APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2003; **17**: 675–681
Published online in Wiley InterScience (www.interscience.wiley.com). DOI:10.1002/aoc.502

Direct evidence for co-binding of cisplatin and cadmium to a native zinc- and cadmium-containing metallothionein

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Received 8 April 2003; Accepted 23 April 2003

Cisplatin is widely used to treat a number of cancers, and its covalent binding to DNA is believed to cause cell death; however, the roles of cisplatin–protein interactions in the mechanisms of action, toxicity, and resistance of the drug largely remain to be elucidated. Here, we investigate the interactions of cisplatin and a native rabbit metallothionein (MT), containing 1.4% zinc and 7.9% cadmium, using nanospray tandem quadrupole time-of-flight mass spectrometry (MS) and size-exclusion high-performance liquid chromatography with inductively coupled plasma MS. At near-neutral pH conditions, reactions between cisplatin and MT resulted in the formation of complexes that contained Cd_4–Pt_n–MT ($n = 1$–7). While zinc was displaced by cisplatin, both platinum and cadmium were bound to the same MT molecule. This is the first report to provide direct evidence for the co-binding of cadmium and platinum to MT, which suggests that the mechanism of the binding of cisplatin to the native MT may not be through the displacement of cadmium as previously proposed. A tandem MS investigation into the binding sites of the platinum and cadmium to MT showed platinum- and cadmium-related fragments, such as $(PtS_2C_2H_7N)^+$ and $(CdS_3C_5H_{17}N_2)^+$, demonstrating the platinum–cysteine and cadmium–cysteine binding. In addition, detection of Cd_4–Pt_7–MT demonstrated more than ten metals bound to a single MT molecule. This finding was extended to the binding of MT with a five-fold excess of $CdCl_2$. As many as 14 metal atoms (13 cadmium and one zinc) were detected bound to a single MT molecule, the complexes being Cd_x–Zn–MT ($x = 5$–13). The high binding capacity of MT for cadmium and platinum is consistent with the role of MT in reduction of metal toxicity and its involvement in drug resistance. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: cisplatin; metallothionein; co-binding; protein binding; nanoelectrospray ionization mass spectrometry; HPLC–ICPMS

INTRODUCTION

Cisplatin, *cis*-$[Pt(NH_3)_2Cl_2]$, is an antitumor agent widely used in the chemotherapeutic treatment of testicular, ovarian, head, neck, bladder, and lung cancers.[1] It is believed to induce apoptosis in cancer cells by covalently binding to the DNA.[2–6] However, the major limitation to the successful treatment of solid tumors with platinum-based chemotherapy is the drug resistance, which could be intrinsic or acquired.[1,2,7] The drug resistance may occur through multiple mechanisms, including reduced accumulation of the drug in tumor cells, inactivation of the drug, increased repair of DNA damage, and increased DNA damage tolerance. Although binding of cisplatin with thiol-containing molecules, such as glutathione (GSH) and metallothionein (MT), has been demonstrated, the roles of platinum–protein interactions in the mechanisms of action, toxicity, and resistance of the drug remain unclear.[1,7]

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Contract/grant sponsor: Natural Sciences and Engineering Research Council of Canada.
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Induction of MT by heavy metals has been suggested as one of the potential mechanisms of tumor cell resistance to cisplatin chemotherapy.[7–14] MT is a low-molecular-weight protein (6000–7000 Da) containing 20 cysteine residues in a total of 61 or 62 amino acids.[15] It is commonly believed that these 20 cysteine residues coordinate with seven metal atoms at a time, such as zinc, cadmium, and copper.[16–20] MT is thought to be an important intracellular storage site for zinc and potentially for other essential trace elements.[10,21,22] This protein is inducible by heavy metals, and in some cases it is preferentially induced in normal cells compared with cancer cells.[23–25] The preferential induction of MT in normal cells is considered to have a potential application in cancer chemotherapy by reducing the toxicity of cisplatin to normal cells while maintaining its efficacy against cancer cells. An understanding of cisplatin interaction with MT is crucial to such an application.

Several groups have studied the interactions of cisplatin and MT using *in vitro* and *in vivo* models.[16–20] These previous studies suggest that cisplatin displaces zinc and cadmium in the native MT, and seven platinum atoms were found to bind with a single MT. These studies were typically carried out using techniques such as atomic absorption spectrometry, chromatography, Fourier transform infrared spectrometry, X-ray photoelectron spectroscopy, and ^{1}H and ^{15}N NMR. Most of these techniques, such as size-exclusion chromatography and atomic absorption spectrometry, may not be able to detect low levels of complexes. The binding stoichiometry of 7 : 1 for cisplatin and MT obtained using these methods is an average number representing the most abundant complexes, and does not provide detailed information on what other species are present.

Nanospray (nESI) tandem mass spectrometry (MS/MS) offers high sensitivity and resolution. No separation is necessary to determine the species in a reaction mixture. Recently, electrospray MS has been used to study a number of metal-containing MTs,[26–32] including zinc–metallothionein,[26,27] cadmium–metallothionein,[26–28] copper–metallothionein[29,30] and mercury–metallothionein.[29] In the present study, we developed nESI tandem quadrupole time-of-flight (Q-TOF) MS and inductively coupled plasma (ICP) MS techniques to study metal–protein interactions. We investigated the interactions of cisplatin with a zinc- and cadmium-bound native rabbit MT. We report here the various stoichiometries of cisplatin–MT binding, including species Cd_4–Pt_n–MT ($n = 1$–7) and Cd_x–Zn–MT ($x = 5$–13) at near-neutral pH. These complexes show that a single MT molecule can bind up to 14 metal atoms. Moreover, we demonstrate the co-binding of platinum and cadmium with MT. Although cisplatin displaced zinc from MT, it did not displace cadmium. This study provides high-resolution molecular mass information and detailed speciation information on complexes that result from the interactions between cisplatin and MT under the conditions that mimic physiological conditions, and contributes to a better understanding of the mechanisms of toxicity and resistance of cisplatin in cancer treatment.

MATERIALS AND METHODS

Materials

A stock solution (4 mM) of cisplatin was prepared by dissolving an appropriate quantity of *cis*-diaminedichloroplatinum(II) (99.999%, Aldrich, Milwaukee, WI, USA) in water. Rabbit liver metallothionein II (MT, 6126.3 Da) was purchased from Sigma (St Louis, MO, USA). This MT consists of 62 amino acids with an acetylated N-terminus. The specified content of zinc was 1.4% and cadmium was 7.9%. The purity of MT was examined using sodium dodecylsulfate–polyacrylamide gel electrophoresis, and a single band was detected when an amount of 10 to 0.01 μg of the protein was loaded on the gel. The purity of MT was confirmed by nESI-MS and the molecular weight of the apo-MT was determined to be 6126.26 Da, which is consistent with the value reported in the literature.[31,33] The apo-MT was obtained by incubating the native zinc- and cadmium-containing MT (Sigma, St Louis, MO, USA) with 1% formic acid for 4 h to remove the metals.

Stock solution (1 mM) was prepared by dissolving 6.1 mg of MT in 1 ml of water. Stock solution (5 mM) of cadmium chloride was prepared by dissolving an appropriate quantity of $CdCl_2$ (99.5%, BDH Chemicals, Mississauga, ON, Canada) in water. All the stock solutions were kept at 4 °C in the dark. High-performance liquid chromatography (HPLC)-grade formic acid, water and methanol (Fisher Scientific, Fair Lawn, NJ, USA) were used throughout the experiments.

Test solutions

The test solutions were prepared to contain a constant concentration (10 μM) of MT and varying concentrations of cisplatin to give [cisplatin]/[MT] molar ratios of 3 : 1 and 20 : 1. For accurate mass analysis, the test solutions were prepared in 20% methanol and 80% water. The reaction mixtures were sampled at various time intervals and acidified by adding formic acid immediately before nESI-MS analysis. These samples contained 0.002% of formic acid to provide pH 6. To detect the maximum number of cadmium binding to MT, fivefold excess of $CdCl_2$ was incubated with MT in water for 4 h at room temperature. The reaction mixture was analyzed using nESI-MS at pH 6. For HPLC–ICP-MS experiments, all the test solutions containing cisplatin and MT were prepared in HPLC-grade deionized water; no methanol or formic acid was added.

Centrifugal filtration for separation of platinum–MT complexes from free cisplatin

Microcon YM-3 centrifugal filters (membrane NMWL 3000; Millipore Corporation, Nepean, ON, Canada) were used for spinning the reaction mixtures for 100 min at 14 000 *g* to remove excess unbound cisplatin and other small molecules. The cisplatin–MT complexes were collected and analyzed to confirm that complexes were present in the solution.

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nESI-MS

MS experiments were performed on an Applied Biosystem/MDS Sciex QSTAR Pulsar i mass spectrometer (Concord, ON, Canada), equipped with a nanospray ionization source. The mass spectrometer was operated in the positive ion mode. Analyte solutions were introduced into the ion source by nanoelectrospray capillaries (NanoES, Protana, Denmark). Analyst QS software (Applied Biosystems, Foster City, CA, USA) was used for the spectrum acquisition and data analysis. Igor Pro software (WaveMetrics, Lake Oswego, OR, USA) was used to plot the spectra.

In the single MS scan mode, the mass measurements (900–2000 amu) were performed using the time-of-flight (TOF) section of the instrument with a resolution of 10 000 (FWHM) at m/z 850 and a 4 ppm mass accuracy using internal standard, while Q1 and Q2 were operating in RF-only mode. Mass spectra were acquired with an electrospray voltage of 1100 V, first declustering potential (DP1) of 65 V, second declustering potential (DP2) of 15 V, and focusing potential (FP) of 215 V. The MS instrument was calibrated daily with two standards: reserpine (M_w 608.7 Da; Applied Biosystems/PE Sciex, Foster City, CA, USA) for low mass range, and apo-MT (M_w 6126.3 Da) for high mass range.

For the nESI-MS experiments, all the test solutions containing cisplatin and MT were analyzed at pH 6. At this pH, the spectrum of the native MT (without cisplatin) contained Cd_4–MT as the major ion for multiple charge states (6+, 5+ and 4+). Therefore, the masses of the native MT–cisplatin complexes (in Table 1) were measured by externally calibrating the corresponding spectrum with respect to the masses of Cd_4–MT species at 6+ (m/z 1097.05) and 5+ (m/z 1316.46) charge states.

In the MS/MS mode, the parent ion was selected by Q1 with a mass window of 1 Da at low resolution, and fragmented in Q2 by collision-induced dissociation with a collisional energy of 35 eV and collision gas setting of 7. The resulting product ions were analyzed by the TOF analyzer. In assigning these product ions, the theoretical fragmentation pattern of MT was generated for comparison using the MS-product tool in Protein Prospector (http://prospector.ucsf.edu).

HPLC–ICP-MS

A Perkin–Elmer 200 series HPLC system (PE Instruments, Shelton, CT, USA) was used. A BioSep-SEC 2000 size-exclusion column (300 × 4.6 mm, Phenomenex, Torrance, CA, USA) was used for separation of the MT-bound from the unbound cisplatin. The chromatographic separation was carried out using isocratic elution with a mobile phase of 5 mM sodium phosphate (pH 7.0) and a flow rate of 0.8 ml min^{-1}. The injection volume was 20 µl. The effluent coming from HPLC was directly detected using an Elan 6100 DRC ^{plus}ICP-MS (PE Sciex, Concord, ON, Canada), with Turbochrom Workstation v.6.1.2 software (PE Instruments, Shelton, CT, USA) for data processing. The operating parameters of ICP-MS were optimized to be RF power 1150 W, plasma gas flow 15 L min^{-1}, auxiliary gas flow 1.2 l min^{-1}, and nebulizer gas flow 0.9 l min^{-1}. Pt, Cd, and Zn were detected using the peak-hopping mode of the ICP-MS at m/z of 195, 114, and 64 respectively.

RESULTS AND DISCUSSION

Characterization of the cisplatin–MT complexes using nESI-MS

Figure 1a presents a typical nanospray mass spectrum of cisplatin–MT complexes formed in a reaction mixture of aqueous cisplatin and native rabbit liver MT (3 : 1) at pH 6. A number of complexes were detected as multiply charged 5+ and 6+ ions, and their accurate mass measurements compared reasonably with the expected mass. The major complexes of cisplatin with MT were identified as Cd_4–Pt_3–MT, Cd_4–Pt_4–MT, Cd_4–Pt_5–MT, and Cd_4–Pt_6–MT, for peaks 3 to 6 respectively, and their sodium adducts Cd_4–Pt_3–MT–Na_4, Cd_4–Pt_4–MT–Na_4, and

Table 1. Expected and experimental molecular masses for adducts (Fig. 2) formed in cisplatin–MT (20 : 1) aqueous mixture by nESI-MS analysis at pH 6

Peak in Fig. 2	MT–Pt adduct identified	Expected molecular mass (Da)	Experimental value: 6+ charge state molecular mass (Da)	Experimental value: 6+ charge state (m/z)	Experimental value: Mass accuracy (ppm)
4	Cd_4–Pt_4–MT	7361.70	7362.18	1227.03	65.20
5	Cd_4–Pt_5–MT	7556.64	7557.12	1259.52	63.52
6	Cd_4–Pt_6–MT	7751.58	7751.16	1291.86	−54.18
7	Cd_4–Pt_7–MT	7946.52	7946.04	1324.34	−60.40
4′	Cd_4–Pt_4–MT–Na_4	7453.73	7454.10	1242.35	49.90
5′	Cd_4–Pt_5–MT–Na_4	7648.67	7648.56	1274.76	−14.12
6′	Cd_4–Pt_6–MT–Na_4	7843.61	7843.81	1307.30	25.70

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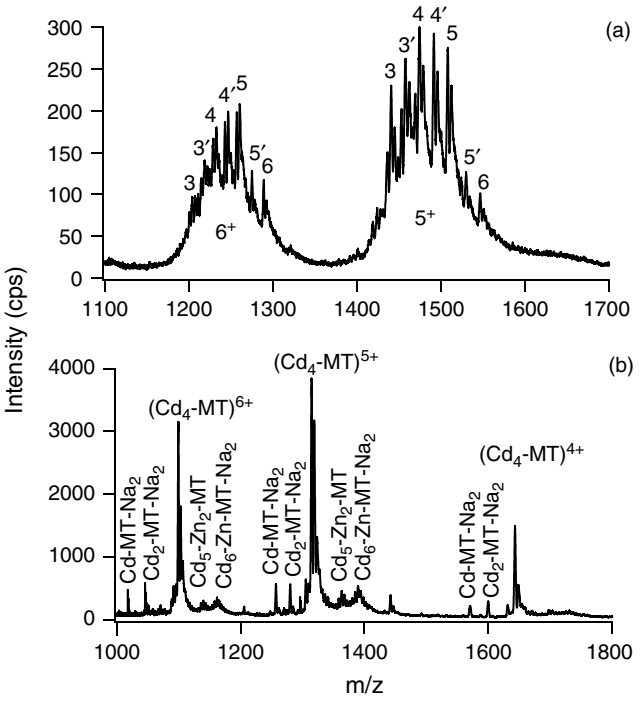

Figure 1. Typical nESI-MS spectra of (a) a mixture containing cisplatin and MT (3 : 1) and (b) the native zinc- and cadmium-containing rabbit MT. Cisplatin (30 μM) and MT (10 μM) prepared in deionized water with 20% methanol was incubated at room temperature for 5 h. The solution was acidified with diluted formic acid to pH 6 immediately prior to nESI-MS analysis. Peak identities are: 3, Cd_4-Pt_3-MT; 3′, $Cd_4-Pt_3-MT-Na_4$; 4, Cd_4-Pt_4-MT; 4′, $Cd_4-Pt_4-MT-Na_4$; 5, Cd_4-Pt_5-MT; 5′, $Cd_4-Pt_5-MT-Na_4$; 6, Cd_4-Pt_6-MT.

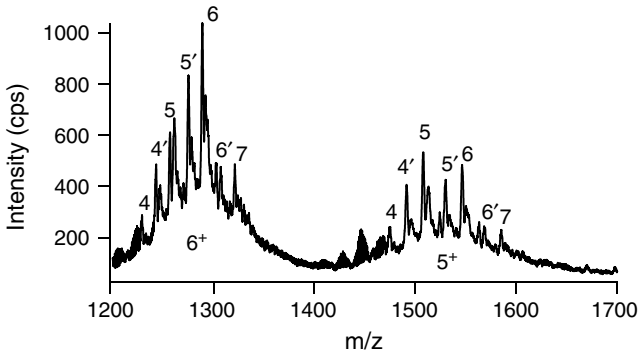

Figure 2. An nESI-MS spectrum of a mixture containing 200 μM of cisplatin and 10 μM of MT. Cisplatin (200 μM) and MT (10 μM) prepared in deionized water with 20% methanol was incubated at room temperature for 7 h. The solution was acidified with diluted formic acid to pH 6 immediately prior to nESI-MS analysis. Peak identities are: 4, Cd_4-Pt_4-MT; 4′, $Cd_4-Pt_4-MT-Na_4$; 5, Cd_4-Pt_5-MT; 5′, $Cd_4-Pt_5-MT-Na_4$; 6, Cd_4-Pt_6-MT; 6′, $Cd_4-Pt_6-MT-Na_4$; 7, Cd_4-Pt_7-MT.

$Cd_4-Pt_5-MT-Na_4$, for peaks 3′ to 5′ respectively. These complexes contained four cadmium atoms and three to six platinum atoms, all bound to the same MT molecule. The native MT without cisplatin was also analyzed using nESI-MS under the same conditions, and its mass spectrum consisted of $(Cd_4-MT)^{6+}$, $(Cd_4-MT)^{5+}$, and $(Cd_4-MT)^{4+}$ as the major ions (Fig. 1b). Other ions present at lower intensities include: $(Cd-MT-Na_2)^{6+}$, $(Cd_2-MT-Na_2)^{6+}$, $(Cd_5-Zn_2-MT)^{6+}$, and $(Cd_6-Zn-MT-Na_2)^{6+}$; $(Cd-MT-Na_2)^{5+}$, $(Cd_2-MT-Na_2)^{5+}$, $(Cd_5-Zn_2-MT)^{5+}$, and $(Cd_6-Zn-MT-Na_2)^{5+}$; and $(Cd-MT-Na_2)^{4+}$ and $(Cd_2-MT-Na_2)^{4+}$. Whether with or without cisplatin, cadmium was bound to MT. These results suggest that cisplatin binding to MT did not lead to the displacement of cadmium. This finding is unique, because the binding of cisplatin to MT was previously thought to take place through the displacement of cadmium.[16–20]

In order to confirm the co-binding of both platinum and cadmium to the same MT (instead of displacement), we further examined reaction mixtures containing a range of cisplatin and MT concentrations. With a cisplatin concentration as high as 20-fold in excess of the MT concentration, we did not observe displacement of cadmium by platinum. Four cadmium atoms remained bound to MT along with the additional four to seven platinum atoms (Fig. 2). Table 1 summarizes the expected and observed masses for MT complexes that were assigned in Fig. 2. The reasonable agreement between the measured and expected masses supports the identification of the MT complexes with platinum and cadmium.

MS/MS analysis of the MT complexes further demonstrated the presence of both platinum–cysteine and cadmium–cysteine fragments, suggesting the binding of both platinum and cadmium to the same MT molecule. Figure 3a shows a typical MS/MS spectrum of Cd_4-Pt_5-MT complex. $(Cd_4-Pt_5-MT)^{6+}$ ion at m/z of 1259.52 was fragmented in the collision cell, resulting in $PtS_2C_2H_7N$, and $CdS_3C_5H_{17}N_2$ ions along with the immonium ions of several amino acids, a_1 $(CH_3CO-C_4SH_9N)^+$ and b_1 $(CH_3CO-C_5SH_9NO)^+$ ions from N-terminal (acetyl), and other fragment ions of MT. The isotopic patterns of the fragments of $PtS_2C_2H_7N$ and $CdS_3C_5H_{17}N_2$, as shown in Fig. 3b, are consistent with those expected for the presence of platinum (32.9% ^{194}Pt, 33.8% ^{195}Pt, 25.3% ^{196}Pt, and 7.2% ^{198}Pt) and cadmium (12.4% ^{110}Cd, 12.8% ^{111}Cd, 24.1% ^{112}Cd, 12.2% ^{113}Cd, 28.7% ^{114}Cd, and 7.5% ^{116}Cd). These results demonstrate the binding of platinum–cysteine and cadmium–cysteine to the same MT molecule.

To confirm that the Cd_4-Pt_n-MT complexes were present in the solution and not formed during the n-ESI process, we separated the MT complexes from the unbound cisplatin in the solution prior to nESI-MS analysis. The nESI-MS analysis of the MT fraction gave a similar mass spectrum to that shown in Fig. 2, demonstrating the presence of Cd_4-Pt_3-MT, Cd_4-Pt_4-MT, Cd_4-Pt_5-MT, Cd_4-Pt_6-MT, and Cd_4-Pt_7-MT species in the solution.

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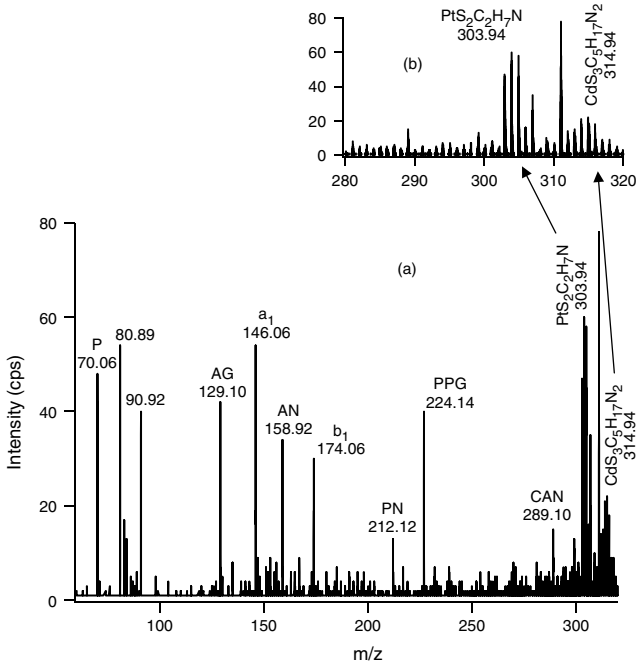

Figure 3. nESI-MS/MS spectra of Cd_4-Pt_5-MT at low mass range (50–350 Da) showing the binding of platinum and cadmium to cysteine. The ion at *m/z* of 1259.52 with 6+ charge state was fragmented in the collision cell, resulting in $PtS_2C_2H_7N$, and $CdS_3C_5H_{17}N_2$ ions along with the immonium ions of several amino acids, a_1 $(CH_3CO-C_4SH_9N)^+$ and b_1 $(CH_3CO-C_5SH_9NO)^+$ ions from N-terminal (acetyl), and the internal fragment b ions (P, AG, AN, PN, PPG, and CAN) of MT. The inset is an expanded region showing the isotopic patterns for the platinum–cysteine and cadmium–cysteine species.

HPLC–ICP-MS evidence for the co-binding of cadmium and cisplatin to MT

Other supporting evidence for the co-binding of platinum and cadmium to MT was obtained from size-exclusion HPLC separation of the MT-bound and unbound metal species followed by ICP-MS detection of the specific metals. Figure 4 shows chromatograms from the simultaneous monitoring of ^{195}Pt, ^{64}Zn, and ^{114}Cd in native MT (Fig. 4a), and MT with three fold (Fig. 4b) and 20-fold (Fig. 4c) excess of cisplatin. As expected, both zinc and cadmium were present in the native MT (Fig. 4a). In the solution containing 3 μM cisplatin and 1 μM MT, 13% of the cisplatin was bound to the MT (Fig. 4b), and both zinc and cadmium also remained bound to the MT (Fig. 4b). The size-exclusion HPLC allowed the separation of the MT-bound platinum (2.5–4.5 min) from the unbound cisplatin (5–6 min). The presence of platinum, zinc, and cadmium in the same MT molecule is evident from the simultaneous monitoring of these three metals in the same chromatographic separation. Increase of cisplatin content to 20 μM (Fig. 4c) resulted in a larger fraction of MT-bound platinum (40%), and displacement of zinc but not of cadmium. Importantly, both platinum and cadmium were present in the MT-bound fraction (2.5–4.5 min). These experiments were conducted in aqueous solutions at neutral pH. The presence of both platinum and cadmium in the MT further supports the co-binding of platinum and cadmium with MT.

The results of both nESI-MS (Figs 1–3) and HPLC–ICP-MS (Fig. 4) investigations have clearly demonstrated that the mechanism of cisplatin binding to the native MT does not occur through the displacement of cadmium, as previously thought.

Binding stoichiometry of cadmium and platinum to MT

The second line of our investigation examined the binding stoichiometry of MT with cisplatin and cadmium. Previous studies[16–20] reported that 20 cysteine residues in MT could bind with seven to ten heavy metal ions, such as Cu^{2+}, Zn^{2+}, and Cd^{2+}. Surprisingly, we consistently observed more than ten metal atoms (platinum and cadmium combined) binding to a single MT when excess amounts of cisplatin or cadmium were present. Cd_4-Pt_7-MT (Fig. 2, peak 7) was clearly detected when cisplatin reacted with MT at a molar ratio of 20 : 1.

We further examined the binding of the zinc- and cadmium-containing MT with five fold excess of $CdCl_2$ after incubating for 4 h at neutral pH (Fig. 5). The nESI-MS analysis (with accurate mass and MS/MS) of the reaction mixture at pH 6 shows the presence of sodium adducts of $Cd_7-Zn-MT$ (peak 7), $Cd_8-Zn-MT$ (peak 8), $Cd_9-Zn-MT$ (peak 9), $Cd_{10}-Zn-MT$ (peak 10), $Cd_{11}-Zn-MT$ (peak 11), $Cd_{12}-Zn-MT$ (peak 12), and $Cd_{13}-Zn-MT$ (peak 13). These species were detected at three charge states: 6+, 5+, and 4+. As many as 14 metal atoms (13 cadmium and one zinc) were detected as bound to a single MT molecule. Palumaa and co-workers[34,35] previously reported the formation of a monomeric MT form containing 13 cadmium ions and inorganic phosphate ($Cd_{13}-(P_i)_2-MT$). Our results are consistent with these findings, suggesting a great degree of structural adaptability of MT in response to various conditions, such as different protein and metal concentrations. The 20 cysteine residues in a single MT molecule have been considered to provide binding sites for up to ten divalent metal atoms. The observation of more than ten platinum and cadmium atoms bound to a single MT molecule suggests that cadmium and platinum may bind to MT through other amino acids in addition to cysteine. The rabbit MT contains aspartic acid, threonine, tyrosine, and serine, which contain carboxyl and hydroxyl groups for metal binding. Palumaa and co-workers[34,35] reported the participation of oxygen and/or nitrogen ligands besides thiolates in the formation of $Cd_{13}-(P_i)_2-MT$. Recent studies on the interactions of cisplatin with ubiquitin demonstrated that cisplatin could bind to ubiquitin at the sites of methionine and histidine.[36–38] Another study on the binding of cisplatin to human serum transferrin indicated that the hydroxyl functional group of threonine was the binding site.[39] Our

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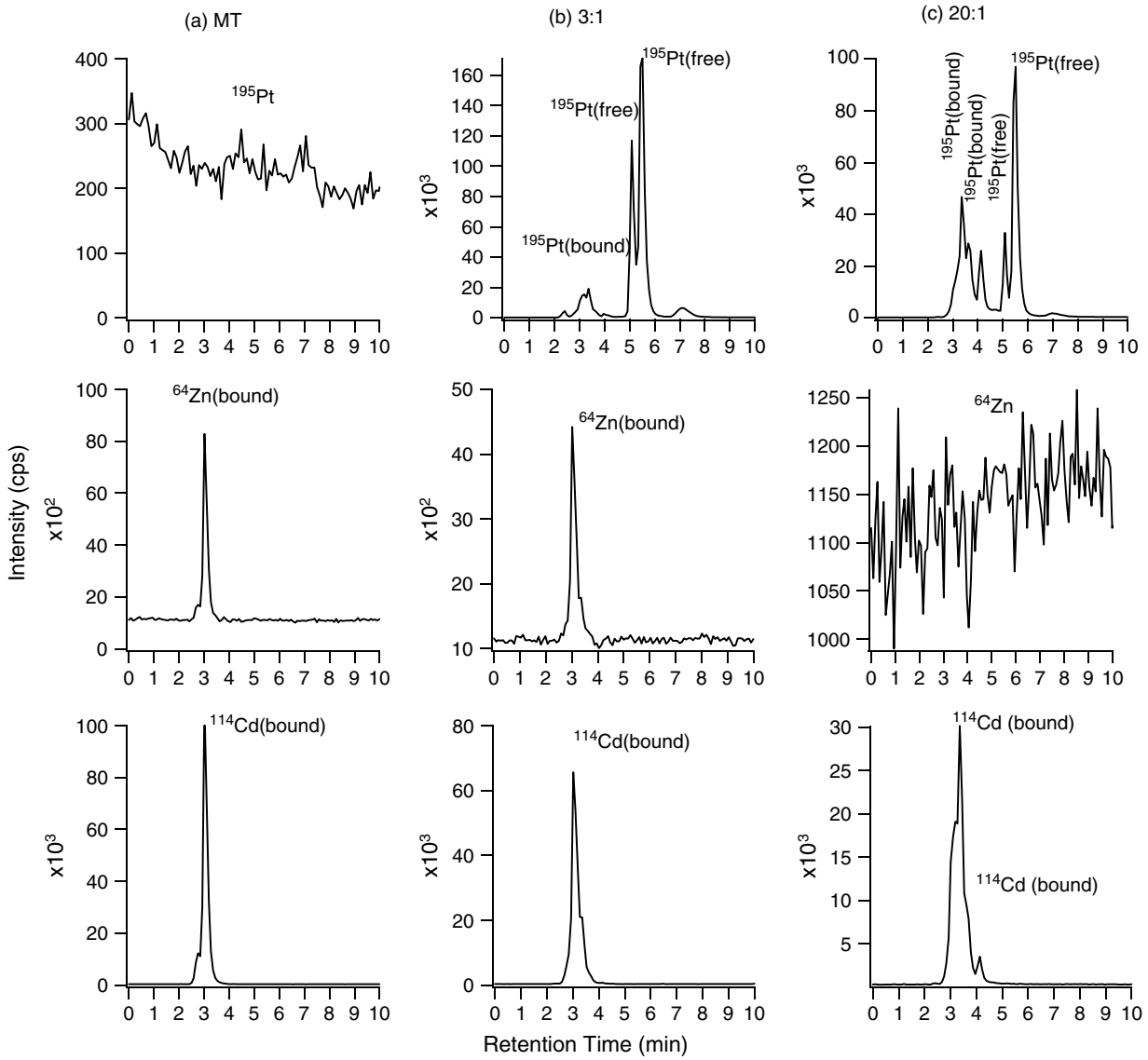

Figure 4. On-line size-exclusion HPLC separation with ICP-MS (HPLC–ICP-MS) detection of platinum, zinc, and cadmium in MT and MT–platinum complexes: (a) 1 μM MT in deionized water; (b) neutral solution containing 1 μM of MT and 3 μM of cisplatin in deionized water; (c) neutral solution containing 1 μM of MT and 20 μM of cisplatin in deionized water. A size-exclusion HPLC column (300 × 4.6 mm, Phenomenex) was used to separate the MT-bound and unbound metal species. The HPLC effluent was introduced directly to an ICP mass spectrometer that was used simultaneously to detect platinum (195), zinc (64), and cadmium (114). Traces in the top row represent chromatograms for platinum-containing species; the middle row is for zinc-containing species; the bottom row is for cadmium-containing species.

findings are consistent with these recent studies, suggesting that cisplatin can bind with cysteine and several other amino acids in proteins.

CONCLUSIONS

We have demonstrated that the combination of size-exclusion HPLC–ICP-MS and nESI-MS provides new information on the adduct formation and potential reaction mechanism of cisplatin with MT. The size-exclusion HPLC with ICP-MS enables the monitoring of cisplatin–MT binding under physiological conditions. nESI-MS/MS offers information on the formation of specific complexes and the characteristics of the binding. This information is useful for a better understanding of drug toxicity and treatment resistance. Previous studies[8,14] have shown that several cancer cell lines, such as SCC-25 and A-253 human head and neck carcinoma, G3361 human melanoma, SW2 human small-cell carcinoma, SL6 human large-cell carcinoma, and L1210

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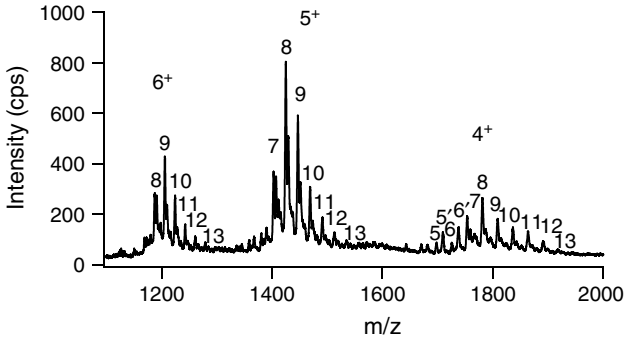

Figure 5. An nESI-MS spectrum of a mixture containing $CdCl_2$ and the zinc- and cadmium-containing native MT. A mixture of $CdCl_2$ (50 μM) and MT (10 μM) prepared in deionized water with 20% methanol was incubated at room temperature for 4 h. The solution was acidified with diluted formic acid to pH 6 immediately prior to nESI-MS analysis. Peak identities 5–13 are Cd_x–Zn–MT–Na (x = 5 to 13 respectively), 5′ is Cd_5–MT–Na and 6′ is Cd_6–MT–Na. All experimental conditions are the same as for Fig. 2.

murine lymphocytic leukemia, with elevated MT levels were resistant to cisplatin treatment; these MT-induced cells showed an increased survival rate compared with cells that contained normal levels of MT. The high binding capacity of MT with cisplatin may contribute to such resistance by reducing the effective dose of the free cisplatin in the cells. Furthermore, the binding of MT to cisplatin could be applied to the reduction of toxicity of the antitumor drug to normal cells. Several studies[40–43] have shown that the MT levels in the tumor and normal cells could be preferentially induced. Taking advantage of the strong binding of MT with cisplatin, one strategy would be to induce the MT levels in normal cells and control the MT levels in tumor cells.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and Alberta Health and Wellness. The authors thank Dr Z. Gong, Ms X. Lu, and Ms R. Kalke for their technical assistance and constructive discussions.

REFERENCES

1. Johnson SW, Stevenson JP, O'Dwyer PJ. In *Cancer: Principles, Practice of Oncology*, 6th edition, DeVita Jr VT, Hellman S, Rosenberg SA (eds). Lippincott Williams & Wilkins: Philadelphia, 2001; 376–388.
2. Chu G. *J. Biol. Chem.* 1994; **269**: 787.
3. Jamieson ER, Lippard SJ. *Chem. Rev.* 1999; **99**: 2467.
4. Ohndorf U-M, Rould MA, He Q, Pabo CO, Lippard SJ. *Nature* 1999; **399**: 708.
5. Marchan V, Moreno V, Pedroso E, Grandas A. *Chem. Eur. J.* 2001; **7**: 808.
6. Wong B, Mass JE, Yen Y-M, Giannikoupolous P, Feigon J, Johnson RC. *Biochemistry* 2002; **41**: 5404.
7. Chao CC. *J. Formos. Med. Assoc.* 1996; **95**: 893.
8. Kelley SL, Basu A, Teicher BA, Hacker MP, Hamer DH, Lazo JS. *Science* 1998; **241**: 1813.
9. Andrews PA, Murphy MP, Howell SB. *Cancer Chemother. Pharmacol.* 1987; **19**: 149.
10. Koropatnick J, Pearson J. *Somat. Cell Mol. Genet.* 1990; **16**: 529.
11. Kondo Y, Satoh M, Imura N, Akimoto M. *Anticancer Res.* 1992; **12**: 2303.
12. Kasahara K, Fujiwara Y, Nishio K, Ohmori T, Sugimoto Y, Komiya K, Matsuda T, Saijo N. *Cancer Res.* 1991; **51**: 3237.
13. Farnworth P, Hillcoat B, Roos I. *Cancer Chemother. Pharmacol.* 1990; **25**: 411.
14. Bakka A, Endresen L, Johnsen ABS, Edminson PD, Rugstad HE. *Toxicol. Appl. Pharmacol.* 1981; **61**: 215.
15. Nordberg M. *Talanta* 1998; **46**: 243.
16. Pattanaik A, Bachowski G, Laib J, Lemkuil D, Shaw CF, Petering DH, Hitchcock A, Saryan L. *J. Biol. Chem.* 1992; **267**: 16 121.
17. Lemkuil DC, Nettesheim D, Shaw CF, Petering DH. *J. Biol. Chem.* 1994; **269**: 24 792.
18. Zhang B, Huang H, Tang W. *J. Inorg. Biochem.* 1995; **58**: 1.
19. Zhang B, Tang W, Gao S, Zhou Y. *J. Inorg. Biochem.* 1995; **58**: 9.
20. Xing B, Zhu H, Shi Y, Tang W. *Biometals* 2001; **14**: 51.
21. Reeves PG, Saari JT. *J. Nutr. Biochem.* 1990; **1**: 374.
22. Heilmaier HE, Drasch GA, Kretschmer E, Summer KH. *Toxicol. Lett.* 1987; **38**: 205.
23. Bauman JW, Liu J, Liu YP, Klaassen CD. *Toxicol. Appl. Pharmacol.* 1991; **110**: 347.
24. Cherian MG, Howell SB, Imura N, Klaassen CD, Koropatnick J, Lazo JS, Waalkes MP. *Toxicol. Appl. Pharmacol.* 1994; **126**: 1.
25. Satoh M, Naganuma A, Imura N. *Cancer Chemother. Pharmacol.* 1988; **21**: 176.
26. Hathout Y, Reynolds KJ, Szilagyi Z, Fenselau C. *J. Inorg. Biochem.* 2002; **88**: 119.
27. Gehrig PM, You C, Dallinger R, Gruber C, Brouwer M, Kagi JHR, Hunziker PE. *Protein Sci.* 2000; **9**: 395.
28. Chassaige H, Lobinski R. *Fresenius J. Anal. Chem.* 1998; **361**: 267.
29. Stillman MJ, Thomas D, Trevithick C, Guo X, Siu M. *J. Inorg. Biochem.* 2000; **79**: 11.
30. Merrifield ME, Huang Z, Kille P, Stillman MJ. *J. Inorg. Biochem.* 2002; **88**: 153.
31. Yu X, Wojciechowski M, Fenselau C. *Anal. Chem.* 1993; **65**: 1355.
32. Nischwitz V, Michalke B, Kettrup A. *Anal. Bioanal. Chem.* 2003; **375**: 145.
33. URL: http://us.expasy.org/sprot/ March, 2003.
34. Palumaa P, Zerbe O, Vasak M. *Biochemistry* 1993; **32**: 2874.
35. Palumaa P, Mackay EA, Vasak M. *Biochemistry* 1992; **31**: 2181.
36. Peleg-Shulman T, Gibson D. *J. Am. Chem. Soc.* 2001; **123**: 3171.
37. Gibson D, Costello CE. *Eur. J. Mass Spectrom.* 1999; **5**: 501.
38. Najajreh Y, Peleg-Shulman T, Moshel O, Farrell N, Gibson D. *J. Biol. Inorg. Chem.* 2003; **8**: 167.
39. Allardyce CS, Dyson PJ, Coffey J, Johnson N. *Rapid Commun. Mass Spectrom.* 2002; **16**: 933.
40. Satoh M, Kondo Y, Mita M, Nakagawa I, Naganuma A, Imura N. *Cancer Res.* 1993; **53**: 4767.
41. Jin R, Bay BH, Chow VT, Tan PH, Dheen T. *Cell Tissue Res.* 2001; **303**: 221.
42. Ebara M, Fukuda H, Hatano R, Saisho H, Nagato Y, Suzuki K, Nakajima K, Yukawa M, Kondo F, Nakayama A, Sakurai H. *J. Hepatol.* 2000; **33**: 415.
43. Janssen AM, van Duijin W, Oostendorp-Van De, Ruit MM, Kruidenier L, Bosman CB, Griffioen G, Lamers CB, van Krieken JH, van De Velde CJ, Verspaget HW. *J. Pathol.* 2000; **192**: 293.

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