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The Anticancer Drug Cisplatin Can Cross-link the Interdomain Zinc Site on Human Albumin

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Supporting Information

Experimental Section

Materials. Recombinant human albumin (rHA) was kindly provided by Delta Biotechnology Ltd (Nottingham, UK) and extensively dialyzed against NH_4HCO_3 buffer (100 mM) and lyophilized before use. Trypsin (sequence grade) was purchased from Promega, guanidine hydrochloride from Amresco, selenocystamine dihydrochloride from Sigma, dithiothreitol (DTT) from Pierce, iodoacetamide and acetonitrile (HPLC grade) from Merck, trifluoroacetic acid (TFA) from Acros, and triethylammonium acetate buffer (TEAA) from Transgenomic. Microcon centrifugal filtration units with a 10 kDa molecular weight cut-off were purchased from Millipore. Aqueous solutions were prepared using MilliQ water (MilliQ Reagent Water System).

HPLC-ESI-MS(/MS). Positive-ion electrospray ionization mass spectra were obtained on a Micromass Q-TOF mass spectrometer (Waters) coupled to a Waters CapLC HPLC system. The tryptic digests of rHA or platinated rHA were separated on a Symmetry-C18 column (10×5mm, 100Å, 3.5 µm, waters). Mobile phases were A: 95% H_2O containing 4.9% acetonitrile and 0.1% formic acid, and B: 95% acetonitrile containing 4.9% H_2O and 0.1% formic acid. The peptides were eluted with a 50 min linear gradient from 1% to 45 % of B at a rate of 30 µL min^{-1}. The eluent was directly infused into the mass spectrometer through the ESI probe. The spray voltage of the mass spectrometer was 3.30 kV and the cone voltage 35 V. The desolvation temperature was 413 K and source temperature 353 K. Nitrogen was used as both cone gas and desolvation gas with a flow rate of 40 L

h^{-1} and 400 L h^{-1}, respectively. The collision energy was set to 10 V. The MS spectra were acquired in the range of 500 – 1800 *m/z*. Collision-induced dissociation MS/MS spectra were obtained in the range of 100 – 1800 *m/z*, and the collision energy was set to 30-40 eV, Ar as a collision gas. Mass Lynx (ver. 4.0) software was used for analysis and post processing.

UV-Vis Spectroscopy. The assay for determining the concentration of rHA-bound zinc was performed as previously described (K. A. McCall and C. A. Fierke, *Biochemistry.*, 2004, **43**, 3979). Aliquots of zinc acetate standard solution (for generation of the calibration curve) or the Zn-rHA complex solution (20 µL) were mixed with a denaturing buffer (160 µL, pH 7.5) containing guanidine hydrochloride (6 M) and MOPS (12.5 mM) and incubated at room temperature for 5 min. Then an aliquot of PAR (20 µL, 1 mM in H_2O) was added to the sample and the absorbance was immediately determined at 515 nm using a SpectraMax M5 spectrophotometer with quartz cuvettes of 1.0 cm pathlength. The concentration of protein-bound Zn was determined by comparison to a calibration curve (0–12 µM).

Platination of rHA. In a typical reaction, rHA (50 µM) in 20 mM TEAA (pH 7.4) was incubated with cisplatin at a molar ratio of 1:5 or 1:10 for 80 h at 310 K. Unbound cisplatin was removed by centrifugal filtration three times with a 10 kDa cut-off filter at 5000 g for 12 min at ambient temperature. The concentration of rHA in the final solution was measured by UV-Vis spectroscopy to be 7.31 g L^{-1}. Then the platinated rHA was analyzed by proteomics approaches.

Proteomics Assay. The rHA or platinated rHA (Pt-rHA) (50 µL, 7.31 g L^{-1}) was mixed with denaturing buffer (450 µL, pH 8.5) containing guanidine hydrochloride (6 M), NH_4HCO_3 (100 mM), DTT (2.5 µL, 1.0 M in 100 mM NH_4HCO_3 buffer, pH 8.2) and selenocystamine dihydrochloride(3 µL, 100 mM in water). The mixture was incubated at 310 K for 5 min to cleave the disulfide bonds. Then iodoacetamide (6 µL, 1.0 M in 1.0 M NaOH solution) was added, and the resulting mixture was incubated in the dark for another 30 min at ambient temperature to alkylate the free thiol groups. The resulting solutions were then dialysed against NH_4HCO_3 buffer (100 mM, 1 L, pH 7.8) for 2 h at room temperature. An aliquot of the dialyzed sample was mixed with trypsin (1 µg), giving a substrate/enzyme ratio of 37:1, and then was incubated at 310 K for 24 h for digestion. The tryptic digests of rHA or platinated rHA were separated on a C18 column and the fractions were analyzed by mass spectrometry.

In Silico Digestion. In silico tryptic digestion of rHA was used to identify peptides. The theoretical *m/z* values of tryptic digestion of rHA were generated by the "MS-Digest" program (http://prospector.ucsf.edu). Cysteines were set as carboxyamidomethyl-cysteines; peptide masses were calculated as monoisotope and multiple charges; no missed cleavages were permitted.

Competition Reactions of Cisplatin vs Zinc for rHA Binding

a) An aliquot of rHA (50 μM) in 20 mM TEAA (pH 7.4) was incubated with cisplatin and zinc acetate at a molar ratio of 1:5:5 for 80 h at 310 K. After removal of unbound cisplatin and zinc by centrifugal filtration, the protein-complex solutions were analyzed by proteomics approaches as described above.

b) Pt-rHA was prepared following the procedure described above with a 1:10 molar ratio of rHA to cisplatin in 20 mM TEAA buffer for 80 h at 310 K. Then an aliquot of Pt-rHA (50 μM) in 20 mM TEAA (pH 7.4) was incubated with zinc acetate at a molar ratio 1:1 for 24 h or 48 h at 310 K. At the same time, the native rHA was incubated with zinc acetate at a 1:1 molar ratio in 20 mM TEAA buffer for 24 h or 48 h at 310 K. After removal of unbound zinc by centrifugal filtration, the concentration of zinc bound to rHA was determined by UV-Vis spectroscopy with using 4-(2-pyridylazo)resorcinol as a zinc indicator.

c) Zn-rHA was prepared following the same method as for Pt-rHA using a 1:1 molar ratio of rHA to zinc acetate in 20 mM TEAA buffer for 48 h at 310 K. Then an aliquot of Zn-rHA (50 μM) in 20 mM TEAA (pH 7.4) was incubated with cisplatin at a molar ratio 1:10 for 80 h at 310 K. After removal of unbound cisplatin and zinc by centrifugal filtration, the concentration of zinc bound to rHA was determined using the zinc indicator 4-(2-pyridylazo)resorcinol.

Modelling. Molecular modelling was performed by a modification of a previously described method (W. B. Hu, Q. Luo, X. Y. Ma, K. Wu, J. A. Liu, Y. Chen, S. X. Xiong, J. P.

Wang, P. J. Sadler and F. Y. Wang, *Chem. Eur. J.*, 2009, **15**, 6586.). The initial molecular structure of rHA was obtained from a published crystal structure (PDB ID 1bm0), and that of cisplatin from the Cambridge Crystallographic Data Centre (Refcode CUKRAB). The fragment $\{(NH_3)_2Pt\}^{2+}$ was docked onto the binding site consisting of amino acid residues His67, Asn99, His247 and Asp249, with the platinum coordinated to the imidazole nitrogen atoms of His-67(N_ε) and His-247(N_δ) residues, forming a four-coordinate square-planar complex. The model was imported into the HyperChem molecular modelling package (Version 7.5, Hypercube, Inc) for structural optimization. A two-step optimization protocol was used. In the first step, the backbone of the above residues in the binding site was fixed and the four nitrogen atoms coordinated to platinum were restricted to maintain a square-planar geometry, but no constraints were applied to their side-chains. The remaining backbone and side-chains of rHA were frozen. This yielded a partially-optimized structure for the fragment $\{(NH_3)_2Pt\}^{2+}$ and its binding site. In the second step, the entire system (rHA and fragment $\{(NH_3)_2Pt\}^{2+}$) was optimized without any structural constraints. The optimizations were carried out using the Molecular Mechanics MM+ force field which is a modified version of the MM2 force field developed by Allinger et al (N. L. Allinger, *J. Am. Chem. Soc.*, 1977, **99**, 8127.). The first optimization took 906 steps, and the second 1209 steps to reach a satisfactory convergence. The steepest descent algorithm and the terminating gradient 0.1 kcal mol^{-1} $Å^{-1}$ were employed during the two optimization steps.

rHA Chemical Modification. Methionine residues were modified by bromoacetic acid.(R. H. L. Marks and R. D. Miller, *Biochem. Biophys. Res. Commun.*, 1979, **88**, 661) The reactions between bromoacetic acid (0.16 M) and rHA solutions (100 μM) were performed in sodium formate buffer (0.2 M, pH 2.9) at 310 K for 24 h. The reaction mixture was dialyzed against water to remove excess bromoacetate and then lyophilized for the following reactions with cisplatin.

Table S1. Identified platinated peptides arising from the trypic digest of Pt-rHA adduct prepared by the incubation of rHA with cisplatin in a 1:10 molar ratio at 310 K for 80h.

Peptide Sequence	Peptide adducts	*m/z*[b]		δ[c] / ppm
		Calculated	Observed	
(65)SLH*TLFGDK(73) (241)VHTEC°C°H*GDLLE C°ADDR(257)	T1-**1′**[a]-T2	667.080^{5+}	667.086^{5+}	9.94
(115)LVRPEVDVMC*TAF HDNEETFLK(136)	T3-**1″**[a]	701.811^{4+}	701.806^{4+}	-7.12
(115)LVRPEVDVMC°TAF H*DNEETFLK(136)	T3′-**1″**	716.066^{4+}	716.086^{4+}	12.5
(287)SHC°IAEVENDEM* PADLPSLAADFVESK(313)	T4-**1″**	797.086^{4+}	797.079^{4+}	8.78

[a]. **1′** = $\{(NH_3)_2Pt\}^{2+}$; **1″** = $\{(NH_3)Pt\}^{2+}$

[b]. The most abundant isotopomer.

[c]. $\delta = (m/z)_{observed} - (m/z)_{theoretical} / (m/z)_{theoretical}$.

°. Carboxyamidomethyl-cysteine.

*. platinated sites.

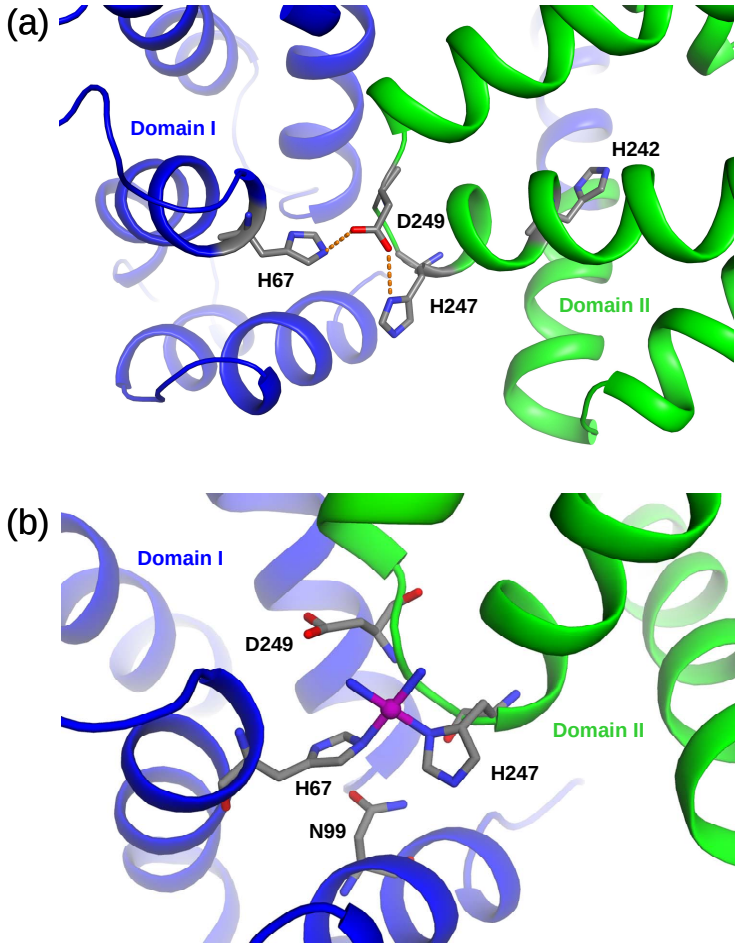

Figure S1. a) The interface of domains I (blue) and II (green) (PDB ID 1bm0) of human serum albumin. His67 and His247 form hydrogen bonds with Asp249, and the buried His242 is located at the opposite side of the interface. b) An energy-minimized model showing $\{(NH_3)_2Pt\}^{2+}$ (CPK coloring) crosslinking His67 and His247 residues. All the figures were prepared using PyMol Ver 0.99rc6.

The MS^2 spectrum of cross-linked platinated peptide T1-**1′**-T2 (**1′** = $\{(NH_3)_2Pt\}^{2+}$) renders assignment of His67 as one of the binding sites in peptide T1. However, no fragment ions containing Pt allowed identification of the coordination site in peptide T2. The three cysteine residues in T2 were all alkylated (Figure 1), precluding these as sites for Pt

coordination. Therefore, His247 and His242 are possible binding sites in T2. The available X-ray crystal structures of HA (e.g. PDB ID 1bm0) show that the solvent-accessible residues His67 and His247 are situated at the interface of domains I and II, and have suitable orientations to coordinate to **1′** simultaneously, whereas the buried His242, located on the opposite side of the interface, does not satisfy this steric requirement. Thus His247 is the preferred binding site for **1′** in T2.

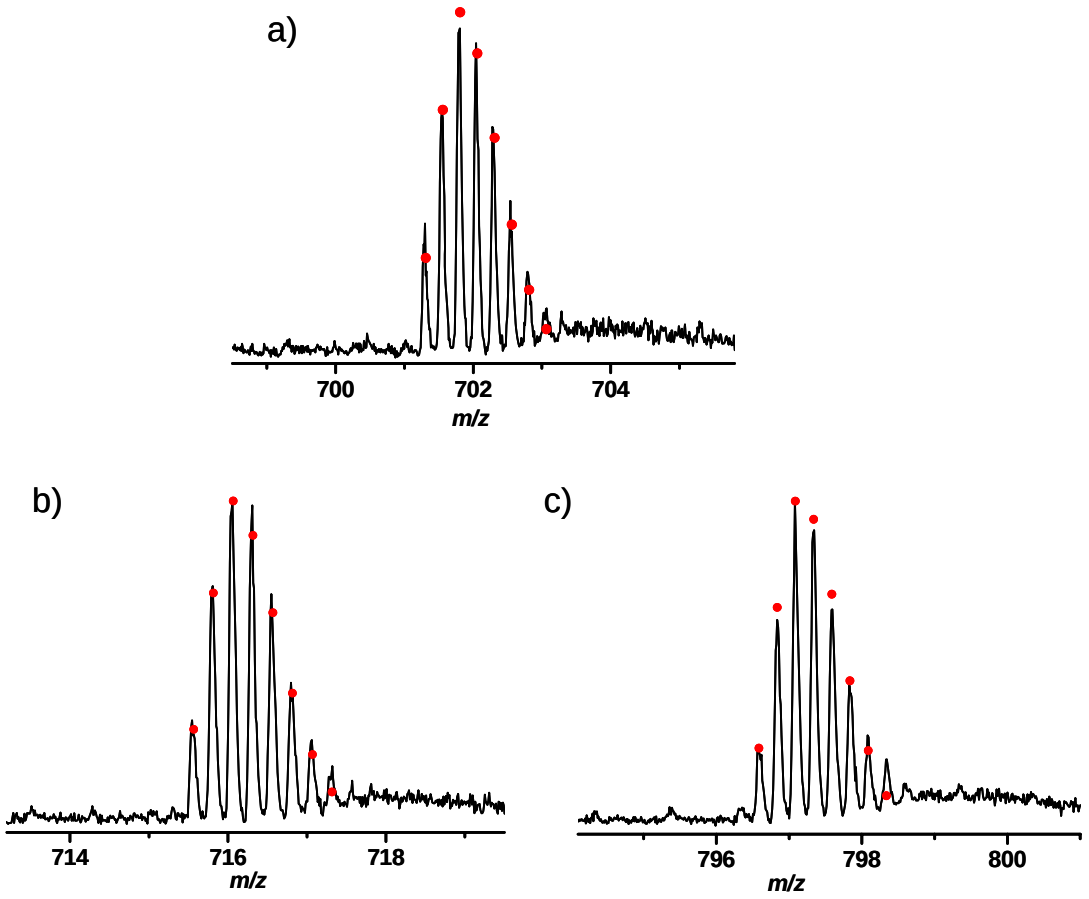

Figure S2. Isotopic models (dots, for which the values of x and y correspond to the *m/z* value and intensity of the respective isotopic ion peak, respectively) and mass spectra (lines) for platinated peptide a) T3-**1″**: (115)LVRPEVDVMC-**1″**TAFFHDNEETFLK(136), b)T3′-**1″**: (115)LVRPEVDVMC°TAFFH-**1″**DNEETFLK(136), c)T4-**1″**: (287)SHC°IAEVENDEM-**1″**PADLPSLAADFVESK(313). Superscript ° indicates the cysteine residue was carboimidomethylated, **1′** = $\{(NH_3)_2Pt\}^{2+}$; **1″** = $\{(NH_3)Pt\}^{2+}$

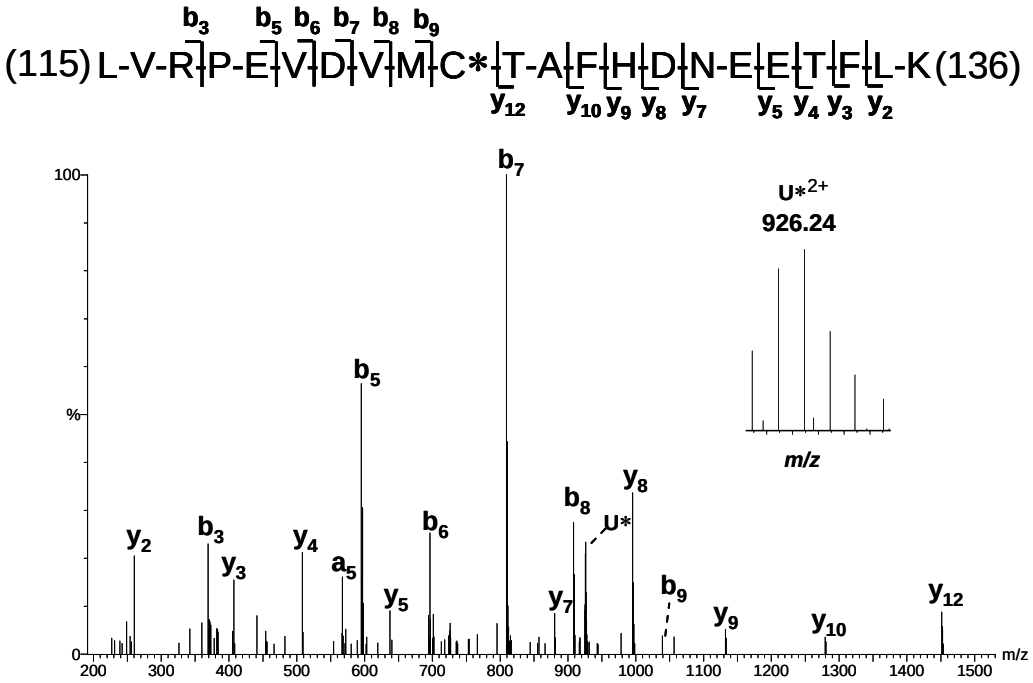

Figure S3. LC-ESI-MS/MS spectrum of the platinated peptide T3-**1″** containing sequence L115-K136 (parent ion at *m/z* 701.806). The inset shows an isotopic pattern of Pt containing ion at *m/z* 923.24, which corresponds to the internal fragment ion **U*** $^{2+}$ = DVMC*TAFHDNEETF, *={$(NH_3)Pt$}$^{2+}$.

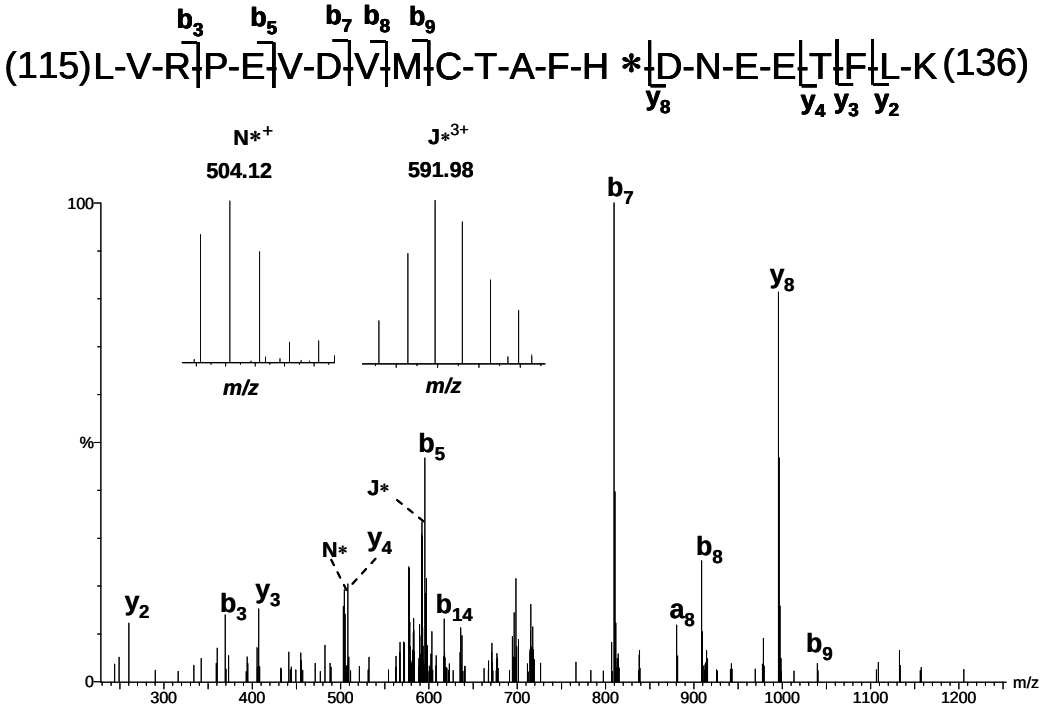

Figure S4. LC-ESI-MS/MS spectrum of the platinated peptides T3′-**1**″ containing sequence L115-K136 (parent ion at *m/z* 716.066). The inset shows the isotopic patterns of Pt-containing ions at *m/z* 504.12 and 591.98, which are assignable to internal fragment ions J^{*3+} = (MCTAFH*DNEETFL – NH_3) and N* = (AFH* – NH_3 - CO), respectively. * = $\{Pt\}^{2+}$, "- NH_3" and "–CO" indicate the neutral loss of an NH_3 group and a CO group, respectively.

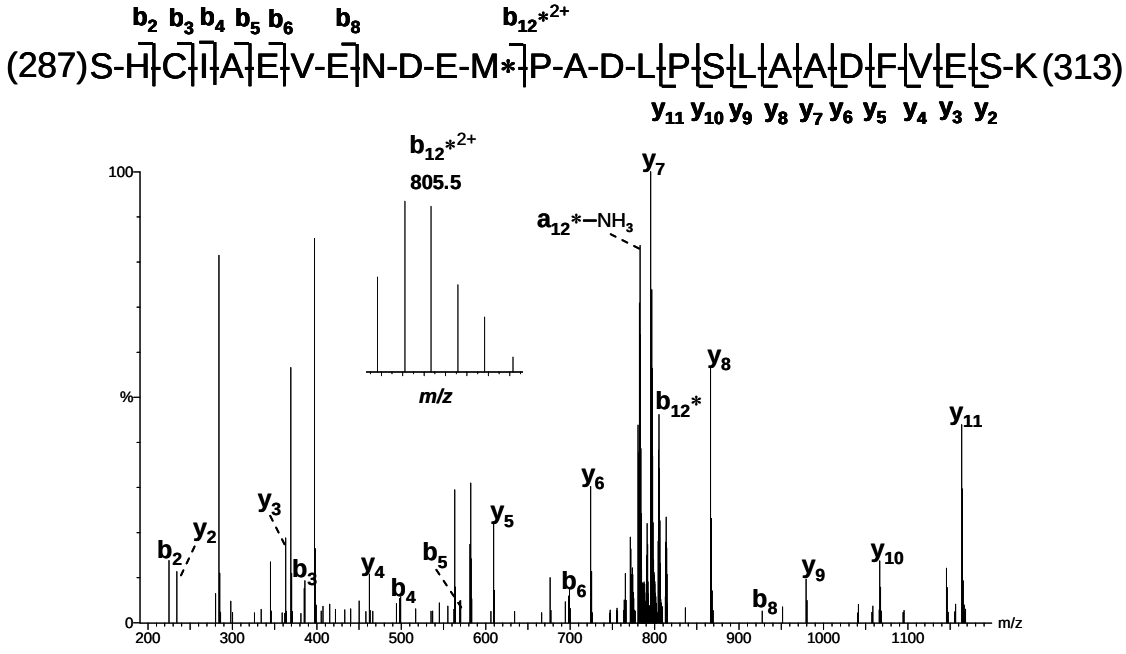

Figure S5. LC-ESI-MS/MS spectrum of the platinated peptides T4-**1″** containing sequence S287-K313 (parent ion at *m/z* 797.079). The inset shows the isotopic pattern of platinum containing fragment ion $b_{12}*^{2+}$ = SHCIAEVENDEM*, *={Pt}$^{2+}$.

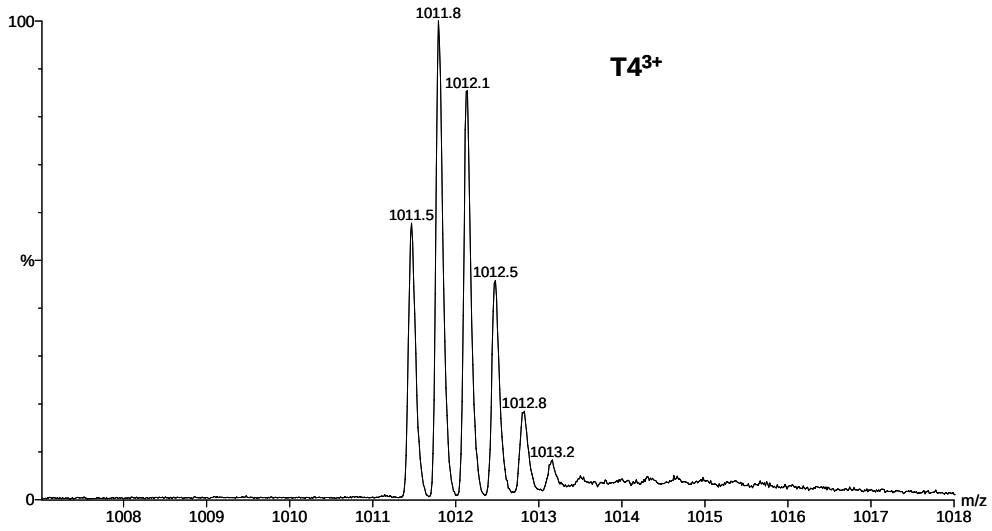

Figure S6. Mass spectrum of peptide T4: (287)SHCIAEVENDE {M-CH_2COOH}PADLPSLAADFVESK(313) arising from the tryptic digest of bromoacetate-modified rHA after incubating with 10 mol equiv cisplatin for 6 d. No platinated T4 was observed while Met298 was alkylated, suggesting that cisplatin coordinated to Met298 in native rHA.

Isotopic modeling (Figure S2a and S2b) revealed that the quadruply-charged peptides at *m/z* 701.811, 716.066 are assignable to platinated peptides T3′-**1″** and T3-**1″** (**1″** = $\{(NH_3)Pt\}^{2+}$), respectively. Both T3′-**1″** and T3-**1″** contain amino acid residues L115-K136, and the 57 Da difference between their masses is attributed to that the Cys124 residue is alkylated in T3-**1″** but not alkylated in T3′-**1″**. Since the Pt-rHA complex was treated with DTT to cleave disulfide bonds of the protein and then reacted with iodoacetamide to block the free thiols prior to tryptic digestion, the observation of non-alkylated Cys124 residue in T3′-**1″** indicates that cisplatin fragment **1″** coordinated to Cys124 in T3′, which was supported by the MS^2 analysis of the $[M + 4H]^{4+}$ parent ion at *m/z* 701.811 (Figure S3). The internal fragment ion **U***$^{2+}$(DVMC*TAFHDNEETF) of T3′-**1″** at *m/z* 926.24 contained cisplatin fragment **1″**. In the native rHA Cys124 and Cys169 form a disulfide bond, therefore, the detection of adduct T3′-**1″** suggests that cisplatin induced the cleavage of the disulfide bond and then coordinates to the reduced Cys124 residue.

In platinated peptide T3-**1″**, Cys124 was alkylated by iodoacetamide (Figure S2b), indicating that Cys124 of peptide T3 was precluded as the binding site for **1″**. The MS/MS spectrum of the $[M + 4H]^{4+}$ parent ion at *m/z* 716.066 (Figure S4) shows two internal fragment ions J*$^{3+}$ (MCTAFH*DNEETFL – NH_3) and N* (AFH* – CO – NH_3) containing the cisplatin fragment $\{Pt^{2+}\}$ and His128 residue, suggesting that His128 is the binding site for platinum in T3-**1″**.

According to the isotopic modeling shown in Figure S2c, the quadruply-charged peptide at *m/z* 797.086 corresponds to the platinated peptide T4-**1″** containing the amino acid residues 287S-313K and the fragment $\{(NH_3)Pt\}^{2+}$. This assignment was confirmed by the MS^2 analysis of $[M + 4H]^{4+}$ parent ion at *m/z* 797.086 as shown in Figure S5. The product ion $b_{12}{}^{*2+}$ that was modified by the coordinated Pt fragment reveals that His288 and Met298 are the probable binding sites (Figure S5). In order to assign the exact binding site, methionine residues of rHA were modified by bromoacetic acid at pH 2.9 prior to incubation with cisplatin; the MS analysis of the Pt-rHA complex (Figure S6) does not show a platinated peptide T4-**1″**, suggesting that platination in T4 occurs at Met298.

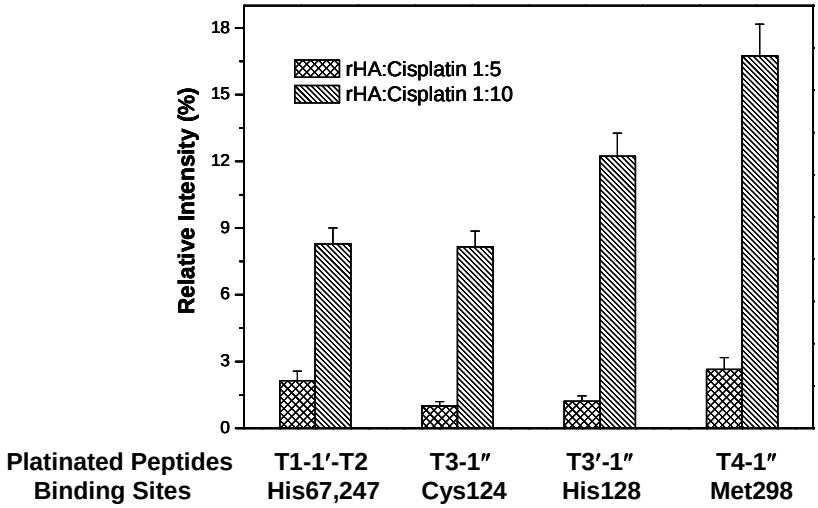

Figure S7. The relative intensities (%) of platinated peptide ions detected in the tryptic digests of Pt-rHA adducts compared with the intensities of non-platinated peptides (373)VFDEFKPLVEEPQNLIK(389), (485)RPCFSALEVDETYVPK(500) and (575)LVAASQAALGL(585) detected in the same tryptic digest of Pt-rHA complex. The Pt-rHA adduct was prepared by incubating rHA with cisplatin at a molar ratio of 1:5 or 1:10 in 20 mM TEAA buffer at 310 K for 80h.