Protein Adducts As Prospective Biomarkers of Nevirapine Toxicity

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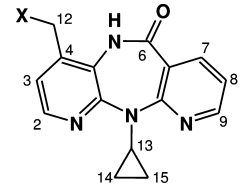
Nevirapine (NVP) is a non-nucleoside reverse transcriptase inhibitor used against human immunodeficiency virus type-1 (HIV-1), mostly to prevent mother-to-child HIV-1 transmission in developing countries. Despite its clinical efficacy, NVP administration is associated with a variety of toxic responses that include hepatotoxicity and skin rash. Although the reasons for the adverse effects of NVP administration are still unclear, increasing evidence supports the involvement of metabolic activation to reactive electrophiles. In particular, Phase II activation of the NVP metabolite 12-hydroxy-NVP is thought to mediate NVP binding to bionucleophiles, which may be at the onset of toxicity. In the present study, we investigated the nature and specific locations of the covalent adducts produced in human serum albumin and human hemoglobin by reaction *in vitro* with the synthetic model electrophile 12-mesyloxy-NVP, used as a surrogate for the Phase II metabolite 12-sulfoxy-NVP. Multiple sites of modification were identified by two different mass spectrometry-based methodologies, liquid chromatography−electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) and matrix-assisted laser desorption ionization tandem mass spectrometry (MALDI-TOF-TOF-MS). These two distinct methodologies, which in some instances afforded complementary information, allowed the identification of multiple adducts involving cysteine, lysine, tryptophan, histidine, serine, and the *N*-terminal valine of hemoglobin. Tryptophan, which is not a common site of covalent protein modification, was the NVP-modified amino acid residue detected in the two proteins and consistently identified by both LC-ESI-MS/MS and MALDI-TOF-TOF-MS. The propensity of tryptophan to react with the NVP-derived electrophile is further emphasized by the fact that human serum albumin possesses a single tryptophan residue, which suggests a remarkable selectivity that may be useful for biomonitoring purposes. Likewise, the NVP adduct with the terminal valine of hemoglobin, detected by LC-ESI-MS/MS after N-alkyl Edman degradation, appears as an easily assessed marker of NVP binding to proteins. Our results demonstrate the merits and complementarity of the two MS-based methodologies for the characterization of protein binding by NVP and suggest a series of plausible biomarkers of NVP toxicity that should be useful in the monitoring of toxicity effects in patients administered NVP.

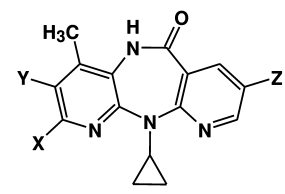
1. Introduction

Nevirapine (11-cyclopropyl-5,11-dihydro-4-methyl-6*H*-dipyrido[3,2-*b*:2′,3′-*e*][1,4]diazepin-6-one (NVP[1]), Scheme 1) was the first non-nucleoside reverse transcriptase inhibitor approved for use in combination therapy of HIV-1 infection (*1*). NVP-based regimens have since gained a significant role in HIV-

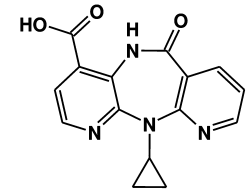
Scheme 1. Structures of NVP, NVP Metabolites, and Other NVP Derivatives Mentioned in the Text



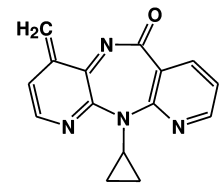
X=H; Nevirapine (NVP)
X=OH; 12-Hydroxy-NVP
X=OSO_3H; 12-Sulfoxy-NVP
X=OSO_2CH_3; 12-Mesyloxy-NVP



X=OH, Y=Z=H; 2-Hydroxy-NVP
X=Z=H, Y=OH; 3-Hydroxy-NVP
X=Y=H, Z=OH; 8-Hydroxy-NVP



4-Carboxy-NVP



NVP quinone methide

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[1] Abreviations: 1D-PAGE, one-dimensional polyacrylamide gel electrophoresis; CID, collision-induced dissociation; ESI, electrospray ionization; Hb, hemoglobin; HIV-1, human immunodeficiency virus type 1; HSA, human serum albumin; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MALDI-TOF-TOF-MS, matrix-assisted laser desorption ionization tandem mass spectrometry; MS/MS, tandem mass spectrometry; NVP, nevirapine; PBS, phosphate-buffered saline; Ph, phenyl.

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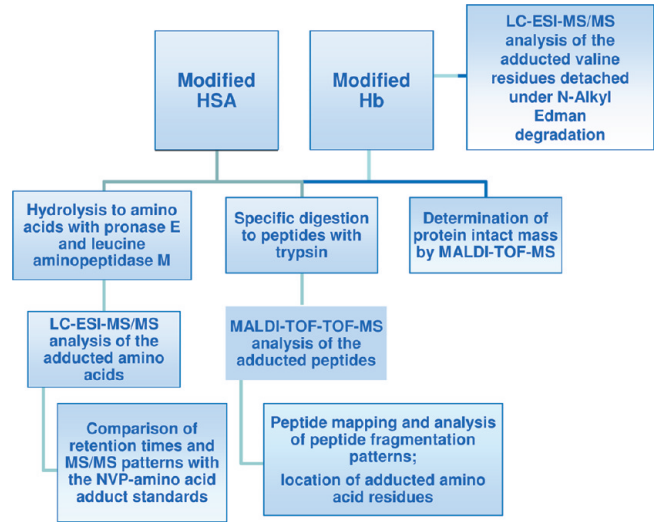
treatment guidelines (*2*) due to the high efficacy levels of the drug, favorable lipid profile, and suitability for use during pregnancy (*3*). NVP is currently one of the most prescribed antiretrovirals in the developing world, both to prevent mother-to-child HIV transmission and in combination therapy (*4*−*7*). Moreover, regimens that include non-nucleoside reverse transcriptase inhibitors allow the holding of protease inhibitors for later use, with the advantage of delaying exposure to some protease inhibitor-associated adverse effects, such as metabolic disease (*8*). Whereas a disadvantage of chronic NVP regimens is a twice-daily dosage schedule, which reduces patient compliance causing treatment failure and drug resistance, prospects of a new extended-release formulation aimed for a once-daily treatment regimen have recently been reported (*9*). Should this more convenient dosing schedule become available, worldwide NVP use will likely increase, especially considering the low cost of the drug (*4*).

Despite its clinical efficacy and although individual susceptibilities to adverse effects differ among patients, NVP produces a variety of toxic responses (*10*−*12*). The most severe is an occasionally fatal hepatotoxicity, while the most common side effect is skin rash, which may be life threatening and lead to the discontinuation of the drug. Although NVP is neither mutagenic nor clastogenic in standard *in vitro* assays, it induces hepato-neoplasias in rodents (*13*). Moreover, whereas no direct correlation between NVP administration and the development of cancer in humans has been reported, an association between the use of non-nucleoside reverse transcriptase inhibitors and an increased incidence of non-AIDS-defining cancers in HIV-1-positive patients has been suggested (*14*). These toxic events raise concerns about the chronic administration of the drug, particularly in perinatal and pediatric settings.

While the reasons for the adverse effects of NVP are still unclear, the involvement of metabolic activation to reactive electrophiles is supported by recent studies, such as the detection of covalent binding of [^{14}C]nevirapine to rat and human liver microsomal proteins *in vitro* and to rat liver tissue and plasma proteins *in vivo* (*15*). In all species investigated, including humans, NVP metabolism consistently involves cytochrome P450-mediated oxidation to 2-, 3-, and 8-hydroxy-NVP, 4-hydroxymethyl-NVP (12-hydroxy-NVP) and 4-carboxy-NVP (Scheme 1); these metabolites typically undergo subsequent glucuronidation and excretion (*16*−*20*). In humans, the formation of 2-hydroxy-NVP is attributed to the CYP3A subfamily, 3-hydroxy-NVP to CYP2B6, 8-hydroxy-NVP to CYP3A4, CYP2B6, and CYP2D6, and 12-hydroxy-NVP to CYP3A4 and possibly CYP2D6 and CYP2C9 (*18*). Phase II pathways other than glucuronidation (e.g., acetylation, sulfonation, or the generation of quinone/semiquinone species) (*21*) are not excluded; such pathways can generate electrophilic metabolites with the potential to react with bionucleophiles and initiate toxic events. Consistent with this interpretation is the evidence for NVP sulfation *in vivo* presented by Chen et al. (*22*), who detected 12-sulfoxy-NVP (Scheme 1) by LC-MS in urine and bile samples from female Brown Norway rats administered NVP. Moreover, the same study provided significant support for the involvement of 12-hydroxy-NVP in an idiosyncratic NVP-induced skin rash observed in this animal model and resembling the rash that occurs in humans treated with NVP (*23*−*25*). A quinone methide metabolite (Scheme 1) was proposed as the reactive intermediate in this process (*22*) and also as the precursor of an NVP−glutathione conjugate through NVP-C12 that was detected upon incubation of NVP with human liver microsomes supplemented with glutathione (*26*).

Scheme 2. Experimental Approach Used to Identify the Adducted Amino Acid Residues in HSA and Hb Following Incubation of the Proteins with 12-Mesyloxy-NVP

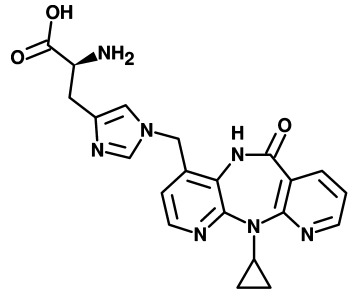


More recently, two NVP mercapturates, through NVP-C3 and NVP-C12, were identified in the bile and urine of NVP-treated rats, as well as in the urine of HIV-positive patients treated with a standard antiretroviral therapeutic regimen that included NVP (*27*). Taken together, these data provide evidence that the phase I metabolite, 12-hydroxy-NVP, has the ability to react with bionucleophiles yielding covalent adducts. Given that sulfotransferases are present in the skin (*28, 29*), one plausible mechanism accounting for the idiosyncratic skin rash associated with NVP administration may involve the sulfonation of 12-hydroxy-NVP, possibly followed by elimination of hydrogen sulfate to yield the quinone methide, subsequent binding to skin proteins, and the onset of an immune response (*30*).

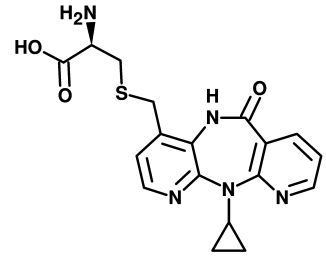
Protein binding by reactive electrophiles has received much attention in recent years (*31, 32*). Although the interpretation of covalent binding data is often confounded by the lack of obvious causal relationships between the occurrence of protein adduction and toxicity, the available data have consistently indicated that protein modification by a specific electrophile tends to be reproducible and highly selective, which may in turn lead to selective organ toxicity (*33*−*36*). Moreover, stable protein adducts are convenient biomarkers of exposure because, unlike DNA adducts, they are not prone to repair and accumulate over the lifespan of the protein (*37*). Abundant blood proteins, such as human serum albumin (HSA) and human hemoglobin (Hb) are used extensively in this context, and mass spectrometry (MS)-based methodologies have become essential tools for qualitative, quantitative, and mechanistic studies of the interaction between reactive metabolites and proteins (*38, 39*).

Using 12-mesyloxy-NVP (Scheme 1) as a synthetic surrogate for 12-sulfoxy-NVP, we have demonstrated the direct reaction *in vitro* with both DNA and amino acids and characterized a series of covalent NVP-DNA and NVP-amino acid adducts (*40, 41*). Moreover, by isolating glutathione and *N*-acetylcysteine conjugates through NVP C12 that were identical to those reported to be formed *in vivo* (*26, 27*), we established the validity of our synthetic model (*41*). The aim of the current study was to explore this model further by characterizing the chemical structures and binding sites of the NVP-amino acid adducts formed upon *in vitro* reaction of HSA and human Hb with 12-mesyloxy-NVP. The strategies adopted toward this goal are summarized in Scheme 2. The approach encompassed (i) enzymatic hydrolysis of the modified proteins to amino acids,

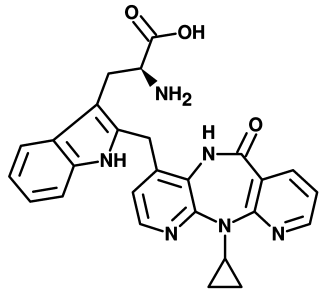
Scheme 3. Structures of the NVP-Amino Acid Adducts Detected in the NVP-Modified Proteins[a]



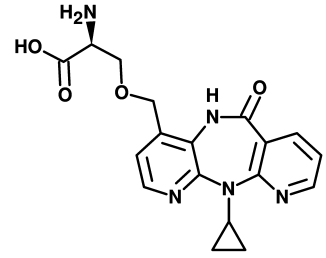
12-(histidin-*N1*'-yl)-NVP



12-(cystein-*S*-yl)-NVP



12-(tryptophan-2'-yl)-NVP



12-(serin-*O*-yl)-NVP

[a] 12-(Histidin-*N1*′-yl)-NVP, 12-(cystein-*S*-yl)-NVP, and 12-(tryptophan-2′-yl)-NVP were identified by LC-ESI-MS/MS in the enzymatic hydrolysate obtained by treatment of NVP-modified HSA with Pronase E and leucine aminopeptidase M. Following a similar treatment, 12-(cystein-*S*-yl)-NVP and 12-(tryptophan-2′-yl)-NVP were also identified in the hydrolysate from NVP-modified Hb. The Hb hydrolysate contained an additional adduct, presumed to be 12-(serin-*O*-yl)-NVP.

followed by liquid chromatography−electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), (ii) specific detachment of *N*-terminal valine adducts in Hb by an N-alkyl Edman procedure, followed by LC-ESI-MS/MS, (iii) determination of the intact protein mass by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and (iv) specific digestion of the modified proteins with trypsin, followed by MALDI-TOF-TOF-MS analysis of the obtained peptides. By using a combination of MS-based methodologies, we sought to identify the amino acids and specific sites in both proteins more prone to react with NVP-derived electrophiles and, ultimately, to establish which adducts and/or tryptic peptides may potentially be used as biomarkers to monitor NVP adduction in blood proteins from NVP-treated patients.

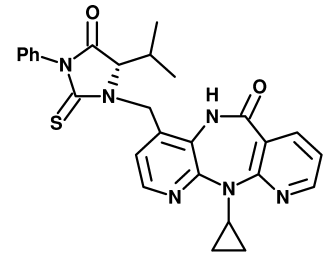
2. Materials and Methods

Caution: *NVP and its derivatives are potentially carcinogenic. They should be handled with protective clothing in a well-ventilated fume hood.*

2.1. Chemicals. NVP was purchased from Cipla (Mumbai, India). HSA, human Hb, and all other commercially available reagents and enzymes were acquired from Sigma-Aldrich Química, S.A. (Madrid, Spain), unless specified otherwise, and used as received. Whenever necessary, solvents were purified by standard methods (*42*).

12-Hydroxy-NVP and 12-mesyloxy-NVP were prepared as described in Antunes et al. (*40*). The NVP-amino acid adduct standards, 12-(histidin-*N1*′-yl)-NVP, 12-(cystein-*S*-yl)-NVP, 12-(tryptophan-2′-yl)-NVP (Scheme 3), and 12-[5-isopropyl-4-oxo-3-phenyl-2-thioxoimidazolidin-1-yl]-NVP (Scheme 4) were prepared by the reaction of 12-mesyloxy-NVP with histidine, *N*-acetyl cysteine, tryptophan, and ethyl valinate, respectively, as described in Antunes et al. (*41*).

Scheme 4. Structure of the Derivatized Valine Adduct, 12-[5-Isopropyl-4-oxo-3-phenyl-2-thioxoimidazolidin-1-yl]-NVP, Identified by LC-ESI-MS/MS in the Ethyl Acetate Extract Obtained after N-Alkyl Edman Degradation of NVP-Modified Hb



12-[5-isopropyl-4-oxo-3-phenyl-2-thioxoimidazolidin-1-yl]-NVP

2.2. Protein Modification Reactions. 2.2.1. Modification of HSA with Two Different Amounts of 12-Mesyloxy-NVP. A solution of 12-mesyloxy-NVP (5 mg, 14 μmol) in THF (500 μL) was added to a solution of HSA (10 mg) in PBS (10 mL). After an overnight incubation at 37 °C, the mixture was dialyzed for 24 h against deionized water (2 L). The resulting solution was dispensed into 1 mL aliquots that were evaporated separately under a nitrogen stream.

In a similar experiment, after an overnight incubation at 37 °C as described above, a second portion of 12-mesyloxy-NVP (5 mg, 14 μmol) in THF (500 μL) was added, and the incubation was continued at 37 °C for 72 h. Nonbonded materials were again removed by dialysis against deionized water (2 L) for 24 h, and the resulting solution was aliquoted and dried as indicated above.

2.2.2. Modification of Hb with 12-Mesyloxy-NVP. A solution of 12-mesyloxy-NVP (6 mg, 17 μmol) in acetonitrile (260 μL) was added to a solution of human Hb (10 mg) in 100 mM phosphate buffer at pH 7.4 (10 mL). The resulting solution was incubated overnight at 37 °C, and the nonbonded materials were subsequently removed by extraction with ethyl acetate (2 × 1 mL).

2.3. Adduct Isolation from the NVP-Modified Proteins. 2.3.1. Detachment of *N*-Terminal Valine Adducts from NVP-Modified Hb by N-Alkyl Edman Degradation. An aliquot (2.5 mL) of the modified Hb solution was lyophilized and then subjected to an N-alkyl Edman procedure (*43, 44*). Briefly, the sample was dissolved in DMF (1 mL), followed by the addition of 1 M NaOH (40 μL) and phenyl isothiocyanate (7 μL, 58.6 μmol). The sample was subsequently stirred for 2 h at 37 °C and then for 1.5 h at 45 °C. Upon cooling to room temperature, water (2 mL) was added, and the adducts were extracted with ethyl acetate (2 × 1 mL). The organic phase was dried under reduced pressure, and the contents were analyzed by LC-ESI-MS.

2.3.2. Hydrolysis of NVP-Modified HSA to Amino Acids. Pronase E (19 μL, 530 μg/mL) and leucine aminopeptidase M (8 μL, 130 μg/mL) were added to aliquots containing approximately 1 mg of NVP-modified HSA in PBS (350 μL). The solution was incubated at 37 °C overnight. The enzymatic hydrolysate thus obtained was analyzed directly by LC-ESI-MS with no further treatment.

2.3.3. Hydrolysis of NVP-Modified Hb to Amino Acids. One aliquot (4 mL) of the modified Hb was hydrolyzed enzymatically to amino acids by treatment with Pronase E (76 μL, 530 μg/mL) and leucine aminopeptidase M (32 μL, 130 μg/mL). The solution was incubated overnight at 37 °C. One aliquot (500 μL) of the enzymatic hydrolysate was analyzed directly by LC-ESI-MS with no further treatment. The remaining portion of the enzymatic hydrolysate (3.5 mL) was purified using a C-18 Sep-Pak cartridge (Waters Associates, Milford, MA). The cartridge was conditioned with methanol (4 mL), followed by water (4 mL). The sample was then loaded, and the cartridge was rinsed with water (2 mL) and methanol (2 mL). The methanolic eluate was concentrated to 300 μL with a nitrogen stream and analyzed by LC-ESI-MS.

2.3.4. In Gel Trypsin Digestion of NVP-Modified HSA and Hb. The NVP-modified proteins were separated by 1D-polyacrylamide gel electrophoresis (1D-PAGE). Specifically, 5 μg of modified HSA and Hb in 1D-PAGE sample buffer [62.5 mM Tris-HCl, pH 6.8, containing 20% (v/v) glycerol, 2% (w/v) β-mercaptoethanol, and a trace of bromophenol blue] were separated on 12% (w/v) polyacrylamide gels and stained with Colloidal Coomassie Blue (*45*). In gel trypsin digestion of the modified proteins was performed according to a protocol described in Gomes et al. (*46*). Briefly, the HSA and Hb gel bands were excised for in gel digestion and destained with 50% (v/v) acetonitrile. After dehydration of the excised gel pieces by treatment with 100% acetonitrile and vacuum-drying, the proteins were reduced with 10 mM dithiothreitol, subsequently alkylated with 55 mM iodoacetamide, and then digested overnight at 37 °C with sequencing-grade modified trypsin (6.7 ng/μL in 50 mM ammonium bicarbonate). The supernatant was then recovered and analyzed by MALDI-TOF-TOF-MS after desalting and concentrating the tryptic peptides (*vide infra*).

2.4. Instrumentation and Analytical Procedures. 2.4.1. Liquid Chromatography−Electrospray Ionization-Tandem Mass Spectrometry (LC-ESI-MS/MS). LC-ESI-MS/MS analyses were performed with a ProStar 410 autosampler, two 210-LC chromatography pumps, a ProStar 335 diode array detector, and a 500-MS ion trap mass spectrometer, with an ESI ion source (Varian, Inc., Palo Alto, CA). Data acquisition and processing were performed using Varian MS Control 6.9 software. The samples were injected onto the column via a Rheodyne injector with a 20 μL loop. Separations were conducted at 30 °C, using a Luna C18 (2) column (150 mm × 2 mm, 3 μm; Phenomenex, Torrance, CA). The mobile phase was delivered at a flow rate of 200 μL/min, using a 5-min isocratic elution with 5% acetonitrile in 0.1% aqueous formic acid, followed by a 30-min linear gradient from 5−70% acetonitrile, a 2-min linear gradient to 100% acetonitrile, and an 8-min isocratic elution with acetonitrile. The mass spectrometer was operated in the positive ESI mode; the optimized operating parameters were ion spray voltage, +5.2 kV; capillary voltage, 20 V; and RF loading, 90%. Nitrogen was used as the nebulizing and drying gas, at pressures of 50 and 30 psi, respectively; the drying gas temperature was 350 °C. MS/MS spectra were obtained with an isolation window of 1.5 Da, excitation energy values between 0.9 and 1.2 V, and an excitation time of 10 ms.

2.4.2. Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS). Desalting and concentration of the NVP-modified proteins and tryptic peptides were performed with homemade chromatographic microcolumns using GELoader tips packed with POROS R1 (for NVP-modified proteins) and POROS R2 (for tryptic peptides) (Applied Biosystems, Foster City, CA). The proteins and peptides were eluted directly from the microcolumns onto the MALDI plate using 50% (v/v) aqueous acetonitrile containing 5% (v/v) formic acid and either 10 mg/mL sinapinic acid (for proteins) or 5 mg/mL α-cyano-4-hydroxy-*trans*-cinnamic acid (for peptides) (*46*). The sample−matrix mixtures were then allowed to air dry.

MS experiments were performed using a MALDI-TOF/TOF 4800 plus mass spectrometer (Applied Biosystems) in the positive linear mode for intact protein mass determinations and the positive reflectron mode for peptide mass measurements. For peptide mass measurements, the mass spectrometer was externally calibrated using des-Arg-Bradykinin (904.468 Da), angiotensin 1 (1296.685 Da), Glu-Fibrinopeptide B (1570.677 Da), adrenocorticotropic hormone (1−17) (2093.087 Da), and adrenocorticotropic hormone (18−39) (2465.199 Da) (4700 Calibration Mix, Applied Biosystems). For intact protein mass measurements, the mass spectrometer was calibrated using bovine serum albumin (66431 and 33216 Da for the 1^+ and 2^+ charge ions, respectively), yeast enolase (46672 and 23336 Da for the 1^+ and 2^+ charge ions, respectively), and trypsinogen (23982 Da for the 1^+ charge ion) (Promix 3 calibration mixture, LaserBio Laboratories, Sophia-Antipolis, France). MS spectra were collected in a result-independent acquisition mode, typically using 1000 laser shots per spectrum and a fixed laser intensity of 3500 V.

The peptides of interest (i.e., having a mass consistent with modification by NVP) were selected for MS/MS experiments. The MS/MS analyses were performed using collision induced dissociation (CID), with 1 kV collision energy and an air pressure of 10^6 Torr. Two thousand laser shots were collected for each MS/MS spectrum using a fixed laser intensity of 4500 V. Raw data were generated by the 4000 Series Explorer Software v3.0 RC1 (Applied Biosystems); contaminant *m/z* tryptic peptide peaks resulting from the autodigestion of trypsin (842.51, 1045.56, 2211.11, and 2225.12 Da) were excluded when generating the peptide mass list used for comparison with the theoretical NVP-modified HSA and Hb tryptic peptide masses. The identification of NVP-modified peptide and amino acid residues was further validated using Peaks Studio 4.5 software (Bioinformatic Solutions Inc., Waterloo, ON, Canada) for auto *de novo* sequencing of the MS/MS spectra, combined with manual inspection of the assigned sequence.

3. Results

We have previously demonstrated the validity of the synthetic model ester, 12-mesyloxy-NVP, as a surrogate for the NVP metabolite, 12-sulfoxy-NVP and/or its putative derivative, NVP quinone methide, and prepared a series of well-characterized NVP-DNA and NVP-amino acid adduct standards (*40, 41*). In the present study, we have further explored the same model electrophile to investigate its reaction *in vitro* with HSA and Hb and determine both the chemical identity of the NVP-modified amino acid residues and their specific locations in each protein by using a combination of MS-based methodologies.

HSA (1 mg/mL in PBS) was incubated with a THF solution of 12-mesyloxy-NVP (10 μg/μL) at a ratio of 1:0.5 (HSA/electrophile, w/w). To investigate if a larger amount of 12-mesyloxy-NVP would lead to more extensive protein modification and/or greater diversity of amino acid targets, a second experiment was performed using a total 1:1 ratio (w/w), with 12-mesyloxy-NVP being added in two sequential equimolar

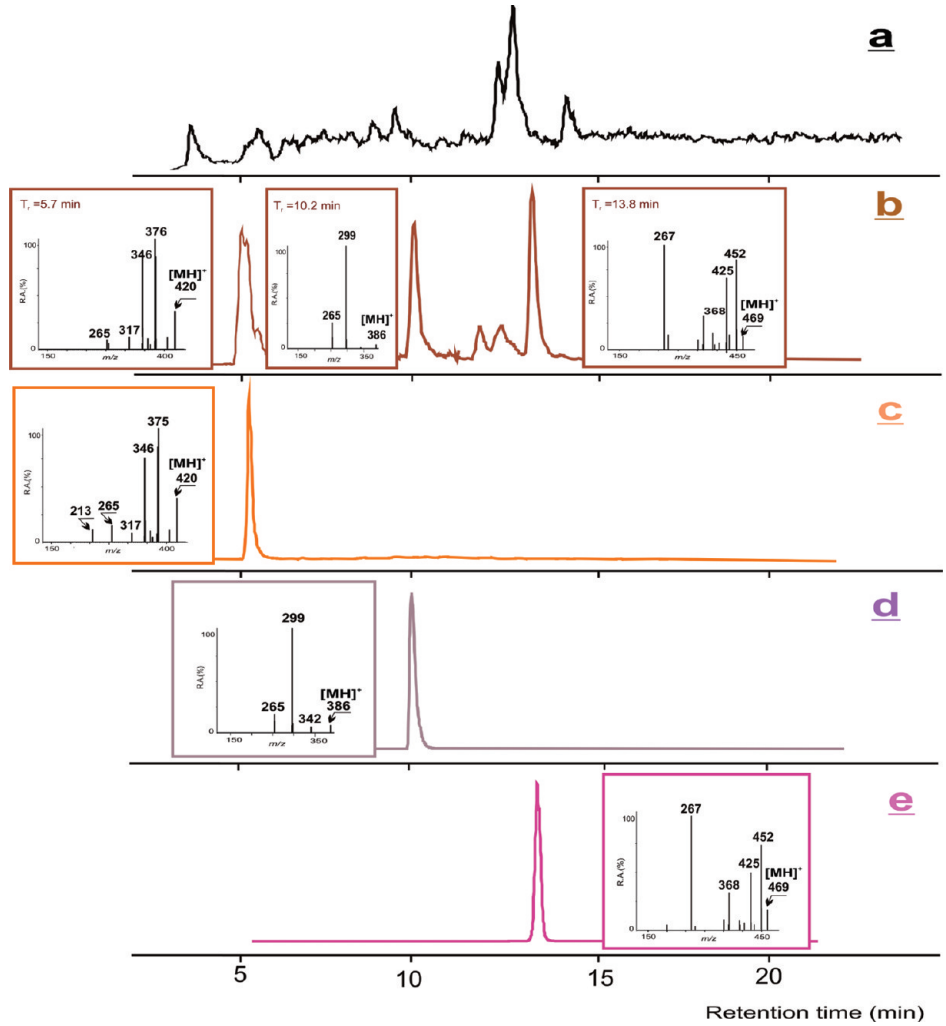


Figure 1. Analysis of the enzymatic hydrolysate of NVP-modified HSA, obtained upon reaction with 12-mesyloxy-NVP and subsequent hydrolysis with Pronase E and leucine aminopeptidase M. (a) HPLC-ESI/MS total ion chromatogram; (b) HPLC-ESI-MS/MS ion chromatograms and product ion mass spectra of the *m*/*z* 420, 386, and 469 ions; (c−e) HPLC-ESI-MS/MS ion chromatograms and product ion mass spectra of the NVP adduct standards with (c) histidine, (d) cysteine, and (e), tryptophan.

fractions to minimize competitive hydrolysis. The modification of Hb required an adaptation of the experimental conditions to prevent protein denaturation. We were able to minimize this effect by adding an acetonitrile solution of 12-mesyloxy-NVP (23 μg/μL) to an Hb solution in 100 mM phosphate at pH 7.4 (1 mg/mL) at a 5:3 ratio (Hb/electrophile, w/w). Upon removal of the nonbonded materials, either by dialysis or ethyl acetate extraction, the covalently bound modifications through the NVP C12 were identified in each protein by two different methodologies: enzymatic hydrolysis of the modified protein to amino acids, followed by LC-ESI-MS/MS analysis, and trypsin digestion of the modified protein to peptides, followed by MALDI-TOF-TOF-MS analysis. Moreover, an N-alkyl Edman degradation of the NVP-modified Hb was also performed, to search for adducted *N*-terminal valine residues by LC-ESI-MS/MS (Scheme 2).

3.1. LC-ESI-MS/MS Identification of the NVP-Modified Amino Acids. The NVP-modified proteins were hydrolyzed to free amino acids by an adaptation of reported methodologies, using a combination of Pronase E and leucine aminopeptidase M, to ensure the endo- and exopeptidase activities required for complete hydrolysis (*47, 48*). The adducts released from the proteins were then characterized by comparison of LC-ESI-MS/MS patterns with those of the corresponding NVP-amino acid adduct standards previously prepared from reaction *in vitro* of 12-mesyloxy-NVP with individual amino acids containing nucleophilic side chains (e.g., histidine, cysteine, and tryptophan) and fully characterized by NMR and MS (*41*).

The enzymatic hydrolysates obtained from HSA modification with two different amounts of 12-mesyloxy-NVP led to very similar results regarding the identity of the NVP-modified amino acid residues. Thus, by comparison with the available synthetic standards (Scheme 3), three distinct adducts, 12-(histidin-*N*1′-yl)-NVP (retention time = 5.7 min), 12-(cystein-*S*-yl)-NVP (retention time = 10.2 min), and 12-(tryptophan-2′-yl)-NVP (retention time = 13.8 min), having protonated molecules at *m*/*z* 420, 386, and 469, respectively, were identified in both mixtures by LC-ESI-MS/MS (Figure 1).

LC-ESI-MS/MS analysis of the NVP-modified Hb hydrolysate (Figure 2) allowed the identification of a tryptophan and a cysteine adduct, both structurally identical to those found in the NVP-modified HSA. One additional putative adduct (retention time = 10.6 min), eluting very close to the NVP-cysteine standard, was detected in the Hb hydrolysate. The extracted ion chromatogram (*m*/*z* 370) suggests a serine adduct, presumably 12-(serin-*O*-yl)-NVP (Scheme 3). This interpretation is also consistent with the similarity of elution characteristics with those of the cysteine adduct, as expected from two structural analogues, differing only by one heteroatom (O vs S) on the amino acid side chain. Nonetheless, due to the lack of a synthetic

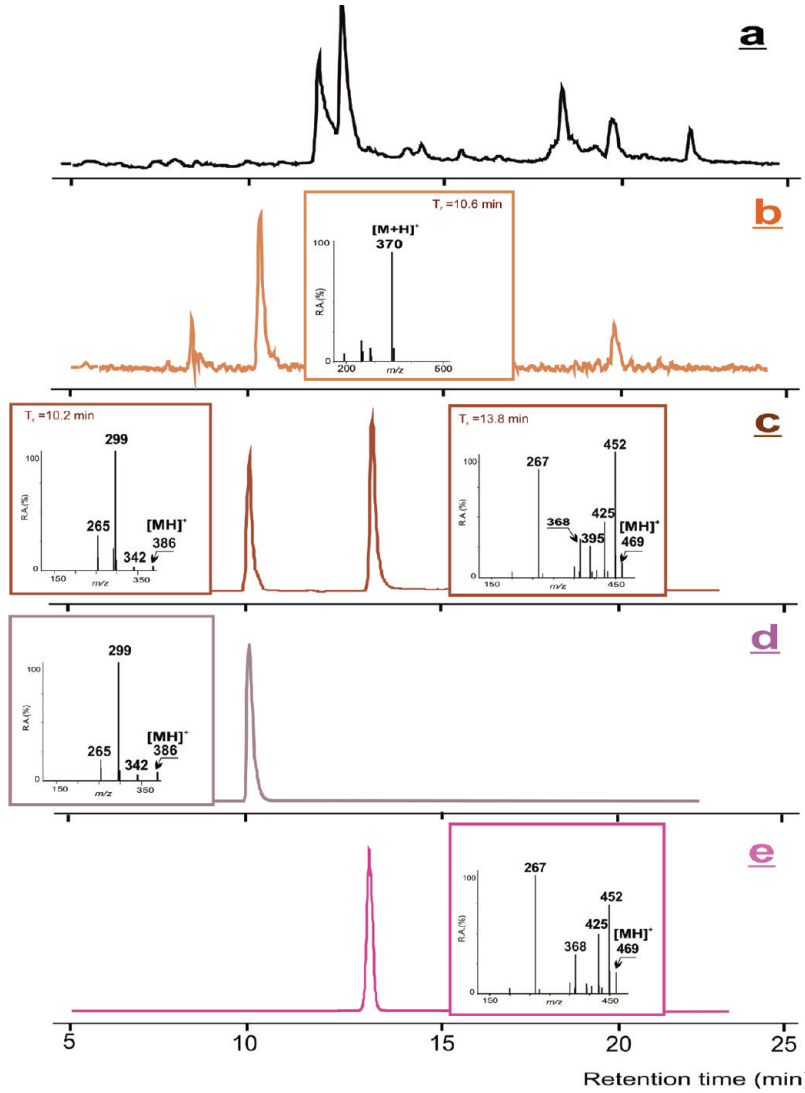


Figure 2. Analysis of the enzymatic hydrolysate of NVP-modified Hb, obtained upon reaction with 12-mesyloxy-NVP and subsequent hydrolysis with Pronase E and leucine aminopeptidase M. (a) HPLC-ESI/MS total ion chromatogram; (b) extracted HPLC-ESI/MS ion chromatogram and mass spectrum of the *m/z* 370 ion, presumed to be a serine adduct; (c) HPLC-ESI-MS/MS ion chromatograms and product ion mass spectra of the *m/z* 386 and 469 ions; (d−e) HPLC-ESI-MS/MS ion chromatograms and product ion mass spectra of the NVP adduct standards with (d) cysteine and (e) tryptophan.

NVP-serine standard, the structural assignment is tentative at this stage. In addition, it should be noted that we obtained the same results when analyzing either the crude Hb hydrolysate or a concentrated methanolic eluate obtained after Sep-Pak cleanup. Thus, concentrating the sample did not lead to the detection of any other adducted amino acids in Hb.

3.2. N-Alkyl Edman Degradation of NVP-Modified Hb and LC-ESI-MS/MS Analysis of the Detached NVP-Valine Residues. The α-nitrogen atoms of the *N*-terminal valine residues in human Hb A and B are primary nucleophilic sites, typically capable of reacting with several classes of electrophiles. The binding products thus formed can be analyzed either as valine adducts, upon total hydrolysis of the protein, or as hydantoins (e.g., phenylthiohydantoins), upon an N-alkyl Edman procedure that leads to the specific detachment of the adducted valine residues from the protein (*38, 43*). This approach was originally developed for GC-MS analysis, which explains the common use of a fluorinated phenyl isothiocyanate, but its application to polar, thermolabile, and high-molecular-weight adducts was limited. However, recent modifications, using LC-ESI-MS/MS for measuring valine adducts after derivatization with phenyl isothiocyanate have successfully broadened the scope of the method (*44*).

Because of the anticipated low volatility of NVP-derived phenylthiohydantoins, LC-ESI-MS/MS is a suitable methodology for the prospective analysis of these species, following *ex vivo* derivatization of NVP-modified Hb. Thus, to determine whether or not the *N*-terminal valines reacted with 12-mesyloxy-NVP, we subjected the NVP-modified Hb to N-alkyl Edman degradation using phenyl isothiocyanate as the derivatization agent (*43*). LC-ESI-MS/MS analysis of the ethyl acetate extract (Figure 3) confirmed the presence of the NVP-valine-derived thiohydantoin (Scheme 4), with a retention time of 28.2 min and *m/z* 499 for the protonated molecule, which was identified by comparison with a fully characterized synthetic standard (*41*).

3.3. Determination of the Intact Mass of the NVP-Modified HSA and Hb by MALDI-TOF-MS. In order to estimate the extent of modification obtained upon the reaction of 12-mesyloxy-NVP with HSA and Hb, we used MALDI-TOF-MS to compare the intact mass of the control and the NVP-modified proteins. The mass spectrum of unmodified HSA had two major *m/z* peaks at 66509 and 33327 Da, corresponding to the single

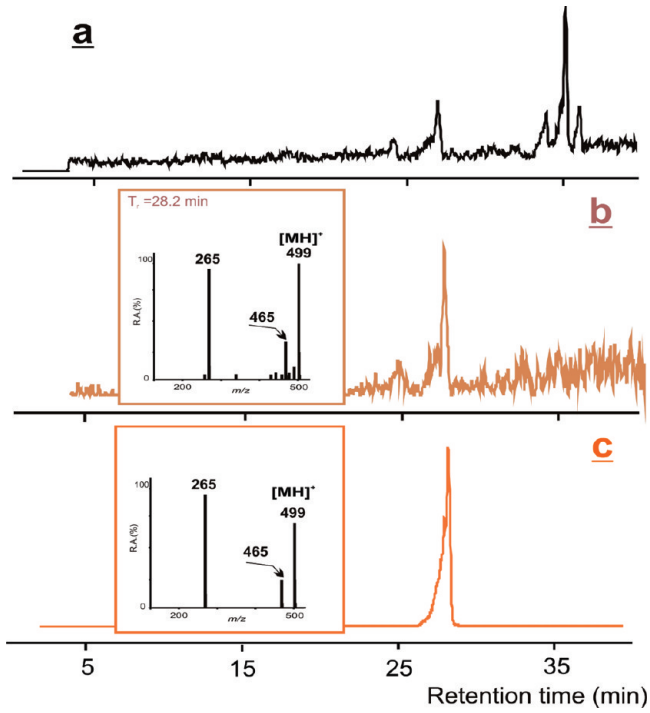


Figure 3. Analysis of the ethyl acetate extract obtained after N-alkyl Edman degradation of the NVP-modified Hb. (a) HPLC-ESI/MS total ion chromatogram; (b) HPLC-ESI-MS/MS ion chromatogram and product ion mass spectrum of the *m/z* 499 ion; (c) HPLC-ESI-MS/MS ion chromatogram and product ion mass spectrum of the derivatized valine adduct standard.

(1^+) and double (2^+) charged molecular ions, respectively. A 1092 Da mass increment, calculated as the difference between the molecular masses of the single charged molecular ions, was detected upon HSA modification with two sequential additions of 12-mesyloxy-NVP; this implies that an average of four amino acid residues were modified since the mass increment imposed by one NVP modification ($C_{15}H_{12}N_4O$) is 264 Da. Incubation with a single addition of 12-mesyloxy-NVP resulted in a mass increment of 711 Da (calculated as indicated above), corresponding to an average of three amino acid modifications (Figure 4a).

A more complex result was obtained for Hb. The mass spectrum of unmodified Hb had two major *m/z* peaks at 15060 and 15811 Da, corresponding to the molecular masses of the Hb A and B polypeptide chains, respectively. By contrast, several new peaks appeared in the MS spectrum of NVP-modified Hb, corresponding to various modified forms of the Hb A and B chains. For instance, the *m/z* peaks at 15331, 15594, and 15856 Da correspond to the incorporation of one, two, and three NVP residues, respectively, on the A chain of Hb (Figure 4b). Likewise, the *m/z* peak series at 16072, 16345, and 16608 Da corresponds to the addition of one, two, and three NVP residues, respectively, to the B chain of Hb (Figure 4b).

3.4. MALDI-TOF-TOF-MS Analysis of the Adducted Peptides. MALDI-TOF-TOF-MS of tryptic digests was used to identify which amino acid residues were bound to NVP upon incubation of HSA and Hb with 12-mesyloxy-NVP. The adopted strategy consisted of (i) comparison of the MS spectra of unmodified and NVP-modified HSA and Hb digests. The presence of new *m/z* peaks in the latter was presumed to correspond to potential NVP-amino acid adducts. (ii) The *m/z* values observed exclusively in the MS spectra of the tryptic digests of the modified proteins were compared with the theoretical tryptic peptide mass list for each protein, taking into account the mass increase characteristic of NVP modification. This would impart a 264.28 Da increment to amino acid residues bearing nucleophilic side chains (i.e., cysteine, serine, histidine, tryptophan, lysine, or threonine); (iii) this information was used to construct an inclusion list of possible NVP-modified peptides to be fragmented by an additional MS/MS experiment using the MALDI-TOF-TOF instrument. The obtained MS/MS spectra were then carefully analyzed with the assistance of Peaks software. The amino acid sequence information thus obtained

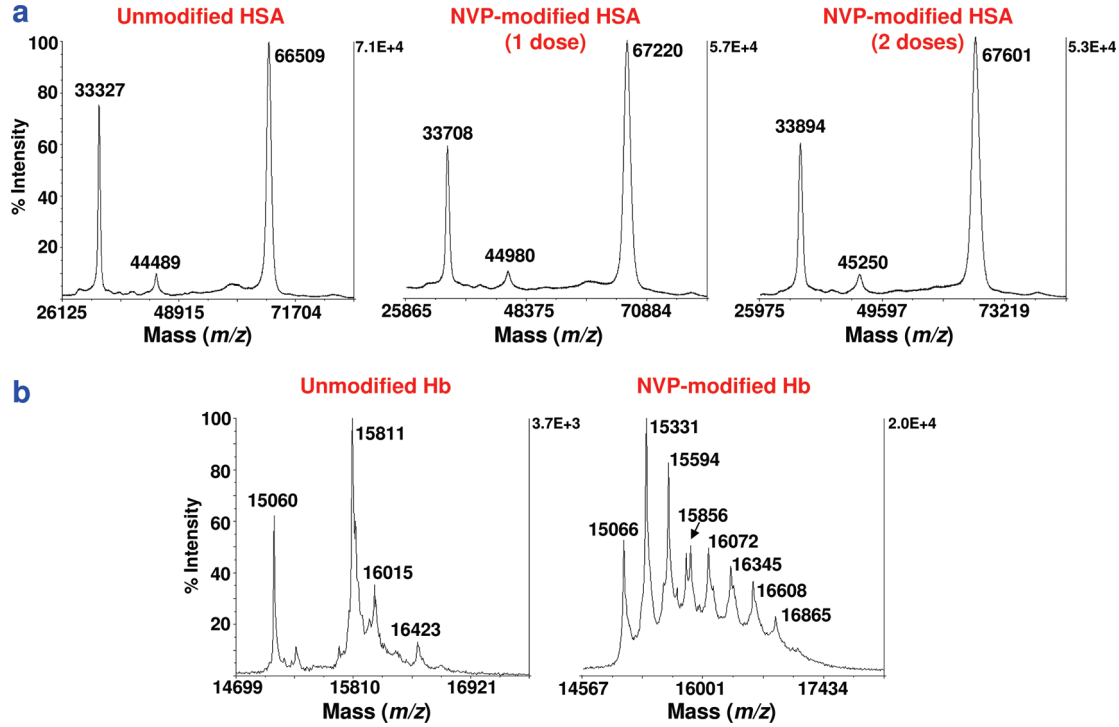


Figure 4. Intact mass analysis of (a) unmodified and NVP-modified HSA and (b) unmodified and NVP-modifed Hb. A clear mass increase is observed upon modification. This increment provides an estimation of the extent of modification.

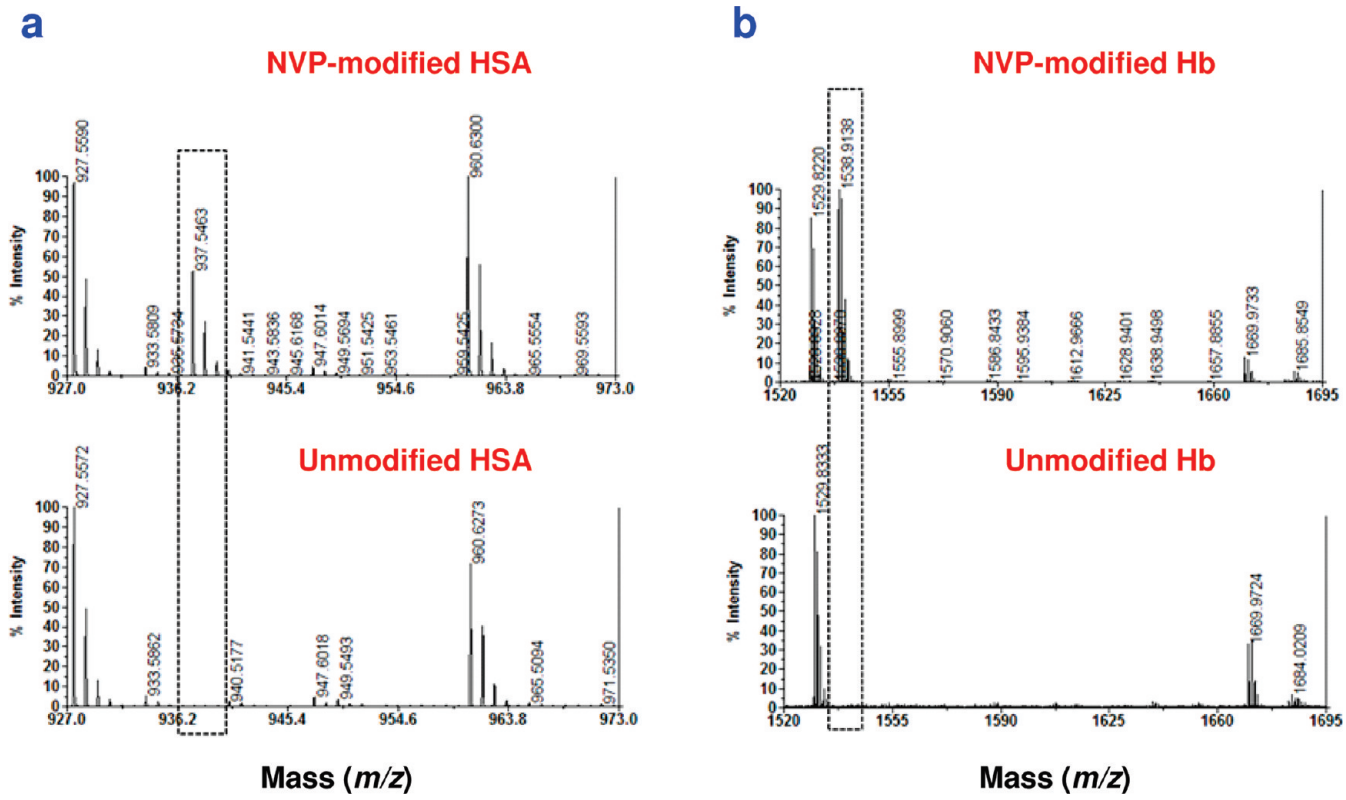


Figure 5. Detection and location of NVP-modified peptides. The panels show representative sections of the MALDI-TOF spectra of tryptic digests of (a) unmodified and NVP-modified HSA, and (b) unmodified and NVP-modified Hb. New *m/z* peaks, absent from the controls, are clearly detected in the mass spectra of the NVP-modified proteins (highlighted in the figure). These new *m/z* values correspond to the mass of a peptide from HSA or Hb plus the mass increment characteristic of NVP modification.

Table 1. Assignment of the NVP-Modified Amino Acid Residues from HSA and Hb That Were Identified by MALDI-TOF-TOF-MS Analyses of the Tryptic Peptides

protein	observed mass[a] (Da)	theoretical mass of the unmodified peptide (Da)	mass increase (Da)	peptide sequence[b]	NVP-modified residue
HSA	937.55	673.38	264.17	A**W**AVAR (237−242)	W238
	1731.99	1467.84	264.15	R**H**PDYSVVLLLR (361−372)	H362
	1782.98	1518.78	264.20	LDELRDEG**K**ASSAK (206−219)	K214
	2047.12	1783.08	264.04	ERQI**KK**QTALVELVK (544−558)	K548 or K 549
Hb	1538.91	1274.73	264.18	LLVVYP**W**TQR (32−41; Hb B)	W38
	1793.93	1529.74	264.19	VGA**H**AGEYGAEALER (18−32; Hb A)	H21
	2793.43	2529.22	264.21	GTFATL**S**ELHCDKLHVDPENFR (84−105; Hb B)	S90

[a] Compared to the theoretical mass of the corresponding unmodified peptide, each peptide had an NVP-specific mass increment (ca. 264.28 Da). [b] The modified amino acid residues are highlighted in each peptide sequence.

allowed the unequivocal identification of NVP-modified peptides and the assignment of the specific NVP-modified amino acid.

A comparative analysis of the tryptic peptide mass spectra from NVP-modified and unmodified proteins revealed noticeable differences, with several peptides appearing exclusively in the modified HSA and Hb (Figure 5 and Table 1). To identify NVP-modified peptides and assign the target amino acid residues, a theoretical trypsin digestion of both proteins under investigation was performed considering up to three missed cleavages (*49*); the mass increment imposed by one NVP modification (264.28 Da) was then added to the theoretically generated peptide masses. Using this approach, several peptides, appearing only in the peptide mass spectra of the modified proteins and showing the NVP-specific mass increment, were observed. For instance, the species at *m/z* of 937.55 in the spectrum of NVP-modified HSA (Figure 5a) appeared to correspond to the 237−242 peptide (AWAVAR), with the observed *m/z* peak (673.38 + 264.17 Da) bearing a mass increment consistent with NVP modification. Given that tryptophan (W) is the only amino acid in this peptide sequence reasonably anticipated to react with electrophiles, this observation strongly suggests that the W238 residue in HSA is a specific target for NVP modification.

To obtain unequivocal confirmation that the new peptides observed in the MS spectra of the modified proteins were in fact modified by NVP, tandem MS experiments were performed to provide sequence information. When using the CID fragmentation technique, bond cleavage occurs mainly through the lowest energy pathways, i.e., the peptide bond. This leads to b-ions, in which the charge is retained by the amino-terminal fragment, and y-ions, in which the charge is retained by the carboxy-terminal fragment. Thus, if an amino acid residue is modified by NVP, the specific complementary y and b ions encompassing the modification site will have the mass value of that particular amino acid increased by 264.28 Da. Considering the peptide with *m/z* 937.55 mentioned above [A(**NVP**)WAVAR], we observed (Figure 6) a b_1 ion corresponding to an alanine (A) residue (*m/z* 72.04); however, the b_2 ion (*m/z* 522.23) did not correspond to the addition of a tryptophan residue (186.08 Da) but rather to the addition of NVP-W (450.19 Da in total). The subsequent b_3−b_6 ions also showed the NVP-

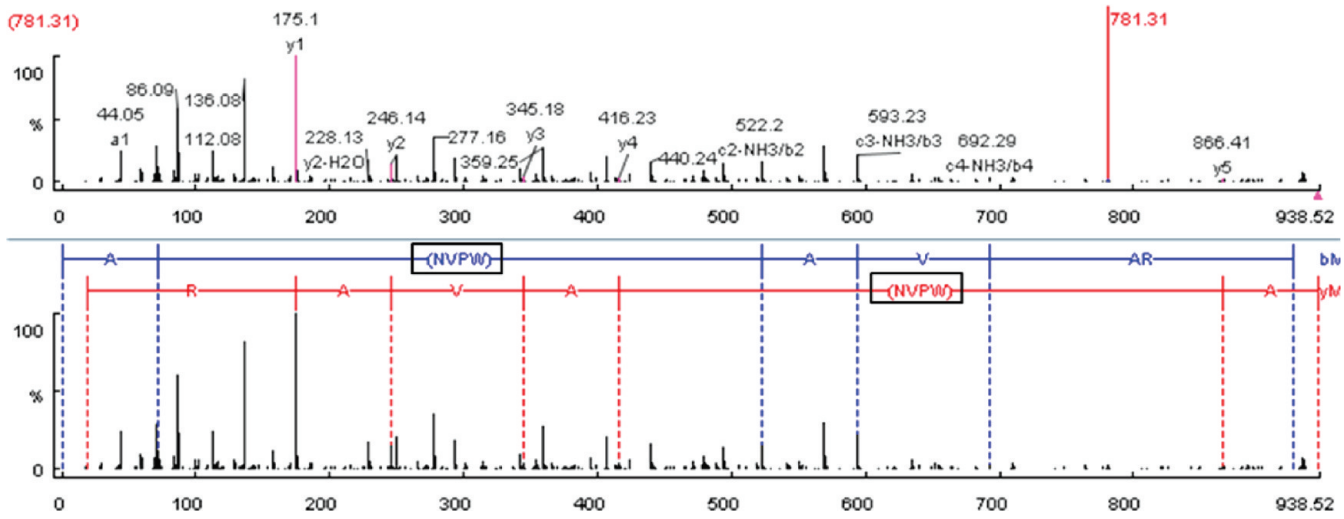


Figure 6. MS/MS spectrum of a representative NVP-modified peptide from HSA. The panels show the y and b fragment ions of the peptide with *m/z* 937.55. The detected fragment ions arise from the amino acid sequence AWAVAR, with an NVP-modification on W.

Table 2. MALDI-TOF-TOF-MS Analysis of the Tryptic Peptide with *m/z* 937.55 Obtained from NVP-Modified HSA

	peptide sequence					
	A	W	A	V	A	R
ion[a]	1	2	3	4	5	6
b	72.04	**522.23**	593.27	692.23	763.37[b]	919.47
y	175.12	246.16	345.22	416.26	**866.44**	937.48

[a] All possible b and y ion masses are shown. The fragment ions encompassing the modification site are highlighted in bold. [b] Not observed.

specific mass increment (Table 2). The same feature was observed for the y ions. Thus, the y_1 ion (*m/z* 175.12) corresponded to an arginine (R) residue, and the y_2-y_4 ions also displayed the *m/z* values expected from a nonmodified peptide; however, the y_5 ion (*m/z* 866.44) had the NVP-characteristic mass increment, as did the y_6 ion (*m/z* 937.48). These results provide unambiguous confirmation that W238 was modified by NVP. The same approach was followed for all the potential NVP-modified HSA- and Hb-derived peptides; the results are summarized in Table 1, and the corresponding spectra are in Supporting Information. Only modified amino acid residues with confirmed sequence information were considered.

Although MALDI-TOF-MS analyses of the intact proteins indicated a dose-dependent extent of HSA modification (*vide supra*), the amino acid targets identified were the same, regardless of the amount of 12-mesyloxy-NVP (one or two additions) used to modify the protein. Thus, four modified amino acid residues were detected in HSA: one tryptophan (W238), one histidine (H362), and two lysines (K214 and K548 or K549). While the modifications of W238, H362, and K214 were identified unequivocally, the adducted lysine in the E544-K558 pentadecapeptide (*m/z* 2047.12) could not be assigned unambiguously. In this instance, the sequential information was insufficient because the y and b ions stemming from peptide bond cleavage between the adjacent lysines (K548 and K549) were not observed in the MS/MS spectrum. A similar MALDI-TOF-TOF-MS analysis of the tryptic digest from NVP-modified Hb indicated one modified tryptophan residue (W38) and one modified serine residue (S90), both in Hb B, in addition to one histidine residue (H21) in Hb A (Table 1).

In summary, the MALDI-TOF-MS results indicate that HSA and Hb are both prone to NVP-induced modification and that tryptophan, histidine, lysine, and serine residues are major targets for reaction. Inspection of 3D structures of the two proteins (Figure 7) shows that all of the amino acids found to be modified are solvent-exposed, which may explain their susceptibility. Although one additional solvent-exposed tryptophan residue is present in both Hb chains (W15A and W16B), the corresponding tryptic peptides (AAWGK and SAVTALWGK, in Hb A and Hb B, respectively) were not detected, possibly due to poor ionization.

4. Discussion

The relationships between protein adduct formation by the reactive metabolites of both endogenous and xenobiotic species and the onset of toxicity have been the focus of an increasing number of studies in the last decades, largely on account of the progress made in highly sensitive analytical methodologies, particularly mass spectrometry (*31*−*39*). Currently, protein adducts are extensively investigated as biomarkers of exposure to carcinogens and allergens, in search of potential dose−toxicity correlations. The abundant blood proteins HSA and Hb are often the models of choice for protein binding studies. Hb has a relatively long and well-controlled lifespan (ca. 120 days in humans); in addition, reactive intermediates have to cross cell membranes to reach the protein, a reason underlying the frequent assessment of Hb adducts as surrogates for DNA adducts of genotoxic carcinogens (*37, 38*). Moreover, despite having a shorter half-life than Hb (ca. 20 days), HSA is a well-characterized protein, and approximately 40% of extravascular HSA is located in the skin (*50*), which supports its use as a tool to investigate protein haptenation mechanisms, particularly by skin allergens.

In the current study, we demonstrate that both HSA and Hb undergo covalent modification upon incubation with 12-mesyloxy-NVP. The two MS-based methodologies selected for the present work afforded similar results regarding the structural identity of multiple amino acid targets for NVP modification; in addition, complementary information was obtained by combining the data from each approach. Thus, although the adopted LC-ESI-MS/MS strategy did not provide sequence information, both techniques indicated that the NVP-modified amino acids in HSA were of the same type, regardless of the amount of 12-mesyloxy-NVP used in the incubations. Likewise, the results from both techniques were in agreement regarding the amino acid residues modified in Hb (*vide infra*).

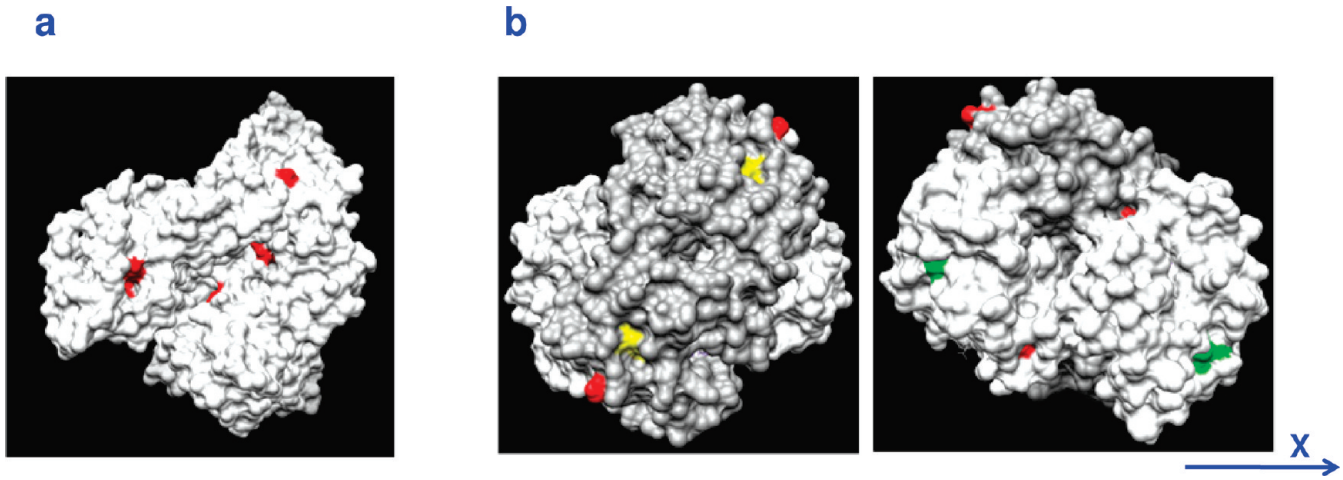


Figure 7. Surface landscape of (a) HSA and (b) Hb, showing the solvent-exposed amino acid residues found to be NVP-modified (red). For Hb, the nonmodified solvent-exposed tryptophan residues are displayed in yellow (chain A) and green (chain B). For greater clarity, the surface of one Hb subunit is shown in dark gray. Two views of the same molecule, rotated by 180° along the indicated axis, are depicted. Atomic coordinates retrieved from the Protein Data Bank archive (http://www.pdb.org) were used to represent the tridimensional structures of HSA (ID 1E78) and Hb (ID 2H35).

Comparison of the MALDI-TOF-MS results obtained for the intact NVP-modified HSA and for the corresponding tryptic peptides revealed a noteworthy consistency. In fact, the assay conducted on the intact protein using the linear mode suggested the modification of, on average, four amino acid residues when the protein was treated with two sequential additions of 12-mesyloxy-NVP; this was confirmed with the MALDI-TOF-TOF-MS experiment, which identified four distinct adducts. Interestingly, the same four amino acid residues were found to be modified after a single addition of 12-mesyloxy-NVP, although the extent of modification corresponded to an average of three amino acid residues per protein molecule. Thus, the reaction with this model ester was not only reproducible but also highly selective.

Tryptophan and histidine modifications in HSA were clearly detected both by LC-ESI-MS/MS and MALDI-TOF-TOF-MS. Previous studies of selective covalent modification of HSA by different chemicals have reported adduction at several key residues, including one cysteine (C34) and several lysines and histidines (*33*). Thus, histidine modification by 12-mesyloxy-NVP was expected. By contrast, the identification of a modified tryptophan residue (W238) was somewhat surprising since W238 is the only tryptophan present in HSA (*51*), and with the exception of binding to this residue in HSA by activated *N*-aryl hydroxamic acids (*52*), reports of tryptophan adducts in proteins are virtually nonexistent. Assuming that 12-mesyloxy-NVP is a good model for the *in vivo* situation, this suggests a remarkable affinity of the indole ring of tryptophan toward NVP-derived electrophiles, which may provide a good biomarker of NVP exposure.

Cysteine modification in HSA was readily identified by LC-ESI-MS/MS but not confirmed by MALDI-TOF-TOF-MS. HSA has 35 cysteine residues, of which 34 are paired in 17 intramolecular disulfide linkages (*33*). Thus, only one cysteine (C34) maintains a free sulfhydryl group, which exists primarily in the highly nucleophilic thiolate form due to the vicinity of three ionizable residues, D38, H39, and Y84, within the tertiary structure of the protein (*53*). As indicated above, this residue is a frequent target for electrophiles (*33*), which makes our detection of cysteine modification by LC-ESI-MS/MS entirely credible. The reasons for our failure to detect the modified cysteine by MALDI-TOF-TOF-MS are not clear. Prior to trypsinization, the protein was subjected to reduction of the disulfide bonds with dithiothreitol and alkylation to prevent reoxidation of the sulfhydryl groups. It is possible that the microenvironment in the vicinity of the adducted C34 catalyzed an interchange of the NVP fragment with dithiothreitol, thereby destroying the adduct. Alternatively, ionization of the tryptic peptide containing the modified cysteine may have been inefficient under the MALDI experimental conditions that were used. By contrast, the LC-ESI-MS/MS analysis did not detect the lysine modifications identified by MALDI-TOF-TOF-MS. This may have been due to deficient hydrolysis at the adducted lysine residues and/or lack of sensitivity of the analytical method when compared to that of MALDI-TOF-TOF-MS. Taken together, the results from a combination of MS methodologies provided complementary information, which allowed the identification of five amino acid targets for NVP modification in HSA: W238, H362, K214, K548 or 549, and C34.

As indicated above, analysis of the intact protein mass by MALDI-TOF-MS gave a complex pattern for Hb, with several clusters of *m*/*z* peaks suggesting that some molecules of each strand were modified with at least up to three NVP residues. However, the results obtained by MALDI-TOF-TOF-MS (Table 1), which identified one modified residue in strand A and two modified residues in strand B, imply that some of the specific adduction sites were not detected.

The ability of tryptophan to be modified by 12-mesyloxy-NVP was further confirmed in the analysis of NVP-modified Hb. Thus, evidence for the presence of 12-(tryptophan-2′-yl)-NVP was achieved unequivocally by LC-ESI-MS/MS through comparison with an authentic standard and corroborated by MALDI-TOF-TOF-MS, which provided the identification of W38 in strand B as a specific site of modification. The MALDI-TOF-TOF-MS results also gave clear indication that a serine residue (S90, in the B chain) was modified in Hb. This confirmed the tentative ascription of serine modification made by LC-ESI-MS/MS; although the lack of a synthetic standard precluded a definite assignment of the adduct structure, it can be reasonably anticipated to involve the binding of the serine oxygen through the NVP-C12 (Scheme 3).

As observed for HSA, the two MS methodologies provided complementary information regarding Hb modification by NVP. Again, cysteine modification was confirmed unequivocally by LC-ESI-MS/MS but not indicated by MALDI-TOF-TOF-MS. In contrast, MALDI-TOF-TOF-MS gave clear indication of

histidine modification (H21 in strand A), but no histidine adducts were detected by LC-ESI-MS/MS. Since histidine modification was readily detected by this technique in the HSA hydrolysate, the failure to detect this adduct in Hb was presumably due to deficient hydrolysis, rather than a lack of sensitivity of the LC-ESI-MS/MS method.

The *N*-terminal valine residues of Hb were an expected modification site since they are primary sites of reaction with several classes of electrophiles (*38*). However, the adopted general procedures, involving Hb hydrolysis to amino acids followed by LC-ESI-MS/MS or MALDI-TOF-TOF-MS of the tryptic peptides, gave no indication of valine modification in Hb. Several factors may have been at play, from inefficient hydrolysis and poor sensitivity of the LC-ESI-MS/MS method to poor ionization under the MALDI-TOF-TOF-MS conditions, as discussed above. However, use of an N-alkyl Edman procedure to detach specifically (and thus enrich) the NVP-modified *N*-terminal valine residues as a phenyl thiohydantoin derivative, followed by LC-ESI-MS/MS analysis, showed unequivocally that modification of the *N*-terminal valine occurred upon treatment of Hb with 12-mesyloxy-NVP. This suggests that similar to what has been extensively demonstrated for numerous other electrophiles (*38*), NVP adducts through the *N*-terminal valine of Hb may be very convenient biomarkers of NVP activation.

In summary, the present work investigated the ability of the synthetic model ester 12-mesyloxy-NVP to bind to the blood proteins, HSA and Hb, *in vitro*. The sites of modification in HSA and Hb were determined by two different MS-based methodologies that involved the enzymatic hydrolysis of the modified proteins to amino-acids, followed by LC-ESI-MS/MS, and trypsin digestion of the modified proteins to peptides, followed by MALDI-TOF-TOF-MS. Multiple sites of Hb and HSA modification through the NVP-C12 were identified, consistently involving adduction at amino acids bearing side chains reasonably anticipated to have nucleophilic character, such as cysteine, lysine, tryptophan, histidine, and serine. With the exception of tryptophan, all other amino acids are common sites of covalent protein modification by electrophilic species (*32*−*39*). Some of the modified residues were identified by the two methodologies, but in other instances, complementary information was obtained by combining the results from both methods. One significant feature is the fact that tryptophan was the adducted amino acid systematically identified in the two proteins by both LC-ESI-MS/MS and MALDI-TOF-TOF-MS. Also noteworthy is the detection of *N*-terminal valine adducts in Hb by LC-ESI-MS/MS following an N-alkyl Edman degradation procedure. The usefulness of the two MS-based methodologies developed in this work is currently being explored in the analysis of serum proteins from rodents administered NVP and 12-hydroxy-NVP to investigate the feasibility of this approach in the assessment of *in vivo* modified samples. Ultimately, the goal is to use these methods in the biomonitoring of NVP-treated patients by taking advantage of both the high sensitivity of MALDI-TOF-TOF-MS to detect protein adducts present in low concentrations and determine their specific location in the protein sequence, and the potential quantitative applications presented by LC-ESI-MS/MS.

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Supporting Information Available: MS/MS spectra of modified peptides from HSA and Hb. This material is available free of charge via the Internet at http://pubs.acs.org.

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