477}	624.24268	624.23996	+2

[a]In red: modifications by DNCB. In blue: modifications by PGE.

Table 3. Peptide Sequence and Confirmed Adducted Site Showing a Correlation between Increased Hapten Concentration and an Increase in the Relative Amount of the Adducted Peptide, Hapten Responsible for the Modification, and Amino Acid Modified, as well as Theoretical, Observed *m/z*, and Charge State of Each Peptide in Hb[a]

Peptide Sequence	Hapten	Adducted Amino acid	Theoretical m/z	Observed m/z	Charge State
VLSPADKTNVK	PGE	Val_1/α	441.25030	441.25029	+3
TYFP**H**FDLSHGSAQVK	PGE	His_{45}/α	661.99155	661.98972	+3
TYFPHFDLS**H**GSAQVK	PGE	His_{50}/α	661.99155	661.98972	+3
VADALTNAVA**H**VDDMP NALSALSDLHAHK	PGE	His_{72}/α	630.11730	630.12055	+5
VHLTPEEK	DNCB	Val_1/β	559.75934	559.75984	+2
GTFATLSELH**C**DK	DNCB	Cys_{93}/β	794.34090	794.33986	+2
L**H**VDPENFR	PGE	His_{97}/β	426.21559	426.21534	+3
LLGNVLV**C**VLAHHFGK	DNCB	Cys_{112}/β	629.32963	629.32905	+3

[a]In red: modifications by DNCB. In blue modifications by PGE.

Lys_{199}, Figures S14 and S15, is also worth mentioning. Adduct formation on these lysines led to missed cleavage by trypsin, and although an increased area of the adducted peptide could be observed, relative quantification could not be performed due to the lack of the equivalent unmodified peptide. Characteristic examples are shown in Figure 3 for HSA, where treatment with a 5-fold molar excess of PGE led to a 112% increase (relative to the unmodified equivalent peptide) of the peptide LQQCPFEDHVK adducted at Cys_{34}, and a 5-fold molar excess of DNCB led to a 4.4% relative increase of the adducted peptide TCVADESAENCDK modified at Cys_{62}.

For Hb, eight adducted sites showed a correlation between an increase in hapten concentration and an increase in the relative amount of the adducted peptide, Table 3 and Figures S16−S21. Two of the sites are N-terminal valines, the Val_1 in subunit α modified by PGE and the Val_1 in subunit β modified by DNCB. Two more sites show an increase in relative amounts when treated with increased DNCB concentrations, Cys_{93} and Cys_{112} of subunit β. For Hb, several of the histidines were found to be adducted by PGE and increased in abundance upon treatment with increased hapten concentration, His_{45}, His_{50}, and His_{72} in subunit α, as well as His_{97} in subunit β. Characteristic examples for Hb adduction are shown in Figure 4. The relative amounts of the peptide containing the Cys_{93} adducted site of the subunit β, GTFATLSELHCDK, increased in abundance when increased molar ratios of DNCB were used. When treated with a 0.1-fold molar ratio, 0.1% was modified, which increased to 23% modified when the ratio was a 5-fold molar excess. Another example is the TYFPHFDLSHGSAQVK peptide containing the His_{45} site, where treatment with PGE leads to an increase in abundance from 13% to 82% when the molar ratio was increased from 0.1- to 5-fold.

If is also worth noting that three of the HSA sites (Cys_{34}, Cys_{62}, and Lys_{190}) and six of the Hb sites (subunit α: Val_1,His_{45}, His_{72}; subunit β: Cys_{93}, His_{97}, and Cys_{112}) were found to be modified already at a 0.1 molar ratio of hapten to protein (Table 4), suggesting that these sites are the most likely sites to be modified *in vivo*.

■ DISCUSSION

ACD has an impact on both quality of life of the people affected,[41] as well as an economic impact for the society.[42] Currently there is no cure for contact allergy, only symptomatic treatment with corticosteroids. Furthermore, disease diagnosis has not progressed much in the last decades. Patients suspected of having contact allergy are diagnosed using the patch test method, in which the 30 most common contact allergens (known as the baseline series) diluted in petrolatum or water as well as other specific series or the patient's own material are applied to the back of the patient. Eczematous reaction at a test site signifies that the patient is allergic to the compound tested.[43] Although the supposition that haptenated proteins trigger the immune system was introduced by Landsteiner and Jacobs already in 1936,[44] not much progress has been made either regarding identification of relevant hapten−protein conjugates or concerning the investigation of the potential of these conjugates as biomarkers for exposure monitoring or development of more effective diagnostic tests for contact allergy. A number of biomarkers have been studied in ACD, including genetic variations and antimicrobial peptides; however, most of them would be characteristic of inflammation in general, and biomarkers specific to ACD are yet to be identified.[45]

The reaction mechanism of MDBGN is not fully understood, and previous attempts to assess the sensitizing capacity of MDBGN have given conflicting results. Results of the GPMT for MDBGN have failed to detect its sensitizing capacity.[46] Additionally, the DPRA has not given useful results,

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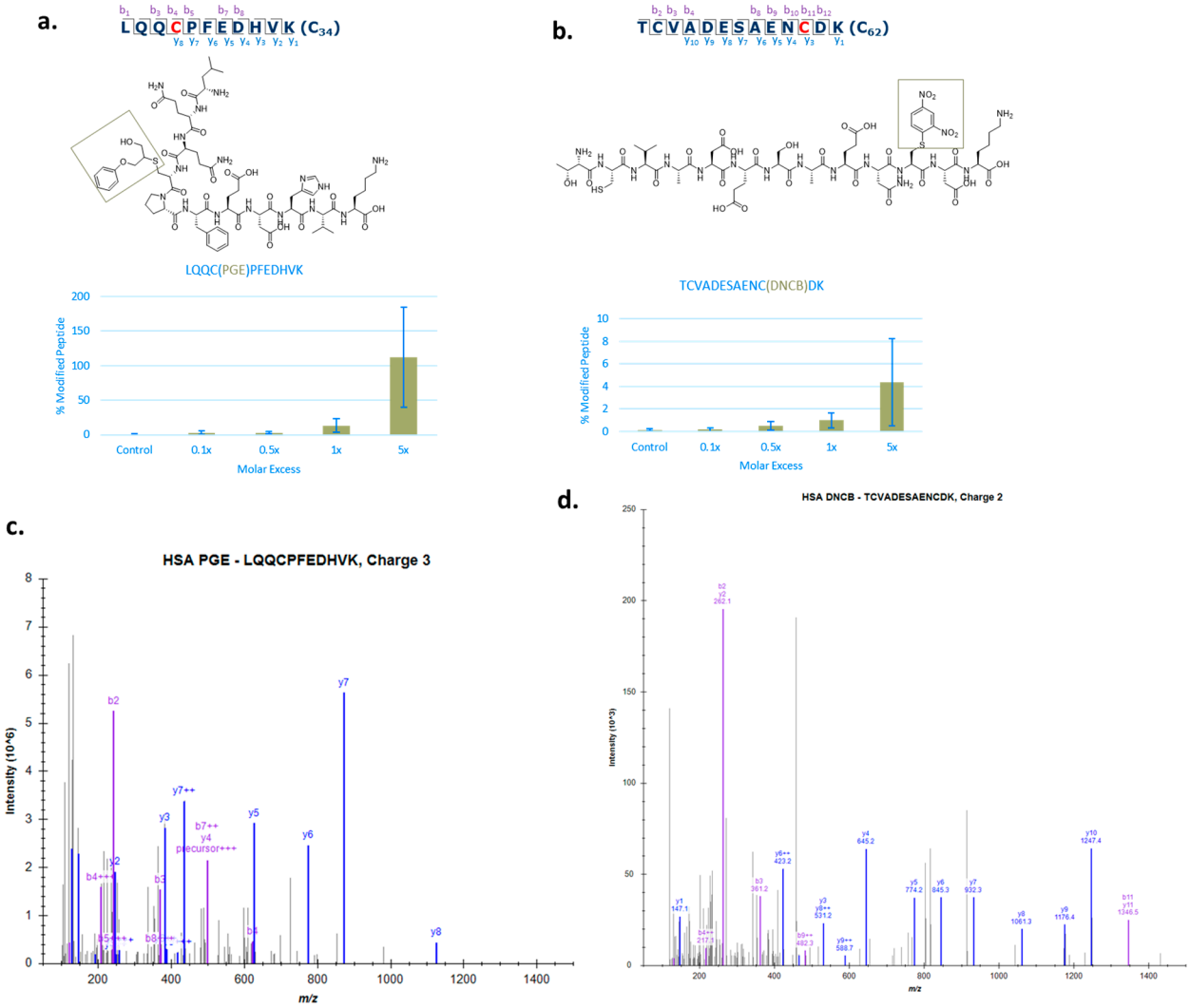

Figure 3. Relative estimation of the peptide containing the Cys_{34} site in HSA modified by PGE (a), relative estimation of the peptide containing the Cys_{62} site in HSA modified by DNCB (b), tandem mass spectra, structure of the adducted peptide identified using PRM analysis for each peptide respectively (c, d).

since complete dimerization of the peptide was observed. Coincubation with propylthiol, a sulfhydryl containing compound, led to the complete disappearance of MDBGN but no peptide-adduct formation.[40] Our experiments are in agreement with the studies performed by Sipes and co-workers who were the first to demonstrate that the sulfhydryl-mediated biotransformation product of MDBGN, 2-MGN, reacts with nucleophilic moieties of biomolecules and that MDBGN does not react directly.[37,38] This finding gives a plausible explanation to the inconsistent results obtained from previous *in vitro* assessments of MDBGN. Indeed, our DPRA assessment of 2-MGN classified it as a moderate sensitizer, which is in agreement with the LLNA classification of MDBGN.

For HSA there is good correlation between the number of sites modified by the different haptens studied and their sensitizing capacity in the LLNA. DNCB is classified as an extreme sensitizer *in vivo* and gives 12 adducted sites at a 5-fold molar excess, whereas PGE, which is classified as a strong sensitizer, gives six adducted sites at a 5-fold molar excess, Figure 2(a). According to the LLNA, MDBGN is a much weaker sensitizer than both DNCB and PGE and is classified as a moderate sensitizer. MDBGN also displayed a much lower reactivity toward HSA compared to DNCB and PGE, and therefore higher molar ratios of MDBGN had to be used in order to obtain adduct levels that could be detected. At a 10-fold molar excess, the number of sites in HSA potentially modified by MDBGN was one, Figure 2(a). A similar correlation between sensitizing potency in the LLNA and the number of adducted sites could not be observed for Hb, for which PGE modified more sites than DNCB, Figure 2(b). The fact that HSA correlates so well with the LLNA could be an indication that haptenation of HSA may actually result in immunogenic hapten−protein conjugates capable of activating T cells. Support for this hypothesis can be found in a study by Jenkinson et al., in which HSA−hapten conjugates were shown to have the ability to induce T cell proliferation in contact allergic patients.[20] In a study of serum from healthy human subjects, IgG and IgM antibodies against xenobiotics conjugated to serum albumin were detected in 8−22% (different percentages for different xenobiotics) of the samples,[47] further supporting the hypothesis that hapten−albumin conjugates may be able to activate the immune system.

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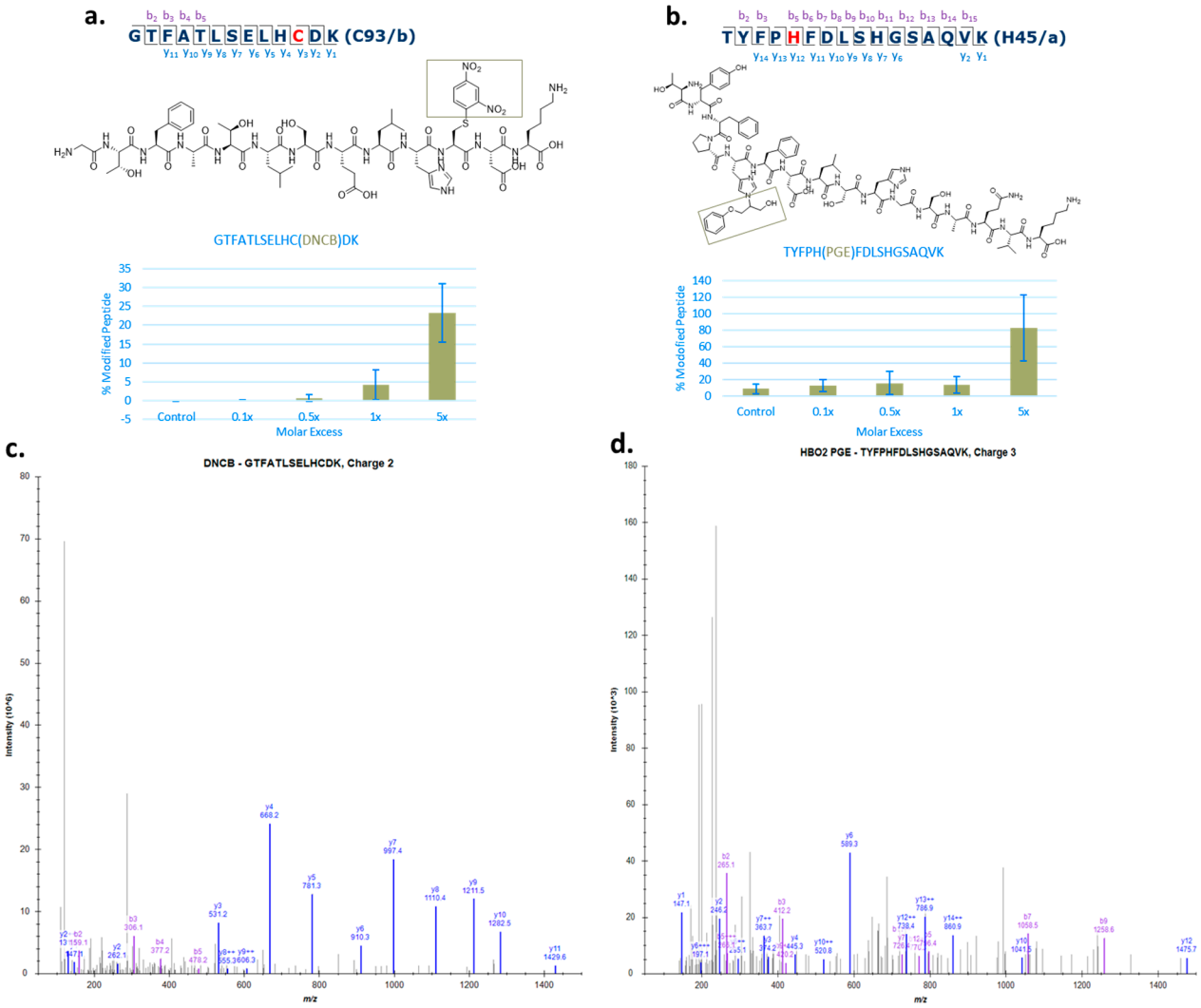

Figure 4. Relative estimation of the peptide containing the Cys_{93} site in the β subunit of Hb modified by DNCB (a), relative estimation of the peptide containing the His_{45} site in the α subunit of Hb modified by PGE (b), tandem mass spectra, structure of the adducted peptide identified using PRM analysis for each peptide, respectively (c, d).

Table 4. Protein Adducted at a 0.1-Fold Molar Excess of Hapten[a]

protein	hapten	peptide sequence	adducted amino acid	replicate(s)
HSA	PGE	LQQ**C**PFEDHVK	**Cys_{34}**	2/3
HSA	DNCB	TCVADESAEN**C**DK	**Cys_{62}**	1/3
HSA	DNCB	DEG**K**ASSAK	**Lys_{190}**	1/3
Hb	PGE	**V**LSPADKTNVK	**Val_1/α**	3/3
Hb	PGE	TYFP**H**FDLSHGSAQVK	**His_{45}/α**	3/3
Hb	PGE	VADALTNAVA**H**VDDMPNALSALSDLHAHK	**His_{72}/α**	3/3
Hb	DNCB	GTFATLSELH**C**DK	**Cys_{93}/β**	3/3
Hb	PGE	L**H**VDPENFR	**His_{97}/β**	2/3
Hb	DNCB	LLGNVLV**C**VLAHHFGK	**Cys_{112}/β**	3/3

[a]Blood protein, hapten responsible for the modification, peptide sequence, confirmed adducted site, and number of replicates in which modification could be observed.

As demonstrated in Figure 5, DNCB seems to modify more sites inside HSA compared to PGE that mostly modifies sites exposed to the surface of the two proteins. Factors, such as steric hindrance as well as the environment of each individual site, depending on the surrounding amino acids, may influence the reactivity of each site and subsequently adduct formation. Modification of cysteine residues, other than Cys_{34}, is another interesting observation, since the aforementioned site is the only free cysteine in HSA. However, this is not the first time that other modified cysteine residues have been linked to DNCB. Parkinson et al.[19] have also reported such cases while also studying the reducing capacity of DNCB. Interestingly, other sites, such as histidines and lysines, are mostly reported in the Parkinson study in which high concentrations of DNCB were used (a 100-fold molar excess). This gives rise to the question if the modified sites reported in the Parkinson study

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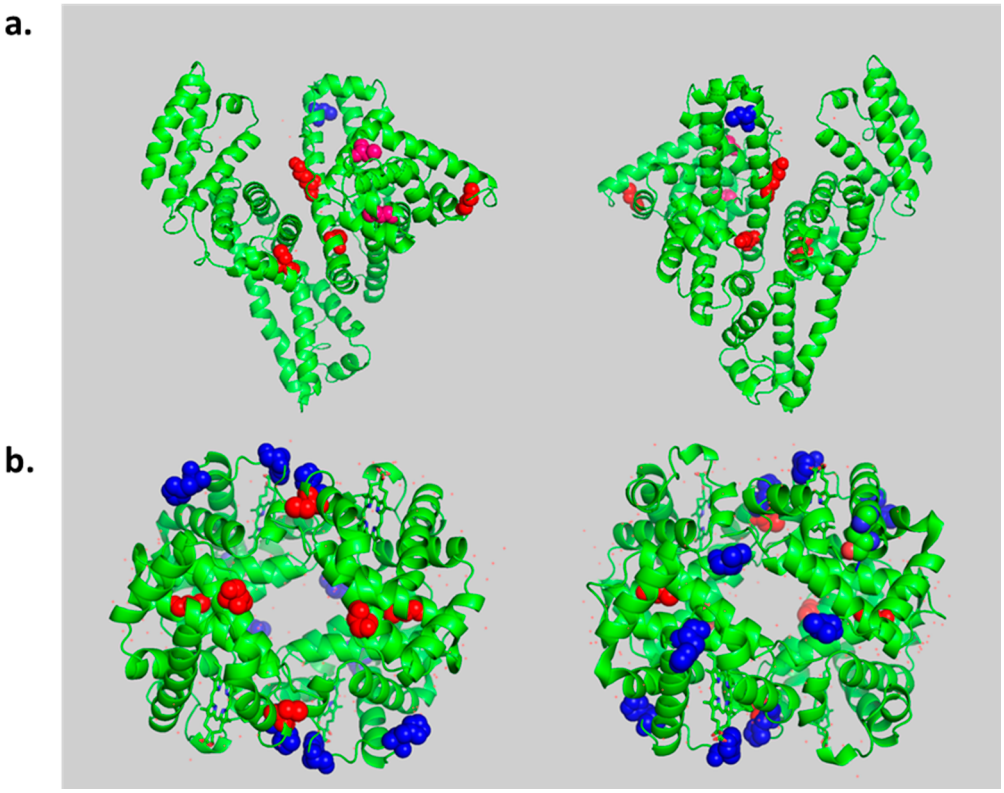

Figure 5. Protein Data Bank (PDB) structure of HSA front and back, with sites modified by DNCB highlighted in red, sites modified by PGE highlighted in blue, and sites modified by both highlighted in pink (a) and the PDB structure of Hb front and back, with sites modified by DNCB highlighted in red and sites modified by PGE highlighted in blue (b).

are an effect of the high exposure level used and not actually representative of modifications occurring at real exposure levels or if the discrepancies with the modified sites identified in the current study are attributed to other experimental differences.

In general, confirmation by targeted analysis of sites suggested from the untargeted analysis is deemed crucial, since a number of sites in the untargeted analysis can be attributed to the incorrect localization of the same adduct to different sites of the same peptide. This can, for example, be seen in the current study in which the decrease seen in the number of adducted sites of subunit β of Hb, when the hapten concentration is increased from equimolar amounts to a 5-fold molar excess of hapten, Figure 2(b), is due to more correct localization of the adduct within a peptide. It is worth noting that although no increase is seen in the number of adducted sites for subunit β of Hb when going from equimolar amounts to a 5-fold excess of hapten, an increase is seen in the relative modification of each site, Figures 4 and S19−S21.

The adductome of HSA has been studied previously for electrophilic contact allergens.[18−21] In many of these cases, experimental factors such as the use of a very high molar excess of the hapten and the use of commercial HSA instead of plasma HSA can lead to findings that are not representative of actual *in vivo* exposure conditions. Underestimation of adduct formation at Cys_{34} due to high levels of mixed disulfide at the specific site is one of the most common disadvantages of using commercial HSA. Another limitation of commercial HSA is the removal of fatty acids and other endogenous ligands that occur during preparation which can alter the protein conformation and thereby affect site reactivity toward some electrophilic compounds.[48] Nonetheless, if the aim of a study is to better understand the mechanistic aspects of adduct formation, in general, or to develop an *in vitro* method that would be able to give a correct classification of a compound's sensitizing potency such as in the study by Parkinson et al.,[19] these deviations from realistic *in vivo* exposure conditions may not be of any significance. The aim of the current study, on the other hand, was to identify sites of HSA and Hb that are likely to become haptenated when an individual is exposed to a contact allergen in order to find potential adduct biomarkers that can be used for exposure monitoring and to develop better diagnostic methods; hence, it was deemed crucial that the incubation conditions are representative of real exposure conditions. Compared to the study by Parkinson et al.,[37] where the adductome of commercial HSA was studied when incubated with DNCB (among other haptens), there is an overlap of the adducted sites with this study. However, the results cannot be directly compared due to the different sources of the HSA in the two studies, commercial HSA compared to plasma HSA in the current study, and the different molar ratios of hapten used, 0.1, 0.5, 1, and 5 in the current study compared to the 100-fold excess of hapten used in the Parkinson study. In addition, there is a difference in the pH of the buffers used to dilute/solubilize HSA; in this study, PBS pH 7.4 was used, whereas triethylammonium bicarbonate pH 8 was used in the study by Parkinson et al. The higher pH used in the Parkinson study results in an increased reactivity of amine nucleophiles such as lysines and histidines. Despite the differences between the two studies, three sites, namely Cys_{34}, Lys_{190}, and Lys_{199}, were found to be adducted in both studies, indicating that haptenation of these sites may be potential biomarkers for this contact allergen. Parkinson et al. also

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showed modification on the Lys_{64} in the peptide TCVADESAENCDK from HSA. A small fragment belonging to that adducted site could be observed in our targeted MS analysis (PRM), though the most intense fragments were observed for the adduct on the Cys_{62}, Figure 3 (d). This also applies to the peptide ETYGEMADCCAK, where Parkinson et al. located the adduct on the Lys_{93}, while in our study the adduct is located on Cys_{90}, Figure S12, with no fragments indicating the presence of an adduct on Lys_{93}. If Lys_{93} was adducted, the y-ions y_1, y_2, and y_3, would also carry the added mass of the modification. Cys_{177} is also a site found to be adducted in both studies, although not by the same hapten. In our study, PGE is found to modify Cys_{177}, whereas Parkinson et al. report a modification of Cys_{177} by DNCB. However, as mentioned previously, the discrepancies between our study and the study by Parkinson et al. might be explained by the differences in hapten concentration, source of HSA, and other experimental differences.

Although there is no available study of Hb adducts with contact allergens, the adductome of Hb has been used extensively to biomonitor exposure to other reactive species, mainly genotoxic compounds.[14] From these studies, Cys_{93} of the subunit β of Hb seems to be one of the most reactive sites.[24,48] Adducts to the N-terminal Val have also been used for monitoring of exposure to many compounds.[49−53] Studies of Hb with styrene-7,8-oxide showed that in addition to the N-terminal Val and Cys_{93}, other sites observed in our study, such as His_{45}, His_{50}, and His_{72} in subunit α, as well as His_{97} and Cys_{112} in the β subunit, are reactive and prone to form adducts with styrene-7,8-oxide.[54,55] In our study, all aforementioned sites were found to be adducted and showed a correlation between an increase in hapten concentration and an increase in the relative amount of the adducted peptide, Table 3.

There are a number of excellent studies conducted concerning albumin- and Hb-adducts of diisocyanates that should be mentioned in this context. Diisocyanates are small molecular chemicals commonly used as cross-linking agents in the production of polyurethane. The most commonly used diisocyanates are 4,4′-methylenediphenyl diisocyanate (MDI), toluene diisocyanate, and 1,6-hexamethylene diisocyanate. The diisocyanates are inherently reactive and can bind covalently to endogenous proteins and cause a number of adverse health effects. Although diisocyanates have the ability to cause contact allergy, the most commonly observed occupational health effect from these compounds is allergic asthma, and the diisocyanates are therefore classified as respiratory allergens rather than contact allergens. Anyhow, respiratory allergens are also haptens that induce harmful health effects by activating the immune system in much the same way as contact allergens, with the main difference being that the symptom upon elicitation is asthma rather than eczema. Albumin and Hb adducts with diisocyanates have been studied both *in vitro* and *in vivo*, and it has been shown that the most abundant diisocyanate adducts are Lys-adducts from albumin[56−60] and N-terminal Val adducts of Hb.[60−63] Diisocyanate-adducts of albumin have been shown to be immunogenic[60,64] and are believed to be involved in the etiology of sensitization to the diisocyanates.[65,66] Further, the levels of MDI-HSA adducts in MDI-exposed individuals have been shown to correlate with symptoms of allergic asthma and with the presence of diisocyanate-specific IgG antibodies.[67] Diisocyanate-adducts of HB are not believed to be immunogenic[60] but are still considered useful for biomonitoring purposes.[62,63] The mean adduct levels of MDI-Lys adducts of HSA in construction workers exposed to MDI were found to be 102 fmol/mg HSA[57] (corresponding to 0.007 mol MDI-Lys/mol HSA), and the mean levels in MDI-exposed individuals suffering from MDI-induced allergic asthma were found to be 501 fmol/mg HSA[67] (corresponding to 0.03 mol MDI-Lys/mol HSA). Hence, very low molar ratios of hapten to protein, such as 0.1 mol hapten/mol protein or even lower, would likely be most relevant. To the best of our knowledge, the *in vivo* levels of hapten−protein adducts in humans exposed to contact allergen is not known, but it is something that we would like to investigate in the future. However, before such studies are possible, it is essential to have analytical methods that can detect very low levels of hapten−protein adducts. Considering the high excess of hapten used in relation to HSA in previous studies (usually 100-fold or more),[18−21] the current study is a big step toward more realistic exposure levels. Indeed, we did observe three HSA sites and six Hb sites to be modified already at 0.1 mol hapten/mol protein (Table 4), indicating that these positions are the most likely sites to be modified *in vivo*, at least by the haptens DNCB and PGE. As mentioned previously, hapten-modification of HSA would appear to be more likely to result in the formation of an immunogenic complex than haptenation of Hb. However, more modified sites could be detected for Hb than HSA at the lowest level of hapten to protein ratio (0.1:1), and in addition, these modifications were seen in all three replicates rather than in just one or two of the replicates, which was the case for HSA (Table 4). These results suggest that Hb-adducts may be more suitable than HSA-adducts for biomonitoring of exposure to contact allergens.

■ SUMMARY AND CONCLUSIONS

In this study, the adduct formation of three different haptens with two major blood proteins, HSA and Hb, was studied. Incubations of the proteins with different molar ratios of haptens, at levels that are an order closer to realistic exposure levels compared to studies conducted in the past, revealed different potential adduction sites. As previously reported by Sipes and co-workers,[37,38] MDBGN was shown to react with cysteine after sulfhydryl-mediated conversion to the dibrominated 2-MGN, a claim further supported by the results obtained from the DPRA analysis.

Targeted (PRM) analysis was used to confirm that the potential modification sites of HSA and Hb found in the untargeted screening were indeed real. The reactivity of two of the haptens (DNCB and PGE) was further evaluated by performing a relative quantification based on the control (nonadducted) peptides. In accordance with the literature, the current study showed that haptens exhibit some degree of specificity, with DNCB reacting mostly with cysteine and lysine side chains in both proteins, whereas PGE reacted mostly with cysteine side chains in HSA and histidine side chains in Hb. In total, seven sites exhibiting an increase in adduction levels with an increase in hapten concentration were confirmed for HSA, and eight sites were confirmed for Hb after treatment with DNCB and PGE. Three of the HSA sites (Cys_{34}, Cys_{62}, and Lys_{190}) and six of the Hb sites (subunit α: Val_1, His_{45}, His_{72}; subunit β: Cys_{93}, His_{97}, and Cys_{112}) were modified already at 0.1 mol hapten/mol protein. Hence, haptenation of these sites has the potential as biomarkers that can be used for monitoring exposure to contact allergens and for development of improved diagnostic methods for contact allergy based on a blood sample.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.0c00271.

Confirmed adducted sites, reaction mechanism of different haptens tested, DPRA for MDBGN, relative quantitative estimation of modified peptides, and PDB structures of adducted proteins (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

2-MGN, 2-methylenepentanedinitrile; ACD, allergic contact dermatitis; ACN, acetonitrile; DCs, dendritic cells; DGEBA, diglycidyl ether of bisphenol A; DMSO, dimethyl sulfoxide; LC, liquid chromatography; DNCB, 1-chloro-2,4-dinitrobenzene; DPRA, direct peptide reactivity assay; EC3, estimated concentration to cause a stimulation index of 3; ERM, epoxy resin monomer; FA, formic acid; FDA, Food and Drug Administration; GPMT, guinea pig maximization test; GSH, glutathione; Hb, hemoglobin; HSA, human serum albumin; LLNA, local lymph node assay; MDBGN, 2-bromo-2-(bromomethyl)glutaronitrile; MDI, 4,4′-methylenediphenyl diisocyanate; MeOH, methanol; OECD, organization for economic cooperation and development; MS, mass spectrometer; PBS, phosphate-buffered saline; PGE, 1,2-epoxy-3-phenoxypropane; PRM, parallel reaction monitoring; SI, stimulation index

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Characterizing Adduct Formation of Electrophilic Skin Allergens with Human Serum Albumin and Hemoglobin

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Confirmed adducted sites

Table S1. Peptide sequence and confirmed adducted site for all haptens tested with HSA, namely DNCB, PGE and MDBGN.

Peptide Sequence	Target Amino acid	DNCB	PGE	MDBGN
LQQ**C**PFEDHVK	**Cys_{34}**	X	X	
TCVADESAEN**C**DK	**Cys_{62}**	X		X
TCVADESAENCD**K**	**Lys_{64}**		X	
L**C**TVATLR	**Cys_{75}**	X		
ETYGEMAD**C**CAK	**Cys_{90}**	X	X	X
ETYGEMADC**C**AK	**Cys_{91}**	X		X
AA**C**LLPK	**Cys_{177}**		X	X
(LDELR)DEG**K**ASSAK	**Lys_{190}**	X	X	
L**K**CASLQKFGER	**Lys_{199}**	X		
VHTE**C**CHGDLLECADDR	**Cys_{245}**			X
I**C**ENQDSISSK	**Cys_{265}**			X
EC**C**EKPLLEK	**Cys_{279}**			X
SH**C**IAEVENDEMPADLPSLAADFVESK	**Cys_{289}**			X
CCAAADPHE**C**Y	**Cys_{369}**			X
TK**K**VPQVSTPTLVEVSR	**Lys_{414}**	X		
YTKKVPQV**S**TPTLVEVSR	**Ser_{419}**	X		
NLG**K**VGSK	**Lys_{432}**	X		
C**C**TESLVNR	**Cys_{477}**	X		

HADI**C**TLSEK	**Cys_{514}**		X
ADDKET**C**FAEEGK	**Cys_{567}**		X

Table S2. Peptide sequence and confirmed adducted site for all haptens tested with Hb, namely DNCB and PGE.

Peptide Sequence	Target Amino acid	DNCB	PGE
VLSPADKTNVK	**Val_1/α**		X
VGA**H**AGEYGAEALER	**His_{20}/α**		X
VGAHAGE**Y**GAEALER	**Tyr_{24}/α**	X	X
TYFP**H**FDLSHGSAQVK	**His_{45}/α**		X
TYFPHFDLS**H**GSAQVK	**His_{50}/α**		X
VADALTNAVA**H**VDDMPNALSALSDLHAHK	**His_{72}/α**		X
VADALTNAVAHVDDMPNALSAL**S**DLHAHK	**Ser_{84}/α**		X
VHLTPEEK	**Val_1/β**	X	
VLGAFSDGLA**H**LDNLK	**His_{77}/β**		X
GTFATL**S**ELHCDK	**Ser_{89}/β**		X
GTFATLSELH**C**DK	**Cys_{93}/β**	X	
GTFATLSELHCD**K**	**Lys_{95}/β**	X	

L**H**VDPENFR	**His$_{97}$/β**		X
LLGNVLV**C**VLAHHFGK	**Cys$_{112}$/β**	X	
LLGNVLVCVLA**H**HFGK	**His$_{116}$/β**		X
VVAGVANALA**H**K	**His$_{143}$/β**		X
VVAGVANALAH**K**	**Lys$_{144}$/β**		X

Reaction mechanism of the different haptens tested

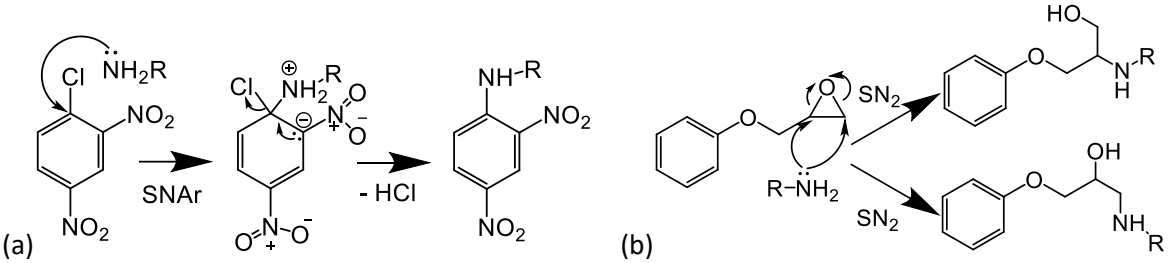

Figure S1. Reaction mechanisms of (a) DNCB and (b) PGE.

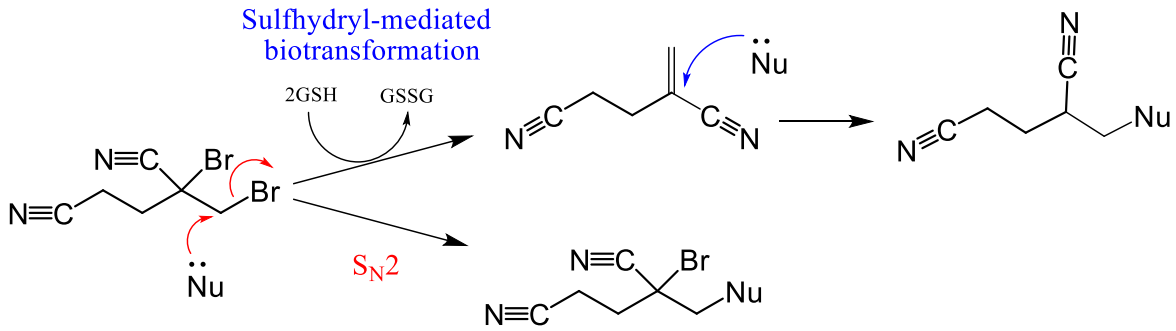

Figure S2. Reaction mechanisms of MDBGN.

Direct Peptide Reactivity Assay (DPRA) for MDBGN

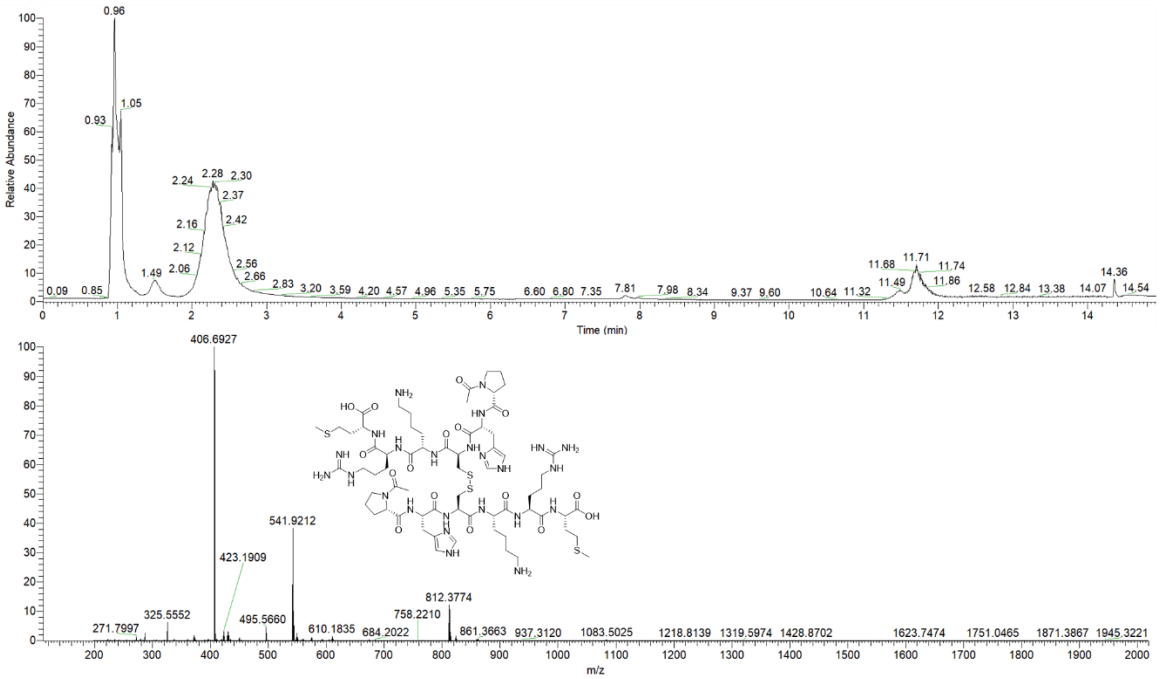

Figure S3. Total Ion Chromatogram (TIC) of the reaction mixture of the peptide Ac-PHCKRM and MDBNG at time point 0, and the MS/MS spectra of the peak corresponding to the peptide showing the complete dimerization of the initial peptide at retention time 2.3 min. The rest of the peaks in the chromatogram are attributed to impurities also observed in the blank and control runs.

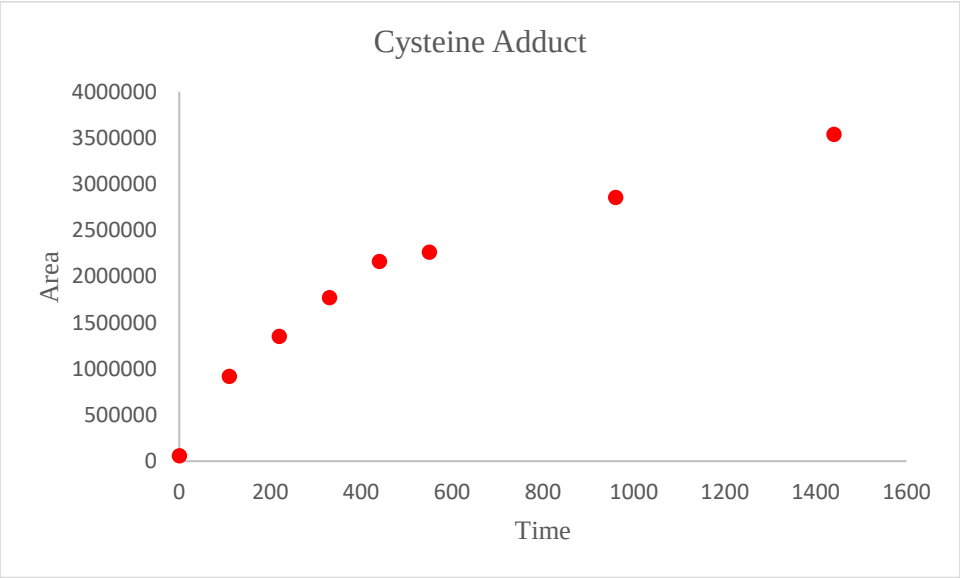

Figure S4. Formation of the adducted peptide Ac-PHCKRM during incubation with standard MDBGN and reduced GSH, over the rate of 24 hours.

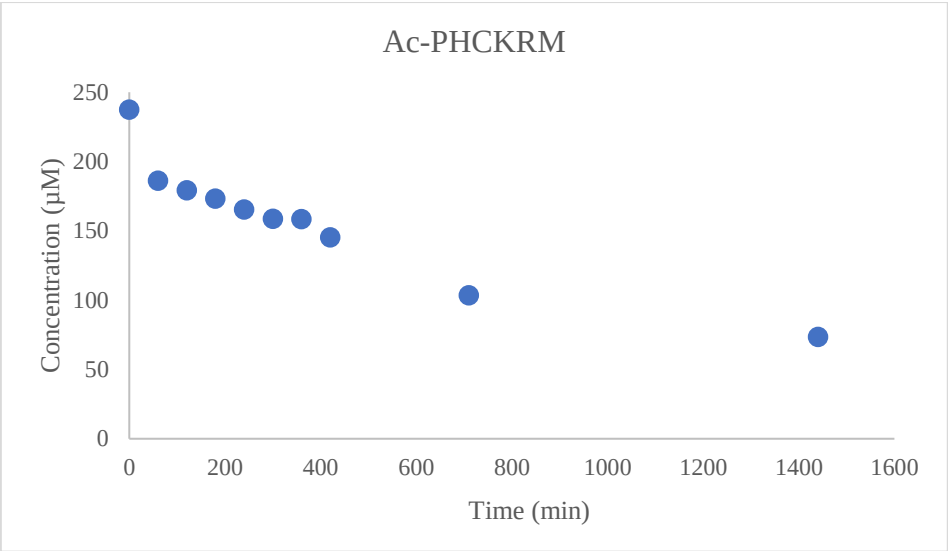

Figure S5. Depletion of the peptide Ac-PHCKRM during incubation with standard 2-MGN, over the course of 24 hours, expressed in µM.

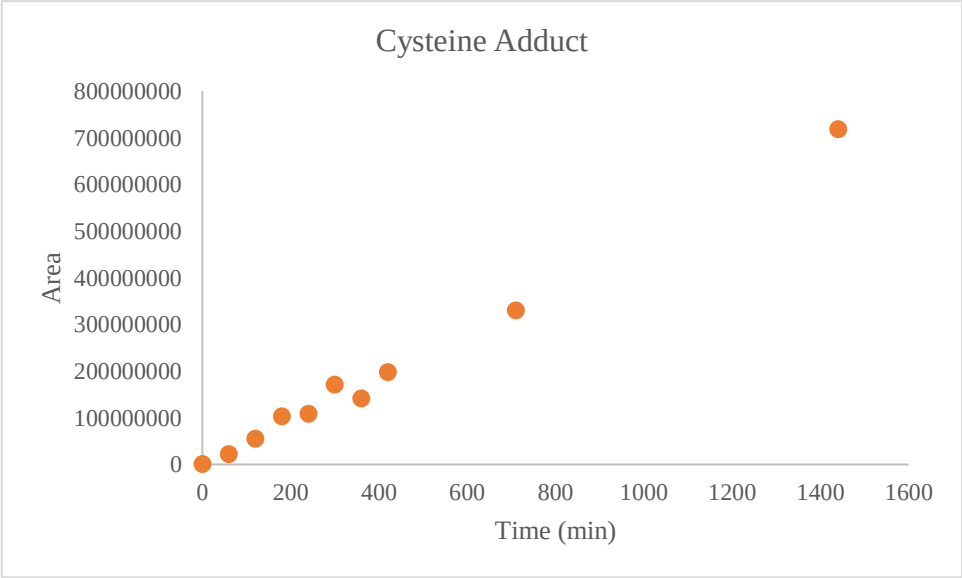

Figure S6. Formation of the adducted peptide Ac-PHCKRM during incubation with standard 2-MGN, over the rate of 24 hours.

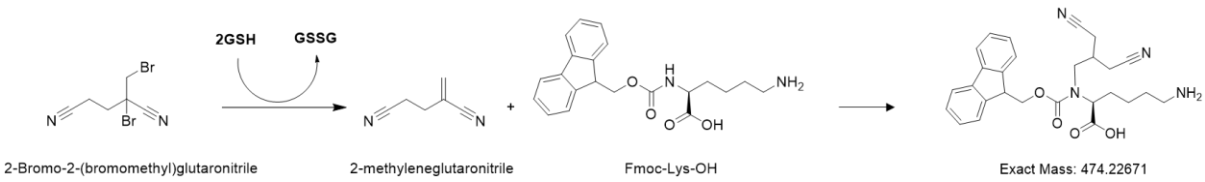

Figure S7. Reaction scheme of MDBGN with F-moc-Lys including activation by GSH.

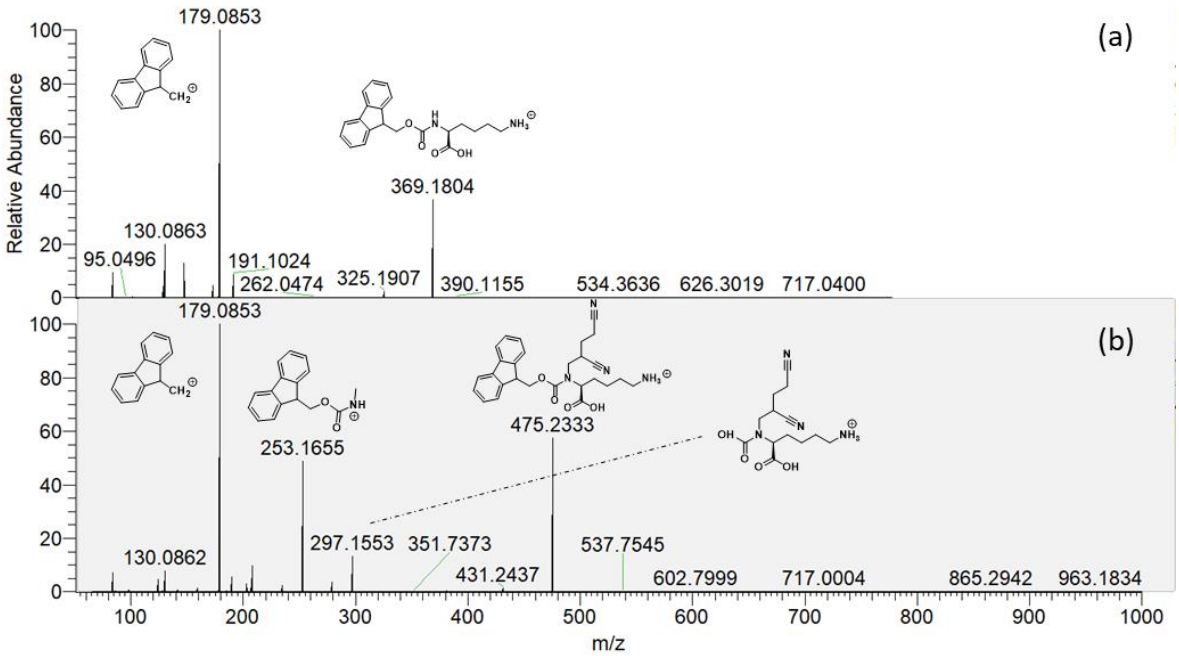

Figure S8. Fragmentation pattern of (a) F-moc-Lys and (b) reaction product of F-moc-Lys with MDBGN activated by GSH.

Relative quantitative estimation of modified peptides

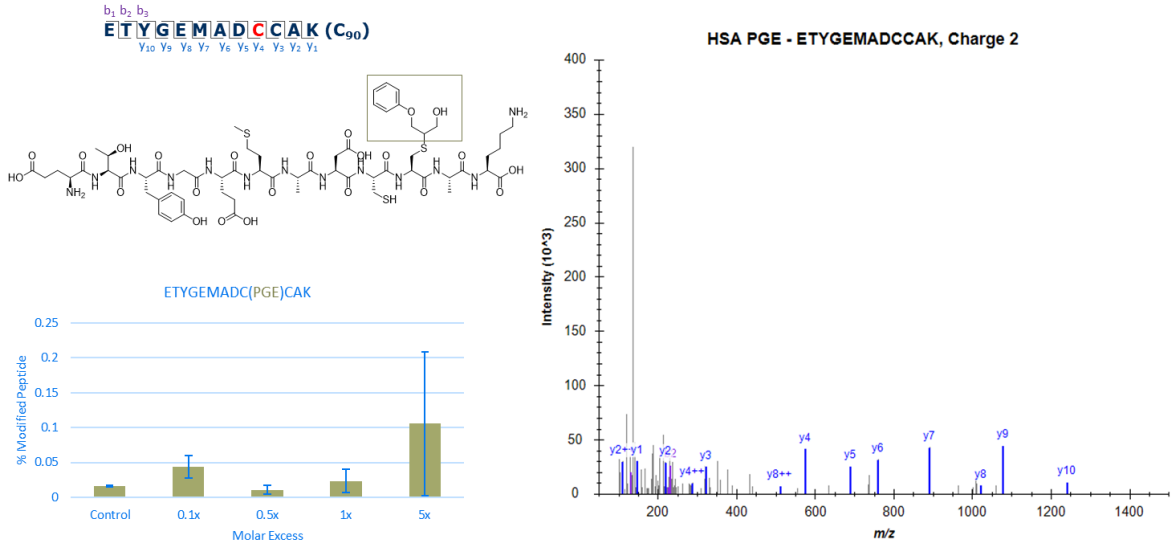

Figure S9. Tandem mass spectra, structure of the adducted peptide identified using PRM analysis, and relative estimation of the peptide containing the Cys_{90} site in HSA modified by PGE.

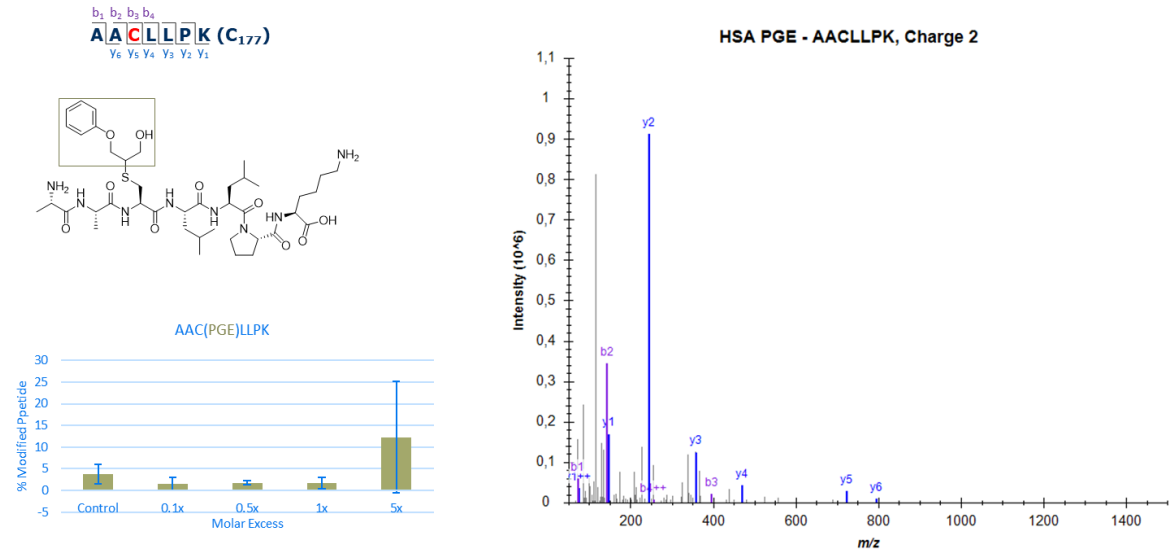

Figure S10. Tandem mass spectra, structure of the adducted peptide identified using PRM analysis, and relative estimation of the peptide containing the Cys_{177} site in HSA modified by PGE.

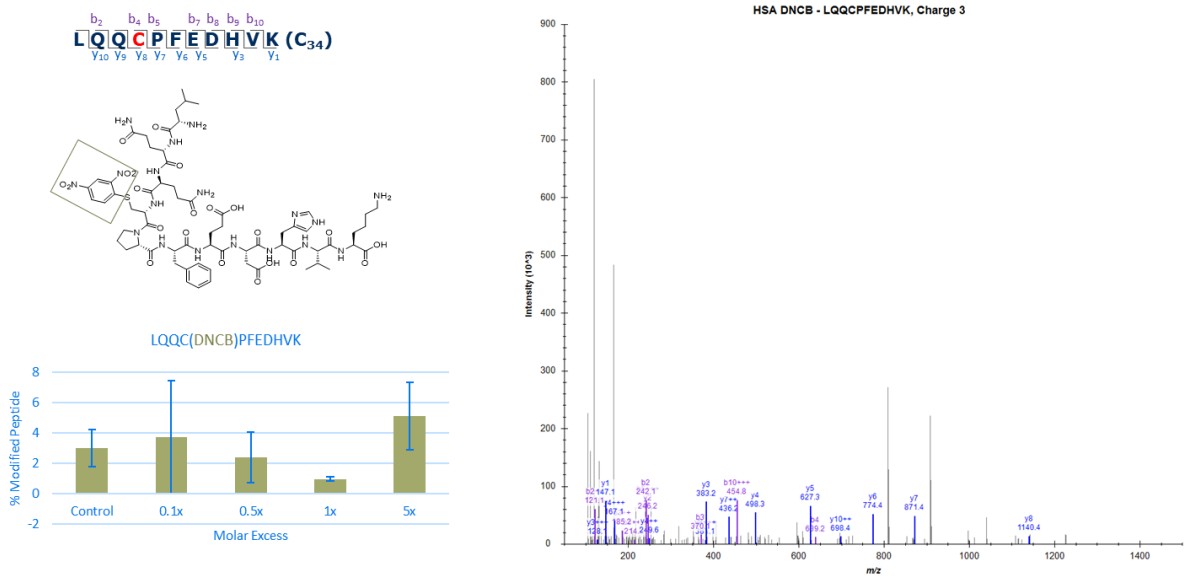

Figure S11. Tandem mass spectra, structure of the adducted peptide identified using PRM analysis, and relative estimation of the peptide containing the Cys_{34} site in HSA modified by DNCB.

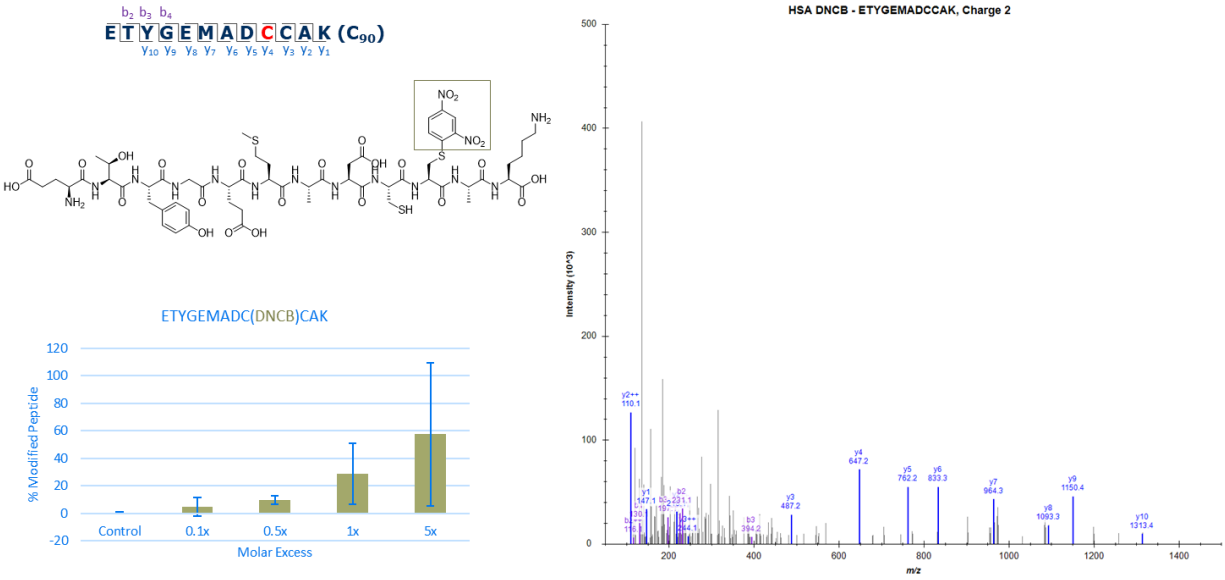

Figure S12. Tandem mass spectra, structure of the adducted peptide identified using PRM analysis, and relative estimation of the peptide containing the Cys_{90} site in HSA modified by DNCB.

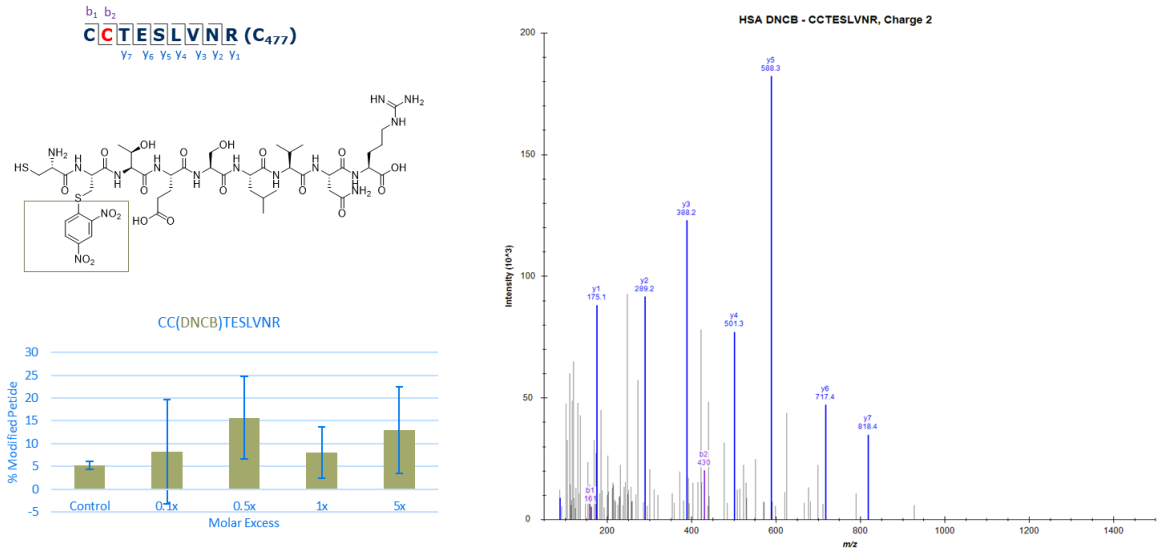

Figure S13. Tandem mass spectra, structure of the adducted peptide identified using PRM analysis, and relative estimation of the peptide containing the Cys_{477} site in HSA modified by DNCB.

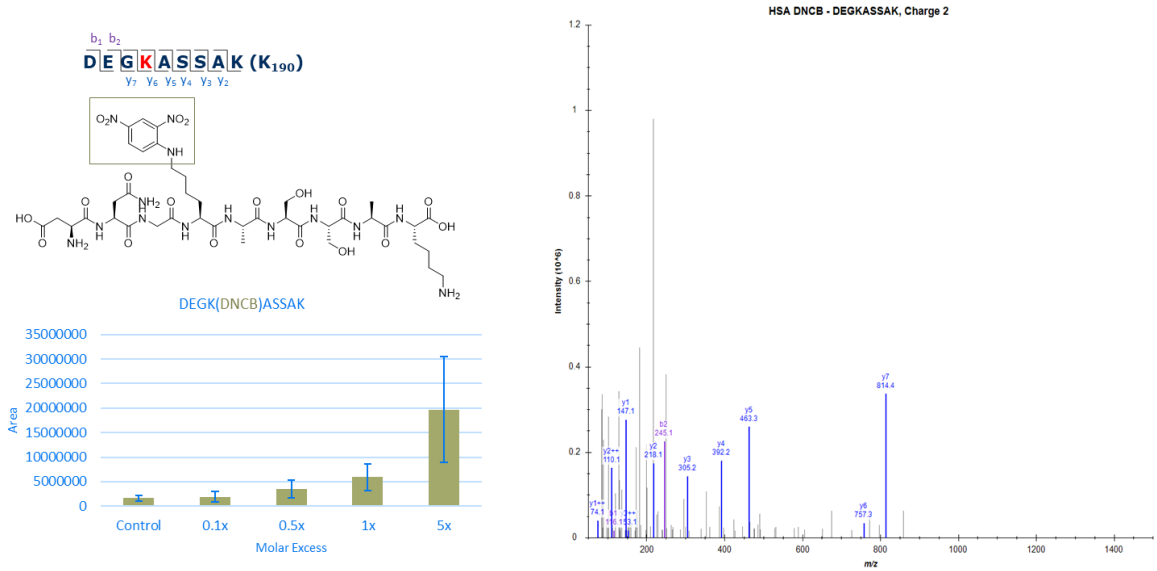

Figure S14. Tandem mass spectra, structure of the adducted peptide identified using PRM analysis containing the Lys190 site in HSA modified by DNCB.

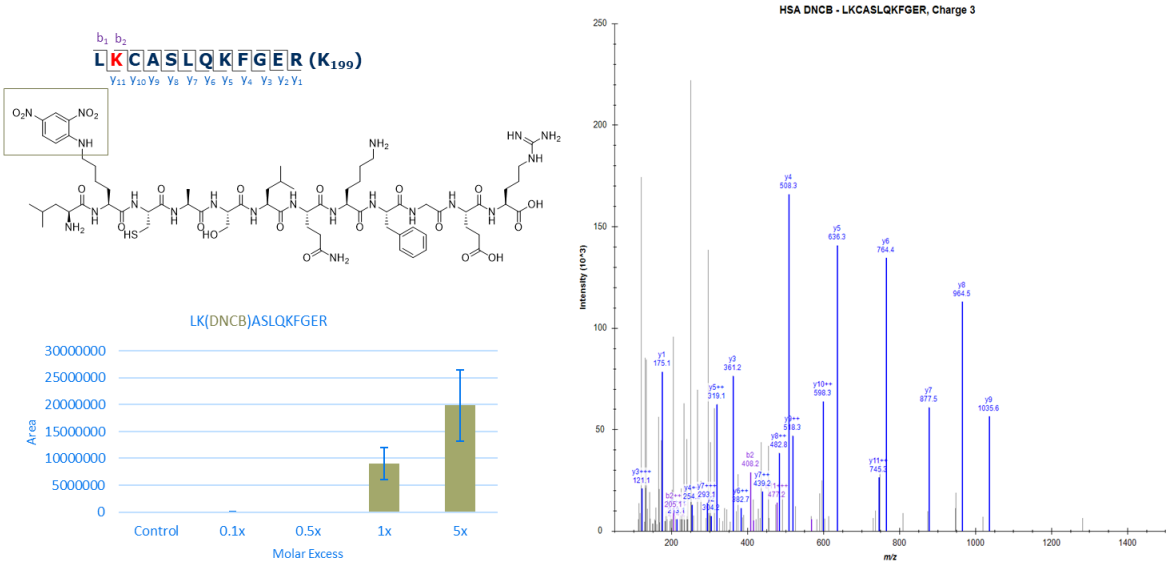

Figure S15. Tandem mass spectra, structure of the adducted peptide identified using PRM analysis containing the Lys199 site in HSA modified by DNCB.

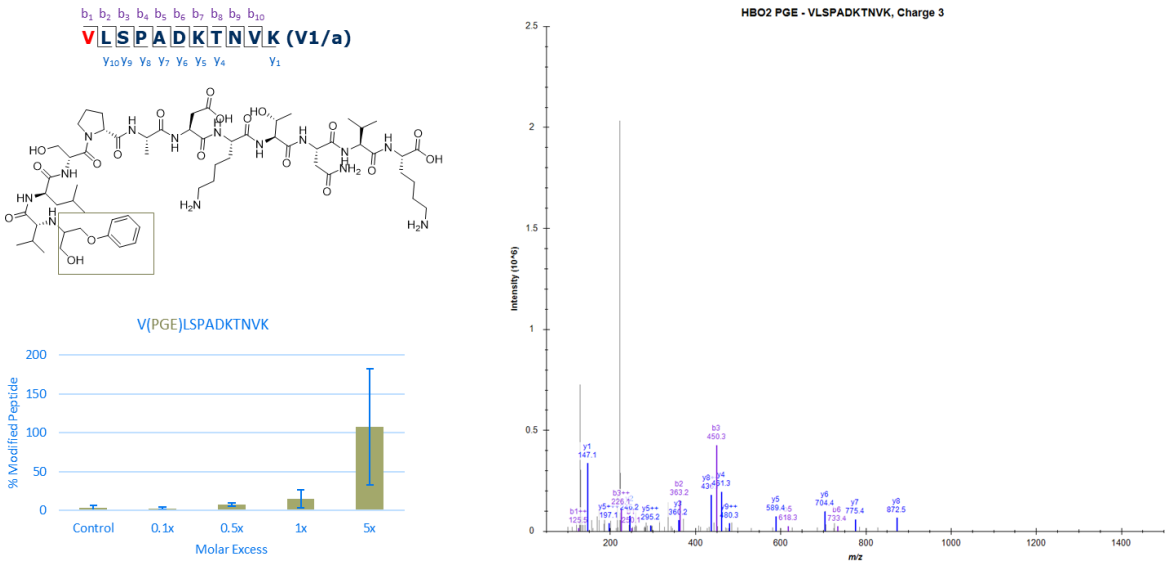

Figure S14. Tandem mass spectra, structure of the adducted peptide identified using PRM analysis, and relative estimation of the peptide containing the Val_1 site in subunit α of Hb modified by PGE.

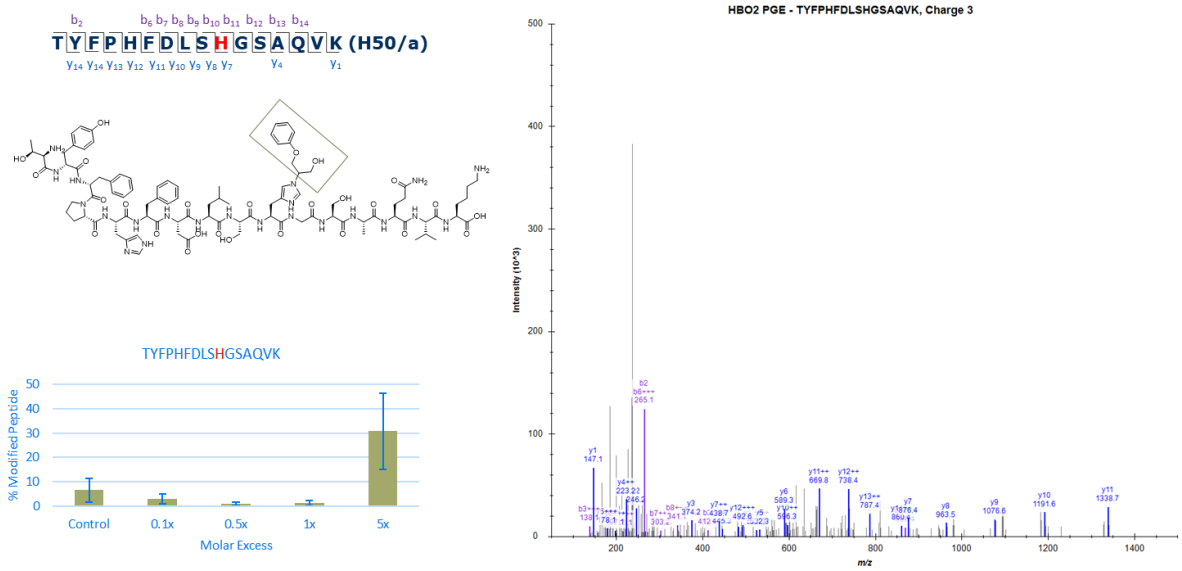

Figure S15. Tandem mass spectra, structure of the adducted peptide identified using PRM analysis, and relative estimation of the peptide containing the His_{50} site in subunit α of Hb modified by PGE.

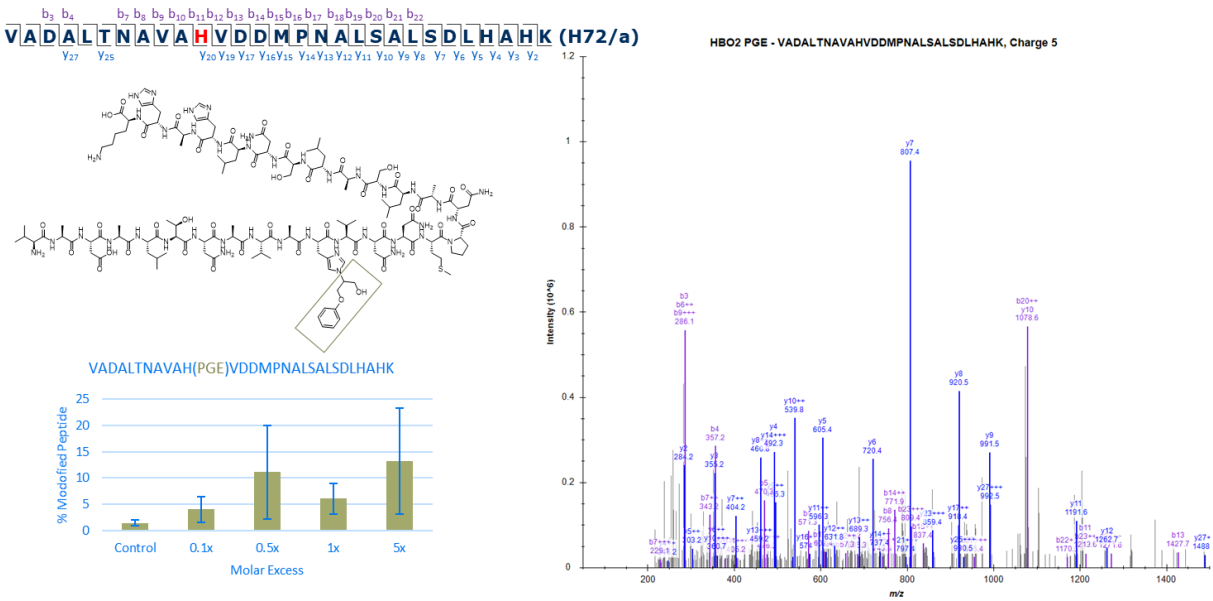

Figure S16. Tandem mass spectra, structure of the adducted peptide identified using PRM analysis, and relative estimation of the peptide containing the His_{72} site in subunit α of Hb modified by PGE.

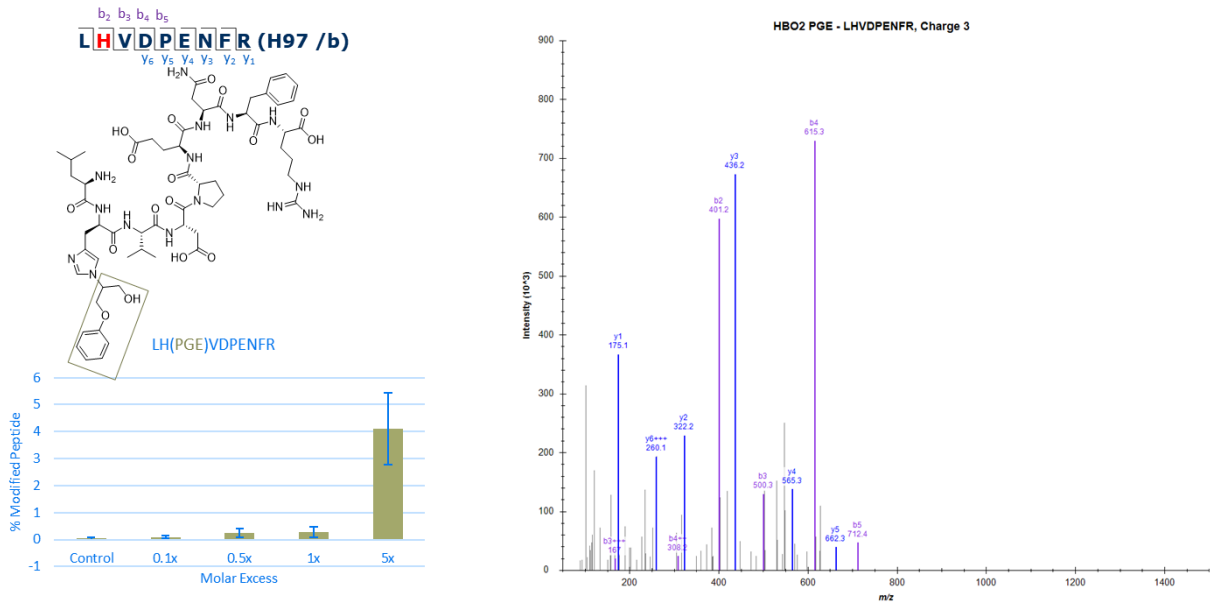

Figure S17. Tandem mass spectra, structure of the adducted peptide identified using PRM analysis, and relative estimation of the peptide containing the His_{97} site in subunit b of Hb modified by PGE.

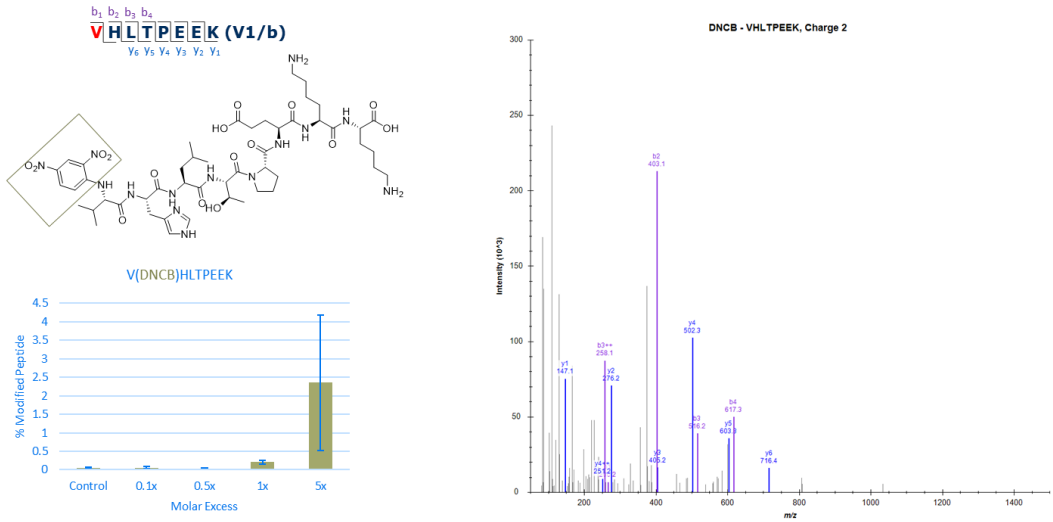

Figure S18. Tandem mass spectra, structure of the adducted peptide identified using PRM analysis, and relative estimation of the peptide containing the Val_1 site in subunit b of Hb modified by DNCB.

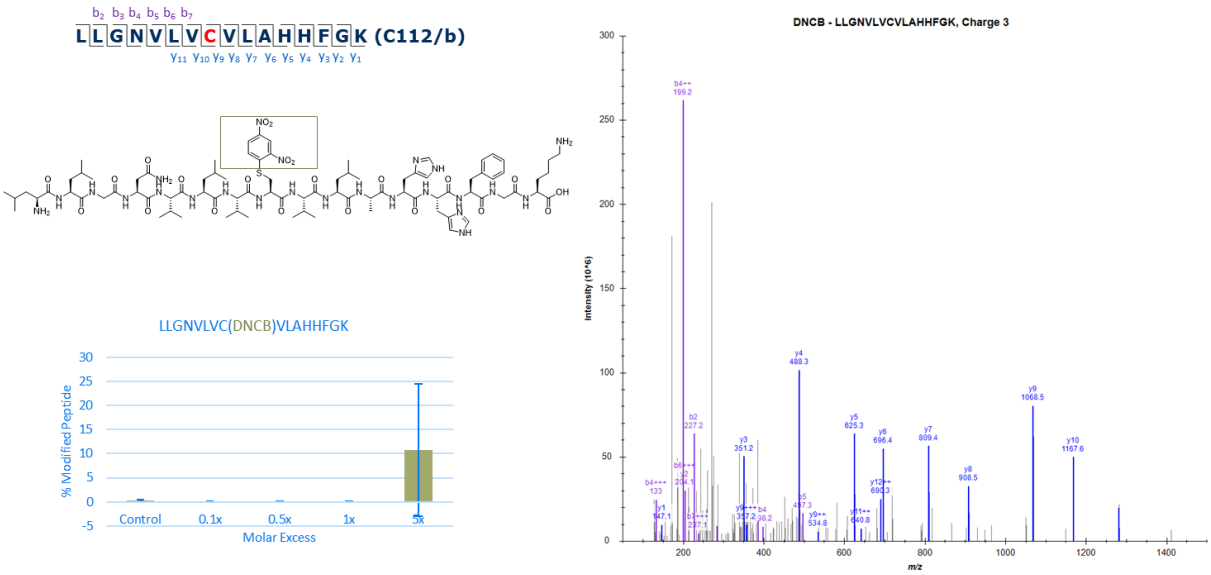

Figure S19. Tandem mass spectra, structure of the adducted peptide identified using PRM analysis, and relative estimation of the peptide containing the Cys_{112} site in subunit b of Hb modified by DNCB.

Protein Data Bank (PDB) structures of the adducted proteins

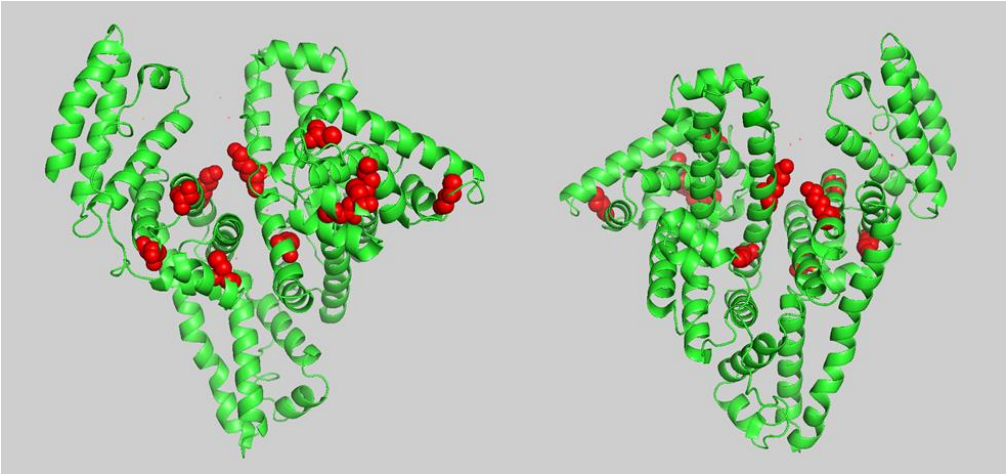

Figure S20. PDB structure of HSA front and back, with sites modified by DNCB highlighted in red.

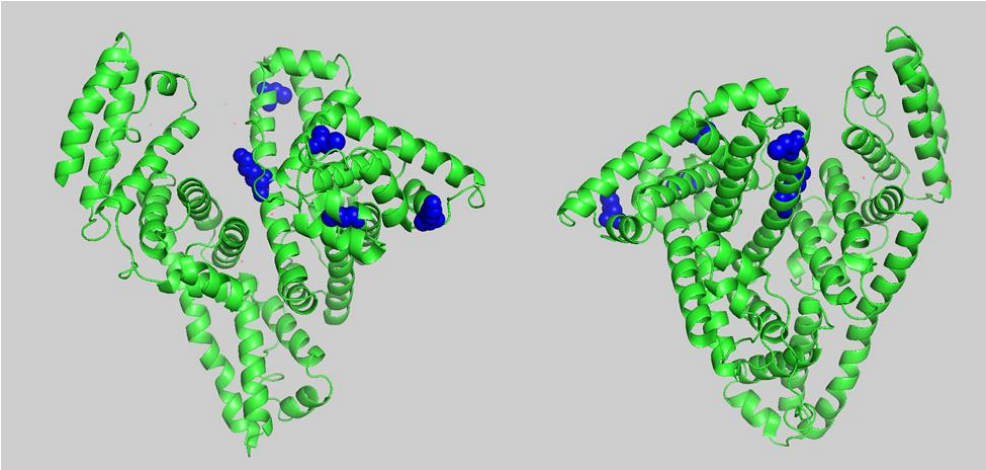

Figure S21. PDB structure of HSA front and back, with sites modified by PGE highlighted in blue.

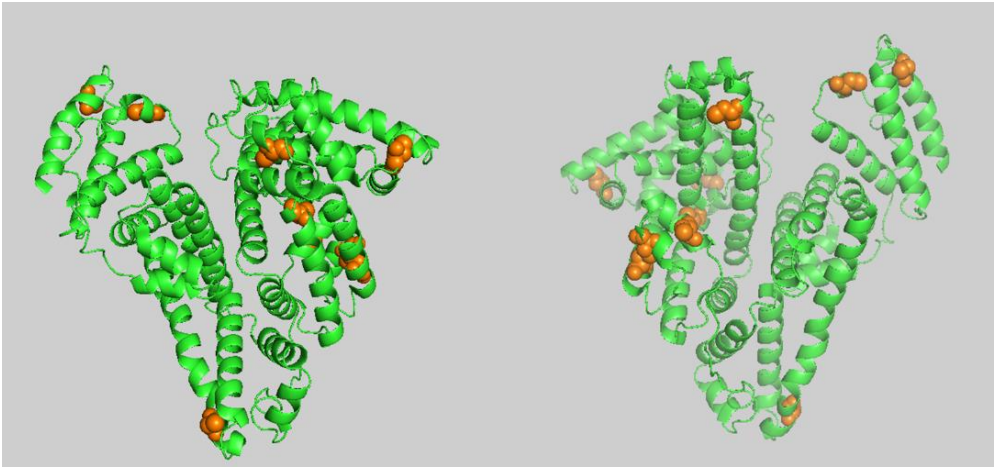

Figure S22. PDB structure of HSA front and back, with sites modified by MDBGN highlighted in orange.

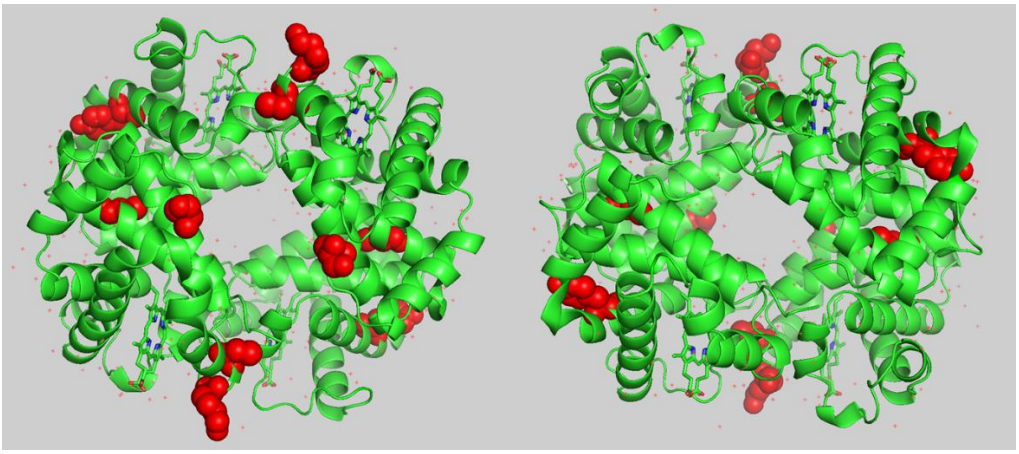

Figure S23. PDB structure of Hb front and back, with sites modified by DNCB highlighted in red.

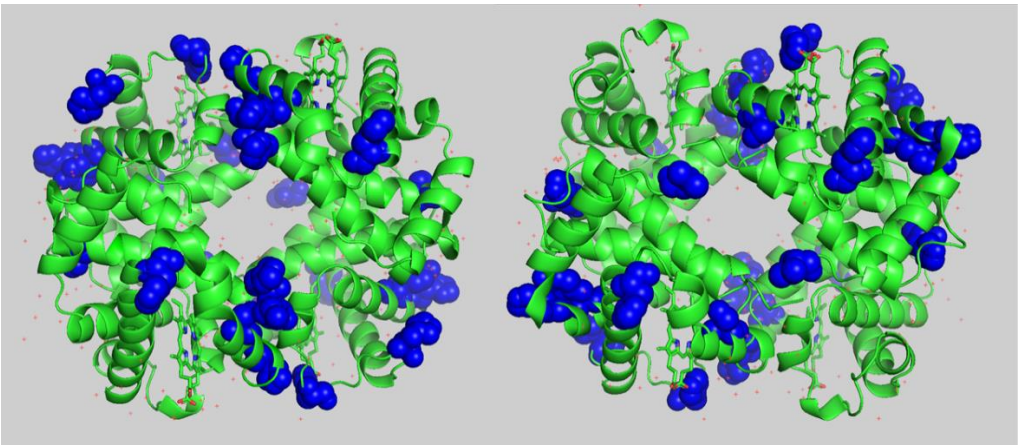

Figure S24. PDB structure of Hb front and back, with sites modified by PGE highlighted in blue.