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Characterization of Platinum Anticancer Drug Protein-Binding Sites Using a Top-Down Mass Spectrometric Approach

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A proof-of-principle study on the application of a top-down electrospray ionization Fourier transform ion cyclotron resonance mass spectrometric approach for characterization of the primary binding sites of the platinum anticancer agents cisplatin, transplatin, and oxaliplatin on ubiquitin is presented. Through employment of different fragmentation techniques, the binding sites of cisplatin and oxaliplatin were found at N-terminal methionine-containing ubiquitin fragments, while transplatin was observed to be attached to ^{19}Pro-Ser-Asp-Thr-Ile-Glu^{24}. The binding to proteins is of particular relevance for the mode of action of metallodrugs with regard to (de)activation, transport, excretion, etc. To the best of our knowledge, this is the first top-down mass spectrometric study on the protein binding site characterization of transition-metal anticancer agents and demonstrates the potential of the applied technique for investigating metal drug–protein interactions.

DNA binding of platinum chemotherapeutic agents (see Chart 1 for the structures of cisplatin and oxaliplatin and the nonactive isomer transplatin) is the main event for exerting their antitumor activity.[1] In contrast, binding to serum proteins is believed to contribute to their side effects, while many investigational drugs, including recently developed ruthenium compounds, are thought to benefit from this type of metabolization.[2−4]

For cisplatin, its nonactive structural isomer transplatin, and oxaliplatin, a distinctly different binding to ubiquitin (Ub; Figure 1) was reported in terms of both binding kinetics and the type of formed adducts.[5−7] Cisplatin was shown, via an indirect determination with oxidized Ub sulfur, to bind primarily at the Met1 sulfur and as a secondary target at His68,[5,6,8] while the transplatin binding to Ub does not appear to be driven by the affinity of platinum for sulfur. This result was concluded from electrospray ionization mass spectrometry (ESI-MS) experiments with oxidized Ub sulfur, which led to a decrease in the reactivity of cisplatin toward Ub,[5,6,8] and from [^{1}H,^{15}N] heteronuclear single quantum coherence NMR spectroscopy.[5] In contrast, for transplatin, no influence on the binding kinetics was found by oxidizing Met1.

The most common way to determine protein binding sites involves a bottom-up approach involving digestion of protein

Figure 1. (Top) Molecular structure of Ub[9] with Met1 and His68 residues highlighted.[10] (Bottom) Amino acid sequence of Ub with the potential metal binding sites highlighted.

Chart 1. Structures of Cisplatin, Transplatin, and Oxaliplatin (from Left to Right)

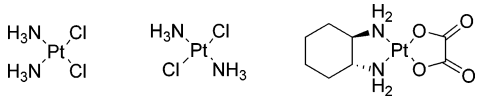

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Table 1. Mass Accuracy of Selected Platinum-Containing Fragments Used for Characterization of Binding Sites in the FT-ICR-MS^2 Spectra

compound	adduct	*m/z* (exptl)[a]	*m/z* (calcd)[a]	error, ppm
cisplatin	$[Pt + H_2N\text{-}^1MQIF^4 - 2H]^+$	713.20836	713.20812	0.34
transplatin	$[Pt(NH_3)_2 + H_2N\text{-}^1M\cdots E^{24}\text{-}NH_2 + 2H]^{4+}$	730.86970	730.86855	1.57
	$[Pt(NH_3)_2Cl + H_2N\text{-}^{19}P\cdots G^{76}\text{-}OH + 8H]^{9+}$	755.94904	755.94910	0.08
oxaliplatin	$[Pt(chxn) + H_2N\text{-}^1MQ^2 - 2H]^+$	567.17119	567.17126	0.12

[a] Most abundant isotopomer.

and liquid chromatography (LC)−MS analysis.[11−13] This method is not always suitable for the study of metal drugs because ammonium bicarbonate buffer induces the release of the platinum adducts.[8] Herein, we present the application of electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FT-ICR-MS) for characterizing directly the binding sites of platinum complexes to Ub using a top-down approach employing FT-ICR-MS/MS. The high resolving power and mass accuracy of FT-ICR-MS have been previously used to study metallodrug−protein interactions,[14,15] although no attempts were made to locate metallodrug binding sites.

Broad-band ESI-FT-ICR-MS spectra of Ub incubated with cisplatin, transplatin, and oxaliplatin demonstrate distinct differences in the extent and composition of the observed species (Figure 2). In accordance with previous studies, a

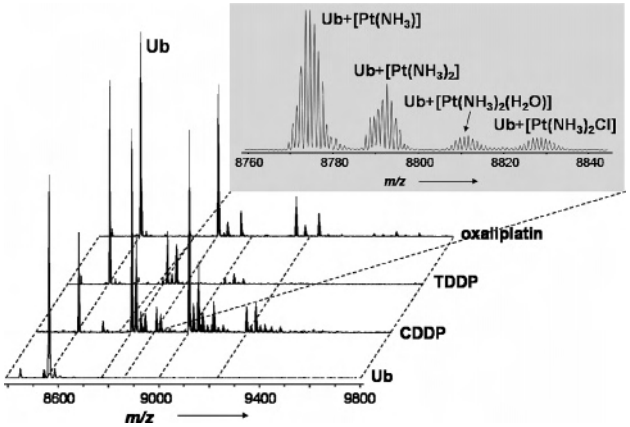

Figure 2. Deconvoluted mass spectra of Ub + cisplatin, Ub + transplatin, and Ub + oxaliplatin (Pt:Ub = 2:1), normalized to the most abundant peak of the respective spectrum (the inset shows a zoom in the monoadduct region of the cisplatin spectrum).

series of mono-, bis-, and also trisadducts were observed for cisplatin, e.g., Ub + $[Pt(NH_3)]$, Ub + $[Pt(NH_3)_2]$, Ub + $[Pt(NH_3)_2Cl]$, Ub + $[Pt(NH_3)_2(H_2O)]$, etc. (compare ref 7). For oxaliplatin up to three platinum moieties and for transplatin up to two platinum moieties per Ub were detected under the applied measurement conditions. In contrast to previous studies,[7] Ub + [Pt(chxn)(ox)] [chxn = (1*R*,2*R*)-cyclohexane-1,2-diamine; ox = oxalato] adducts were detectable for the oxaliplatin incubation, presumably because of the higher performance of the instrument.

The location of the primary platinum−protein binding sites of prominent adducts (for cisplatin Ub + $[Pt(NH_3)]$, for transplatin Ub + $[Pt(NH_3)_2Cl]$, and for oxaliplatin Ub + [Pt(chxn)]) was probed by slow-heating fragmentation methods, collision-induced dissociation (CID)[16] and infrared multiphoton dissociation (IRMPD).[17] CID and IRMPD FT-ICR-MS^2 produced many singly and doubly charged products for all three Ub−Pt systems. The most obvious difference for cisplatin and oxaliplatin versus transplatin is the appearance of low-molecular-weight fragments containing the characteristic platinum isotope pattern, giving the first evidence for potential binding sites. Surprisingly, "soft" ion activation/dissociation by electron capture (ECD),[18] which is known to preserve labile protein modifications,[19] did not provide information on the binding sites, and no low-molecular-weight platinum-containing fragments were observed. It is possible that platinum-containing ECD products are generated but as neutral entities due to electron capture by the platinum ion.

For oxaliplatin, CID MS^2 of $[Ub + Pt(chxn)]^{11+}$, *m/z* 807, resulted in the elucidation of the binding site to a resolution of two amino acids (Table 1 and Figure 3). Oxaliplatin was

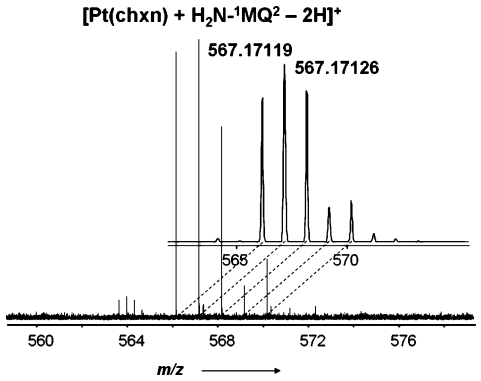

Figure 3. CID FT-ICR-MS of Ub + oxaliplatin [parent ion, *m/z* 807.0, Ub + Pt(chxn)]. The inset shows the theoretical isotopic pattern and the mass of the most abundant isotopomer.

found attached to the N-terminal ^{1}Met-Gln^2 sequence in which the chxn ligand remained bound to the platinum moiety. The affinity of platinum to the soft donor sulfur

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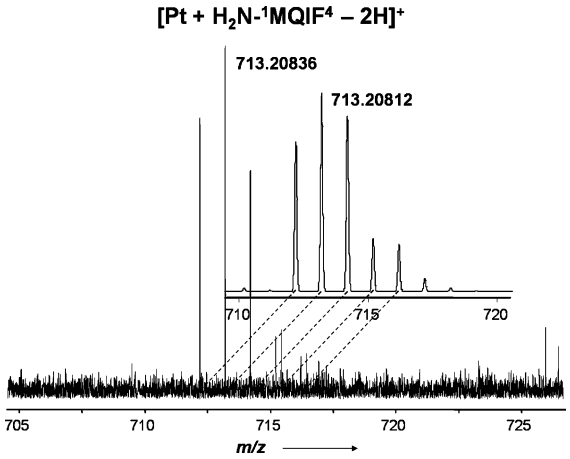

Figure 4. IRMPD FT-ICR-MS of Ub + cisplatin [parent ion, *m*/*z* 1096.2, Ub + [Pt(NH_3)]. The inset shows the theoretical isotopic cluster and the mass of the most abundant isotopomer.

demonstrates that Met1 is involved in binding. Furthermore, a series of multiply charged fragments were assigned to peptide sequences and were, when containing a platinum moiety, exclusively located at the N terminus of Ub.

Compared to the MS^2 characterization of oxaliplatin with a limited number of different adducts, for cisplatin and transplatin, the MS^2 spectral analysis was considerably more complicated. Nevertheless, the cisplatin binding site for the $\{Ub + [Pt(NH_3)]\}^{9+}$ ion (*m*/*z* 1096.2) could be determined by IRMPD FT-ICR-MS^2. The smallest platinum-containing fragment was characterized as Pt-[^{1}Met-Gln-Ile-Phe^4] (Figure 4 and Table 1). Again the platinum moiety is probably bound to the methionine residue, in agreement with previous data.[6] In addition, a series of fragments were identified, confirming the N-terminal attachment of the platinum center. Notably, fragmentation of Ub + [Pt(NH_3)] resulted in the cleavage of the ammine ligands from the platinum center.

In contrast to both cisplatin and oxaliplatin, the main binding site for transplatin was found to be located not at the N terminus but, surprisingly, at the ^{19}Pro-Ser-Asp-Thr-Ile-Glu^{24} peptide (a few platinum-modified C-terminal peptides of low abundance were also observed), a sequence also being accessible at the protein surface and containing a number of potential donor atoms. Both N- and C-terminal fragments were identified for $\{Ub + [Pt(NH_3)_2Cl]\}^{11+}$ (*m*/*z* 803.0) by CID to contain the platinum-modified peptide sequence (Figure 5 and Table 1). The sequence found contains a number of amino acids, i.e., Ser, Asp, Thr, and Glu, that could potentially coordinate to platinum so the precise binding site can only be speculated.

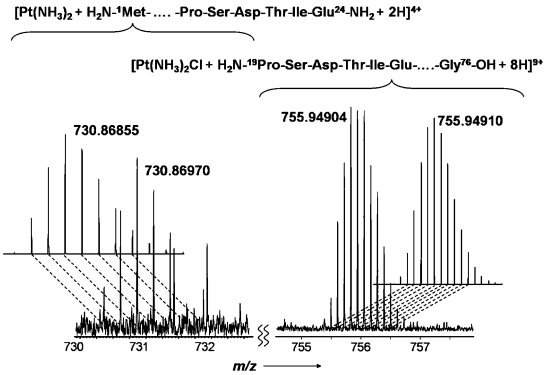

Figure 5. CID FT-ICR-MS of Ub + transplatin (parent ion, *m*/*z* 803, $\{Ub + [Pt(NH_3)_2Cl]\}^{11+}$). The insets show the theoretical isotopic clusters and the masses of the most abundant isotopomers.

This finding is partly in agreement with previous reports, where it was suggested that His68, but not Met1, might be the target of transplatin.[6] Note that the N-terminal fragments always contain a $Pt(NH_3)_2$ moiety, whereas the majority of the C-terminal fragments carry a $Pt(NH_3)_2Cl$ adduct.

The measured *m*/*z* values are in excellent agreement with the calculated values (Table 1), and in general, the experimental and theoretical isotopic patterns of the platinum−peptide complexes correlate well. For all of the Pt−Ub adducts, additional fragments were successfully characterized, confirming the proposed binding sites.

Herein, we have shown that for cisplatin, transplatin, and oxaliplatin the primary binding sites on Ub could be directly determined by top-down FT-ICR-MS and the uncertainties arising from postdigestion reactions from the bottom-up approach are eliminated. Specifically, cisplatin and oxaliplatin were observed to bind to an N-terminal fragment containing Met1, whereas transplatin is bound to the peptide sequence ^{19}Pro-Ser-Asp-Thr-Ile-Glu^{24}. Notably, no fragments attributable to the direct interaction of His68 with the platinum complexes were identified. This top-down approach is likely to become the standard method for determining metal binding sites to proteins, especially in the domain of metal-based drugs, and it should ultimately provide data that facilitate drug design and discovery.

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Supporting Information Available: Experimental details, data processing, mass spectra, and peak assignment. This material is available free of charge via the Internet at http://pubs.acs.org.

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