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COMMUNICATION

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

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Cisplatin can crosslink residues His67 of domain I and His247 of domain II in recombinant human albumin (rHA), occupying the major binding site of the essential metal zinc on the protein.

Cisplatin is a widely used anticancer agent, particularly effective for treating solid tumors such as ovarian, testicular, bladder, head and neck cancers.[1] In vivo, cisplatin is converted to its active forms by aquation,[2] being highly reactive toward biomolecules such as DNA[3] and proteins.[4] Although DNA is probably the crucial target for cisplatin inducing apoptosis and cell death,[5] it is now increasingly recognized that an understanding of its reactions with proteins is very important for understanding its metabolism and side-effects. One day after intravenous administration, 65 to 98% of cisplatin is bound to blood plasma proteins,[6] and most of the Pt (50-61%) from cisplatin added to human blood plasma at physiologically-relevant doses is bound to albumin.[7] Albumin, a 66 kDa single-chain protein consisting of 3 isostructural domains,[8] and present in blood at ca. 0.6 mM, can bind and transport a variety of endogenic and exogenic substances, such as fatty acids, bilirubin, pharmaceuticals and metal ions.[9] Despite numerous reports on the interaction of cisplatin with albumin, few Pt binding sites have been characterized unambiguously. According to NMR spectroscopic studies,[10] a Met298 S,N-macrochelate is a major cisplatin binding site, and monofunctional adducts involving Cys34 and surface histidine residues are also be formed. Recently, Cys34, Met329, Tyr150 (or Tyr148) and Asp375 (or Glu376) were identified as cisplatin binding sites using multidimensional liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), after incubating cisplatin with blood serum for 3 h.[11] In the present study, we have used a bottom-up MS approach to reveal for the first time that the reaction of cisplatin with recombinant human albumin (rHA) gives rise to a bifunctional cisplatin-rHA adduct involving intramolecular interdomain crosslinking with platinum occupying the major zinc site on albumin, with implications for the cause of abnormalities in zinc levels reported for patients undergoing cisplatin therapy.[12]

Cisplatin was incubated with rHA in a 10:1 molar ratio in 20 mM TEAA buffer (pH 7.4), at 310 K for 80 h, followed by ultrafiltration (10 kDa cut-off) to remove unbound platinum at ambient temperature. Four platinated peptides (Table S1 in supporting information (SI)) were observed by analyzing the tryptic peptides of the platinated rHA (Pt-rHA) complex by LC-ESI-MS (experimental details are given in SI). Isotopic modeling (Fig. 1a) indicated that the quintuply-charged peptide at *m/z* 667.080 is assignable to the platinum-cross-linked peptide T1-**1′**-T2 (**1′** = $\{(NH_3)_2Pt\}^{2+}$) containing amino acid residues S65-K73 (T1) and V241-R257 (T2). This assignment was verified by the MS/MS spectrum (Fig. 1b) which shows that the product ions from the $[M + 5H]^{5+}$ parent ion at m/z 667.080 correspond to partial b and y ion series of T1 and T2 peptides. The detection of the b_3*′ ion (Fig. 1c) containing Pt revealed that platination of T1 occurs at His67. Although no fragment ions containing Pt allowed identification of the coordination site in peptide T2, the three cysteine residues in T2 were all alkylated upon carboxyamidomethylation of the sample (Fig. 1), precluding these as sites for Pt coordination. Analysis of the X-ray crystal structure of rHA (PDB ID 1bm0) suggested that only His247 of T2 adopts a suitable orientation to occupy the second coordination site available in **1′**; thus His247 is the likely binding site for **1′** in T2 (for more details see Fig. S1 in SI).

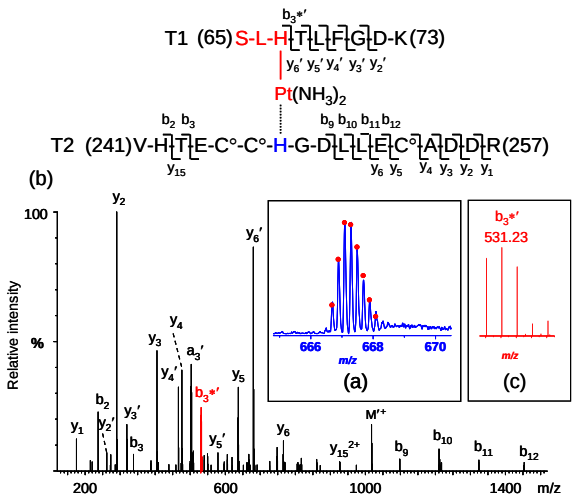

Fig. 1 (Top) Sequence and fragmentation pattern of the platinated peptide T1-1′-T2, 1′ = $\{(NH_3)_2Pt\}^{2+}$. superscript ° indicates cysteine residue was carboxyamidomethylated. (a) Mass spectrum of the parent ion of the quintuply charged peptide T1-1′-T2 at *m/z* 667.086, and isotopic model (dots correspond to the m/z value and intensity of the respective isotopic ion peak); (b) LC-ESI-MS/MS spectrum of the platinated peptide T1-1′-T2; (c) the isotopic pattern for b_3*′ fragmentation ion (* = $\{Pt\}^{2+}$).

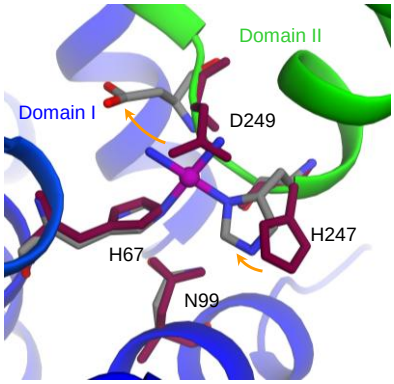

Fig. 2 Superposition of a model of the His67-His247 adduct of human albumin with *cis*-$\{(NH_3)_2Pt\}^{2+}$ (energy-minimized, $\{(NH_3)_2Pt\}$ in CPK coloring) and the X-ray structure of unliganded rHA (PDB ID 1bm0, magenta) showing crosslinking of domains I and II by Pt. Orange arrows indicate direction of rotation of side chains of His247 and Asp249 induced by Pt coordination. Figure prepared using PyMol Ver 0.99rc6.

Based on isotopic simulations and the MS/MS spectra of three other platinated peptides, residues Cys124[13] and His128 in peptide T3 covering the sequence L115 to K136, and Met298 in peptide T4 containing amino acid residues S287 - K313 were also identified as binding sites for cisplatin (for details see Fig. S2-S6 in SI).

The relative intensities of the platinated peptides were compared with those of three non-platinated peptides detected from the same tryptic digest of Pt-rHA complex. These data showed that at a 1 : 5 molar ratio of rHA to Pt, cisplatin binding to His67-His247 and Met298 predominated slightly compared to binding to Cys124 and His128, while at a 1 : 10 molar ratio, cisplatin preferentially bound to His128 and Met298 (Fig. S7 in SI).

The cross-linking of His67 and His247 by *cis*-$\{(NH_3)_2Pt\}^{2+}$ is highly significant since these residues form part of the proposed major zinc site on albumin,[9] the major transport system for zinc in blood. On the basis of the X-ray crystal structures of rHA (PDB ID 1bm0) and cisplatin, a molecular model was constructed for coordination of **1′** to His67 and His274 (Fig. 2). On coordination, the imidazole group of His247 moves towards His67, and the side-chain of residue Asp249 rotates significantly away from its initial position.

Zinc coordination to albumin is also proposed to involve the side-chains of Asn99 and Asp249, and a fifth exogenous ligand (e.g. H_2O).[9] Interestingly His67 and His247 belong to two different domains of albumin, domains I and II, respectively (Fig. 2) and the binding of long-chain fatty acids in a nearby site (fatty acid binding site 2) disengages the two halves of this zinc site.[9] We can therefore postulate that the fatty acid status of albumin may also affect platination at this site.

Next we investigated the competitive binding of zinc and cisplatin to confirm that cisplatin does indeed bind to the zinc site. rHA was reacted with cisplatin and zinc acetate in a molar ratio of 1:5:5. MS data (Fig. 3a) showed that the extent of Pt coordination to His67 and His274 decreased by 82% as compared with the reaction of cisplatin with rHA in the absence of Zn^{2+}. In contrast, the extent of platination of the other three platinated peptides did not change significantly (data not shown).

Further experiments were carried out to investigate whether

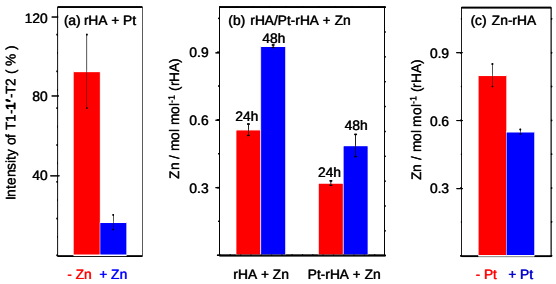

Fig. 3 Competitive binding of cisplatin and zinc to human albumin. (a) Relative intensities (%) of *cis*-$\{(NH3)_2Pt\}^{2+}$ (1′) His67-His247 crosslinked platinated peptide ion $\{T1\text{-}1'\text{-}T2\}^{5+}$ in the tryptic digests of Pt-rHA adducts formed in the absence or presence of Zn^{2+} (rHA:cPt:Zn = 1:5:5, cPt = cisplatin,) in 20 mM TEAA buffer at 310 K for 80 h. The average value (n = 3) of the relative signal intensity of $\{T1\text{-}1'\text{-}T2\}^{5+}$ in the absence of Zn was set as 100%; (b) Zinc bound to rHA determined from reaction mixtures of zinc acetate with native rHA or Pt-rHA complex in 20 mM TEAA buffer at 310 K for 24 h or 48 h (rHA:Zn or Pt-rHA:Zn = 0.05:0.05 mM); (c) Zinc bound to rHA in 20 mM TEAA buffer (pH 7.4) incubated in the absence or presence of 10-fold excess of cisplatin at 310 K for 80 h. Zn content is the mean from three independent determinations.

cisplatin coordination to rHA would reduce the binding of zinc to rHA. Firstly, rHA was incubated with cisplatin at a molar ratio of 1 : 10 (rHA:Pt) to allow Pt-rHA adducts to form, and then the resulting Pt-rHA complex was reacted with 1 mol equiv of Zn^{2+}. The results (Fig. 3b) show that the extent[14] of Zn binding to Pt-rHA decreased by ca. 45%, compared to the reaction of Zn^{2+} with native rHA. On the other hand, when zinc acetate was first reacted with 1 mol equiv of rHA to form Zn-rHA, and then this complex was incubated with a 10-fold molar excess of cisplatin in 20 mM TEAA buffer (pH 7.4) at 310 K for 80 h, the amount of Zn bound to rHA was reduced by ~31% (Fig. 3c). These results indicate that cisplatin coordination to rHA hinders Zn binding and that cisplatin can displace the bound zinc from rHA, in agreement with the hypothesis that the albumin-bound Zn is exchangeable.[9] The strong binding of platinum to albumin is also evident from the retention of Pt by albumin peptides on digestion and chromatography. The extent of binding of cisplatin to albumin is more likely to be controlled by slow kinetics than that of zinc.

The repeated admininistration of cisplatin might be expected to lead to cumulative effects on zinc transport and metabolism, Plasma albumin has a long half-life (>19 days for healthy and young people, > 30 days for patients),[15] and the clinical use of cisplatin has been reported to lead to accumulation and long term retention of Pt in patients.[16]

Interestingly, as early as 1985, cisplatin administration to rats was found to reduce cellular Zn levels significantly, particularly in the kidneys. Intracellular, cytosolic zinc decreased by as much as 64%.[17] Sweeney and co-workers also reported that cisplatin administration may result in hyperzincuria and could potentially precipitate a symptomatic zinc deficiency state in patients.[12] Hence, the identification of cisplatin coordination to the major binding site of zinc on albumin provides for the first time a possible mechanism which links the clinical effects of cisplatin with hyperzincuria and hypozincemia in patients.

It has been reported that cisplatin can also displace zinc from metallothioneins [18] and zinc finger proteins such as the HIV

nucleocapsid NCp7[19] and DNA polymerase I.[20] However, these metal exchanges are intracellular and affect the intracellular storage and distribution of zinc, whereas albumin binding will affect the extracellular transport and delivery of zinc.

His67 and His247 are thought to be involved in the pH-induced N-to-B conformational transition of albumin.[9] Therefore coordination of these residues to Pt may trigger conformational changes and allosteric effects in albumin, and have a direct influence on the binding of other endogenous and exogenous molecules to the protein, for instance, fatty acids. Long-chain fatty acid site FA2 is formed by two half-sites, one in each of the domains I and II.[9, 21] Zinc binding causes a significant change in the conformation of the site FA2.[9, 21] Therefore, the binding of cisplatin to the interdomain Zn site may also inhibit fatty acid binding to site FA2.

In conclusion, the combination of tryptic digestion with LC-MS/MS analysis has provided an effective strategy for identifying the binding sites for the anticancer drug cisplatin on human albumin. Importantly cisplatin can crosslink His67 and His247 at the interface between domains I and II, part of the major zinc binding site on albumin. These findings have potential physiological implications, providing for example a link between cisplatin and hyperzincuria and hypozincemia which are known to develop in patients treated with cisplatin.

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† Electronic Supplementary Information (ESI) available: experimental details of HPLC and ESI-MS/MS analysis, tryptic digestion and molecular modeling; Table S1 and Figure S1-S7. See DOI: 10.1039/b000000x/

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