Cisplatin−Protein Adducts Are Efficiently Removed by Glutathione but Not by 5′-Guanosine Monophosphate

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Cisplatin, *cis*-[$Pt(NH_3)_2Cl_2$], a widely used antitumor agent in the treatment of testicular and ovarian cancers, is believed to induce apoptosis in cancer cells by covalently binding to the DNA.[1] It is administered intravenously, and within 1 day, 65−98% of the drug is bound to blood plasma proteins.[2] While it is widely accepted that Pt−DNA adducts are responsible for the drug's cytotoxicity, the role of Pt−protein adducts in the mechanism of action of the drug remains to be elucidated. On one hand it has been postulated that cisplatin binding to proteins is the likely cause of many of the drug's side effects[3,4] while on the other hand several reports suggest that Pt−HSA (HSA, human serum albumin) adducts may be important for the activity of the drug.[5] When preformed Pt−HSA adducts were administered clinically they increased the survival time of the patients. Also, patients with low levels of HSA did not respond well to cisplatin-based chemotherapy.[6]

But how does this electrophile (cisplatin) that has a high affinity for sulfur atoms survive the onslaught of the plethora of extra- and intracelluar platinophiles and reach the DNA? Studies with small model compounds demonstrate the clear kinetic preference of Pt(dien)Cl to the sulfur atoms of methionine and cysteine over the N7 of guanine and the subsequent transfer of the platinum moiety from the methionine thioether (but not from the cysteine thiolate) to the N7 of the guanine of ss and ds oligonucleotides.[7−9] The authors suggest that protein−Pt adducts may serve as a reservoir for DNA platination.[10] The relevance of this hypothesis to large biological systems such as proteins is not known.

It is important to understand the basic principles that govern the formation and reactivity of protein−Pt adducts since these adducts may be important in defining the therapeutic profile of the drug. We chose ubiquitin (Ub) as a model protein to study the formation and reactivity of its adducts with cisplatin. Ub is a small (MW = 8565 amu), tightly folded protein whose structure is well characterized by both X-ray crystallography and NMR.[11,12] It has two potential platinum binding sites: one *N*-terminal methionine (Met1) and one histidine (His68).[11] The Met1 sulfur is somewhat buried in the *N*-terminus of the protein, while His68 is exposed on the surface.

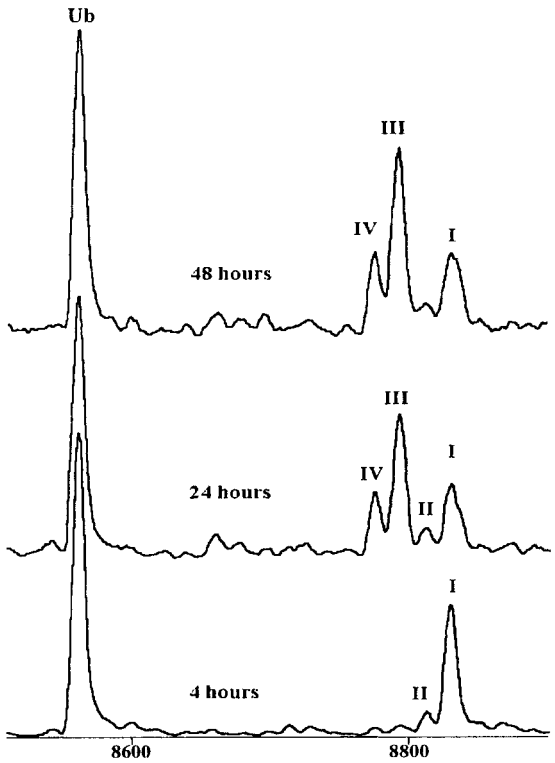

Figure 1. ESI-MS of a 1:1 reaction of cisplatin with native ubiquitin; the formation of four types of adducts is clearly visible: $Pt(NH_3)_2(Ub)Cl$ [I, 8830 amu], $Pt(NH_3)_2(Ub)(H_2O)$ [II, 8812 amu], $Pt(NH_3)_2(Ub)$ or $Pt(NH_3)(H_2O)Ub$ [III, 8792 amu], and $Pt(NH_3)(Ub)$ [IV, 8775 amu].

We have shown that Electrospray Ionization Mass Spectrometry (ESI-MS) is extremely useful in studying the interactions of cisplatin with proteins,[13] and provides direct information on the nature of the adducts that are formed. [^{1}H,^{15}N]-HSQC NMR spectroscopy has been utilized in the analysis of ^{15}N-labeled platinum ammine complexes providing information on the nature of the ligands that are trans to the labeled ammine.[14] The combination of the two techniques provides a powerful tool for simultaneously studying the modifications of the protein (ESI-MS) and the changes in the platinum coordination sphere (HSQC). In this study, we utilized both ESI-MS and [^{1}H,^{15}N]-HSQC NMR spectroscopy to determine the binding sites for cisplatin on ubiquitin, and the reactivity of the adducts toward some biological nucleophiles.

When a reaction of 1 mM cisplatin with 1 mM native ubiquitin (in 1 mM phosphate buffer, pH 6.4, at 37 °C) is monitored by ESI-MS, the formation of four types of adducts[13] is clearly visible (Figure 1). The masses of the adducts correspond to the monofuntional adducts $Pt(NH_3)_2(Ub)Cl$ [I, 8830 amu] and $Pt(NH_3)_2(Ub)(H_2O)$ [II, 8812 amu], the bifunctional adducts $Pt(NH_3)_2(Ub)$ or $Pt(NH_3)(H_2O)Ub$ [III, 8792 amu], and trifunctional adducts $Pt(NH_3)(Ub)$ [IV, 8775 amu]. The ESI-MS allows us to observe the interconversion of the mono- to the bi- and trifunctional adducts (Figure 1). It is noteworthy that contrary to expectations based on model compounds, the chloride ligand of the monofunctional adduct $Pt(NH_3)_2(Ub)Cl$ (MW = 8830 amu) does not undergo rapid aquation and the diamminemonochloro adduct is stable for weeks. This may be due to a protective effect by the protein.

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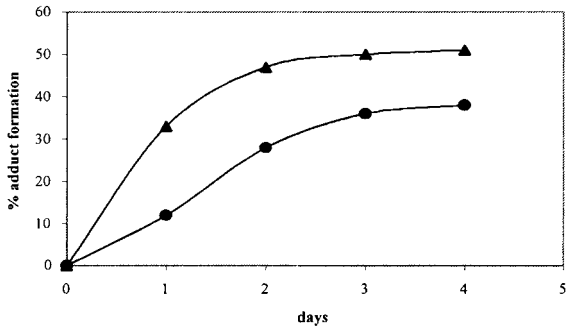

Figure 2. The percent of ubiquitin modified by cisplatin in the 1:1 reaction between ubiquitin and cisplatin (▲) and oxidized ubiquitin and cisplatin (●).

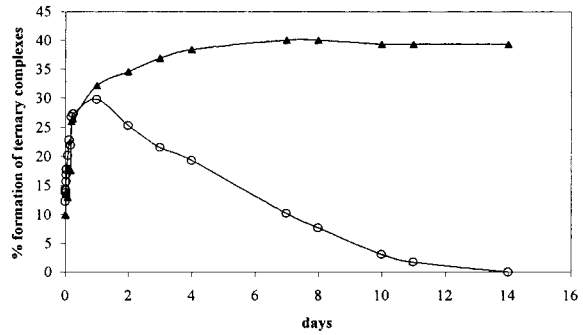

Figure 3. The formation/dissociation of the ternary complexes Ub-Pt-GSH (○) and Ub−Pt−(5′-GMP) (▲). The percent of the ternary complexes is relative to the total Ub−Pt adducts.

Pt has high affinity for sulfur and since the *N*-terminal Met1 can easily form bifunctional adducts with cisplatin in the form of an (N_α,S) chelate, and since the [^{1}H,^{15}N]-HSQC NMR data of this reaction reveal a cross-peak at −40.0/4.46 ppm [which is in the ammine trans to the sulfur region[14]], it seems reasonable to conclude that Met1 is the major binding site. To verify this conclusion and to try and distinguish Met1 binding from His68 binding, Met1 was prevented from binding Pt by selectively oxidizing the residue's thioether to a sulfone with performic acid.[15] The initial binding of cisplatin to the oxidized Ub is considerably slower (Figure 2) and after 1 day only 10% of the oxidized proteins were platinated compared to about 35% for the native Ub. After 4 days the adduct levels of the oxidized and native Ub reached 35% and 50%, respectively. The difference in the protein modification levels in the first day implies that most of the adducts that cisplatin forms with the native protein are due to cisplatin binding to Met1. Comparison of the aromatic regions of the ^{1}H NMR spectra of the native Ub and of the platinated Ub reveals that the His68 C_4H resonance in the native protein (δ 8.01 ppm)[16] moves in the platinated protein suggesting the involvement of His68 in Pt binding.

The main binding site of cisplatin on ubiquitin is Met1 and the secondary is His68 indicating that when possible, cisplatin prefers the thioether ligand to the imidazole group of histidine. The binding of cisplatin to Ub is slow (it takes 3 days for 50% of the Ub to be modified) relative to the binding of cisplatin to methionine (the reaction is complete in 18 h) and relative to the reported reaction of cisplatin with HSA (complete disappearance of cisplatin after 17 h).[17]

We assessed the reactivity of the cisplatin−ubiquitin adducts toward 5′-GMP and glutathione (GSH) by ultrafiltration of the Ub−cisplatin reaction mixture (55% of the Ub was platinated) to remove any excess cisplatin, and subsequent reaction with a 5-fold excess of either 5′-GMP or GSH under similar reaction conditions to those reported above. The reactions were monitored by ESI-MS. In the case of GSH, a ternary complex of the type $Pt(NH_3)$(Ub)(GSH) (MW = 9083 amu), whereby glutathione is bound to cisplatin in a monodentate fashion, was formed. The ternary complex was detected from the very early stages of the reaction and reached a peak after 1−2 days; subsequently the Pt−GSH moiety began to come off the protein. After about 13 days the native protein was completely recovered (Figure 3). The first Ub−Pt adducts to react are the $Pt(NH_3)_2$(Ub)(H_2O) [II, 8812 amu] followed by $Pt(NH_3)_2$(Ub)Cl [I, 8830 amu] and the bifunctional adducts [III, 8792 amu]. The ternary complex with GMP also formed rapidly but in contrast to the Ub−Pt−GSH cross-link, the Ub−Pt−GMP adduct is stable for weeks and there is no evidence for the transfer of the Pt moiety from the protein to GMP. The facile formation of the ternary adducts with both GSH and 5-GMP suggests that the steric bulk of the protein does not significantly hinder the binding of GSH and 5′-GMP to the Pt. The complete removal of the platinum from the protein by GSH but not by GMP may indicate that the thiolate ligand in GSH is able to *trans*-labilize the Pt moiety and discharge it from the protein.

Glutathione is present in cells at varying concentrations (0.5−10 mM) and is known to function as a detoxification agent of chemotherapeutic drugs. GSH was recently shown to have a protective effect against cisplatin-induced toxicity in animal models, and is also known to modulate cisplatin cell sensitivity.[10,18] Ternary complexes formed between the cisplatin−protein adducts and GSH may serve as intermediates in the discharge of free cisplatin from proteins back into the blood stream. In cells, there is a large excess of GSH relative to protein−Pt adducts (IC_{50} of cisplatin is $\sim 10^{-7}$ M) and therefore we used an excess of GSH for these studies. This coupled with the fact that GSH is extremely efficient in recovering the native protein from its platinum adducts makes it unlikely that the Pt−methionine adducts, though stable in their own right, act as a reservoir for the sustained release of the drug for the ultimate platination of cellular DNA.

In conclusion, cisplatin reacts with both Met1 and His68 of ubiquitin to form a variety of mono-, di-, and trifunctional 1:1 adducts. The monofunctional diamminemonochloro adducts are very stable to aquation. The rate of reaction of cisplatin with ubiquitin is slower than that reported for the reaction of cisplatin with HSA and with protected amino acids. The protein−Pt adducts are stable in the lack of nucleophiles but readily form long-lived cross-links with GMP. All the protein−Pt adducts are eventually removed by GSH via the rapid formation of protein−Pt−GSH intermediates.

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