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In vitro binding of an orally active platinum antitumor drug, JM216 to metallothionein

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Abstract

The *in vitro* binding of an orally active anticancer drug JM216 to metallothionein is firstly investigated in this paper. It is revealed that a redox reaction following a substitution reaction from JM216 with rabbit liver Zn_7MT-II is presented. The reaction feature, the metal binding stoichiometry and the oxidation states of platinum and sulfur in the products are studied by UV-visible, chromatography and X-ray photoelectron spectroscopy methods. Parts of MT are oxidized to precipitate products with intra and intermolecular CyS-SCy disulfides linkages. Pt(IV) is reduced to its Pt(II) counterpart. And the reduced Pt(II) replace the metal ions in native MTs. Meanwhile it can also cause the dimerization of MT. Increasing the reaction ratio of JM216 to MT leads to a concomitant increase in the apparent yield of the precipitate and dimeric products and the elevation of the binding stoichiometry of Pt to the protein. Based on the experimental data, the reaction mechanism between JM216 and Zn_7MT-II *in vitro* are discussed.

Introduction

Metallothioneins (MTs) are low-molecule-weight, cysteine-rich and metal-binding proteins found in a variety of mammalian, invertebrates, birds, fish and microorganisms (Hamer 1986; Sadler 1991; Palumaa *et al.* 1993) Mammalian MTs are composed of a string of 61 or 62 amino acids, 20 of which are conserved cysteine residues. Such a large amount of sulhydryl groups in this protein contributes a great deal to its high affinity for various heavy metal cations and electrophilic agents including platinum complexes (Templeton *et al.* 1991; Chen *et al.* 1996; Nielson *et al.* 1985).

Platinum complexes including cisplatin and carboplatin are widely used in treating a number of human cancers (Mellish *et al.* 1993; Loehrer *et al.* 1984). Many studies have been made to investigate the reactions of Pt(II) complexes, especially cis-dichlorodiammine platinum (II) (CDDP) with MTs *in vivo* and *in vitro* (Zelazowski *et al.* 1984; Pattanaik *et al.* 1992; Bongers *et al.* 1988, 1991; Zhang *et al.* 1994, 1995). It appears that Pt-MT can be formed when Pt(II) complexes react with Zn_7MT, Cd-MT or apoMT *in vitro*, in which Pt(II) has tetrathiolate coordination (Pattanaik *et al.* 1992; Lemkuil *et al.* 1994). Bongers reported that when the native MT reacted with K_2PtCl_4, about half of the starting material was recovered as monomeric products and half as oligomeric products (Bongers *et al.* 1991). Comparing with the details of the reactions of the Pt(II) complexes with MTs, limited information exists with respect to the interactions between Pt(IV) complexes and MTs *in vivo* and *in vitro*. Nielson reported that Pt(IV) ion could give a positive indication of binding to metallothionein, but the stoichiometry was unclear (Nielson *et al.* 1985). Our experimental groups have reported the *in vivo* and *in vitro* reaction of native MTs with K_2PtCl_6 and Iproplatin (Zhong *et al.* 1997; Zhong *et al.* 1997). A few Pt(IV) complexes have been considered to extend the application of platinum chemotherapy. For example, JM216{*trans*-bis-

acetato-*cis*-dichloroammine (cyclohexylamine) platinum (IV)}, a new orally active platinum antitumor drug, has currently entered phase II clinical trials in the USA, Japan and Europe (Mckeage *et al.* 1995). We have investigated the properties of JM216 to induce the biosynthesis of metallothionein *in vivo* by oral administrations and injections s.c. (Zhong *et al.* 1997). In order to obtain more information on the reaction properties of metallothionein with JM216, the orally active platinum antitumor drug, JM216 was synthesized and the *in vitro* binding of JM216 to MT was studied in this paper. Based on the experiment data, the mechanism of the reactions between JM216 and Zn_7MT-II are discussed.

Experimental

Chemicals

JM216 was synthesized from K_2PtCl_4 by multi-step procedures as previously described (Talman *et al.* 1997; Giandomenico *et al.* 1995), and determined by element analysis (wt%), found: C 24.27, H 4.72, N 5.42 (Calc.$PtC_{10}H_{22}Cl_2\ N_2O_4$: C 24.01, H 4.43, N 5.60). Sephadex G-50 and G-25 were purchased from Pharmacia, DEAE-Cellulose DE-52 from Whatman, Tris base and standard proteins: bovine superoxide dismutase (MW 33, 000 Da), cytochrome C (MW 14,000 Da) and native rabbit liver Zn_7MT (MW 6500 Da) from Sigma. All chemicals were reagent grade or better and deionized water was used.

Preparation and characterization of MT

Rabbit liver Zn_7MT-II was isolated and purified according to the literature reported (Comeau *et al.* 1992), and was checked by measuring the contents of sulfur and metal ions by inductively coupled plasma (ICP) spectrometric determination using an emission line of S (181.978 nm), Pt (224.552 nm) and Zn (213.856 nm) performed on a JOBIN YVON JY38S ICP spectrometer according the method reported in previous literature (Bongers *et al.* 1988). The concentration of the protein was determined by the absorbance at 220 nm of apoMT at pH 2 ($\varepsilon = 47,300\ M^{-1}\ cm^{-1}$) (Buhler *et al.* 1974).

UV-Visible spectrum assay

Zn_7MT-II and stock solutions of JM216 in water were used for all studies. The reactions occurred in 0.01 M potassium phosphate solution, pH 7.40. All solutions were degassed on a vacuum line before using and all experimental procedures were performed in a nitrogen atmosphere.

A series of deaerated native Zn_7MT-II solution containing different molar equivalents of JM216 were prepared to study the reaction kinetic properties by UV-Visible spectral experiments. An aliquot (50 μl) of 0.42 mM native Zn_7MT-II solution was added to an appropriate volume of 0.01 M potassium phosphate solution, pH 7.40, which was used to maintain the final volume of 3 ml (final MT concentration in all experiments is 7 μM). These solutions were mixed with an appropriate volume of 1.25 mM JM216 solution to produce samples containing 2, 3, 4, 7, 10 and 15 molar equivalents of JM216. The samples were transferred to a 1-cm cuvette and sealed with parafilm. The UV-Visible spectra were recorded on a shimadzu UV-3100 spectrometer. The reference solution contained all the regents except JM216.

Chromatographic separation and stoichiometry determination

All the reactions were carried out under an anaerobic condition at 37 °C. The reaction mixture of a series of the deaerated Zn_7MT-II in 3.6 mM potassium phosphate solution, pH 7.40, containing 2, 4, 7 molar ratios of JM216 which were prepared similarly to those mentioned above (final MT concentration was 0.2 mM), were left standing for 3 h at 37 °C, and were centrifuged to remove the precipitate. Then the supernatant was introduced into a Sephadex G-50 column (1.1 × 55 cm), eluted with 3.6 mM potassium phosphate solution, pH 7.40 at 4 °C and monitored at 254 nm. Fractions (2 ml/tube) were pooled and analyzed for S, Pt and Zn by ICP spectrometry.

X-Ray Photoelectron Spectroscopy Measurement

The X-Ray photoelectron spectroscopy (XPS) measurement was performed on an ESCALAB MK II electron spectrometer using Al-$K\alpha$ radiation (1486.6 eV) as the X-Ray excitation source. The C(1s) line from oil contamination (binding energy 285 eV) was used as internal standard for calibrating the spectra. The powdered samples were produced from lyophilized fractions corresponding to various MTs from the Sephadex G-50 column following desalting by the Sephadex G-25 column (1.6 × 50 cm) using water as eluant.

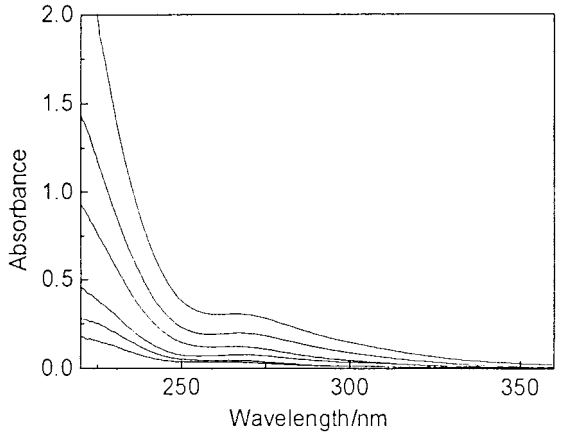

Fig. 1. UV-visible spectra of rabbit liver Zn_7MT-II with JM216 in 0.01 mol.l^{-1}, pH 7.40, potassium phosphate solution for 5 h at 37 °C. From top to bottom are: the molar ratio of JM216 to MT: 15:1,10:1, 7:1, 4:1, 3:1, 2:1(concentration of MT was 7.0×10^{-6} mol.l^{-1}).

Results and discussion

UV-Visible Spectrum

Figure 1 shows the UV-visible spectra recorded for a series of native Zn_7MT-II solutions (5 μM protein) incubated with 2, 3, 4, 7, 10 and 15 molar equivalents of JM216 at pH 7.40, 37 °C for 5 h. It can be seen that a broad absorbance band around 280 nm appeared when native Zn_7MT-II reacted with JM216. This characteristic absorbance can be due to the formation of platinum-thiolate as reference reported (Bongers *et al.* 1991).

Binding stoichiometry of metals to metallothionein

After removing the precipitate, which may be Pt-containing poorly resolved products of higher oligomers, the products from a set of reactions of Zn_7MT-II with 2, 4 and 7 molar ratios of JM216 in 3.6 mM potassium phosphate solution, pH 7.40 for 3 h at 37 °C were fractionated on a Sephadex G-50 column. A typical elution profile monitored at 254 nm is shown in Figure 2. Incubation of Zn_7MT-II with 2 molar equivalent of JM216 results in three peaks corresponding to high-molecule-weight component (HMW) from 7 $\sim$ 13 fractions, low molecular weight (LMW) component from 14 to 20 fractions and a small molecular component from 21 $\sim$ 30. After pre-calibration with standard proteins, cyt c (MW 12,400 Da), bovine superoxide dismutase (MW 33,000 Da) and rabbit liver Zn_7MT-II (6,500 Da). The

Table 1. Binding energies for 4f levels of platinum and 2p level for sulfur[a]

Compounds	Binding Energies (eV)		
	4f(7 / 2)	4f(5 / 2)	2p
K_2PtCl_6	75.7	79.0	–
K_2PtCl_4	73.2	76.4	–
Cysteine	–	–	163.2
Methionine	–	–	162.8
Cystine	–	–	64.2
Pt(II)-MT[b]	73.0	76.4	163.2
7JM216:MT	72.0	75.4	162.4
$4K_2PtCl_6$:Zn_7MT[b]	72.6	75.9	163.1
Precipitate	72.2	75.6	162.7 164.4

[a] Binding energies are accurate to $\pm$ 0.2 eV.
[b] The result was from the reaction of Zn_7MT with K_2PtCl_6 (Buhler RHO *et al.*).

LMW component was considered to be monomeric MT form. The HMW component was dimeric MT. The distribution pattern of the metal ion for two molar ratio of JM216 to MT (Figure 3a) was similar to the absorption elution profile. There were also three peaks which corresponded to the monomeric and dimeric products as well as small molecules, respectively. The ICP measurement showed that the monomeric MT contained 6.05 ± 0.03 Zn and 1.13 ± 0.23 Pt per mole protein, and the dimeric form contained 5.10 ± 0.14 Zn and 1.39 ± 0.43 Pt per monomeric unit (Table 2). (The precipitate containing higher oligomeic products was not analyzed).

In the experiment with 4 molar equivalent of JM216 to MT, there were also three peaks corresponding to high, low-molecule-weight components and small molecules in the absorption profile. In this case, the peak intensity of the monomeric MT form decreased and became a shoulder. Whereas the peak intensity of dimeric MT increased (Figure 2b). The amount of the precipitate which may be platinum-containing higher oligomeric products and the small molecular component containing Zn, Pt and other inorganic ions was also increased. The metal distribution pattern was similar to the corresponding absorption elution profile too (Figure 3). After measuring by ICP spectrometry, it was found that the monomeric product contained about 6.63 ± 0.14 Zn and 1.01 ± 0.42 Pt per mole of protein, and the dimeric product contained about 4.80 ± 0.06 Zn and 2.97 ± 0.27 Pt per monomeric unit in dimer.

When the reaction molar ratio of JM216 to MT was up to 7:1. More precipitate product was generated. Af-

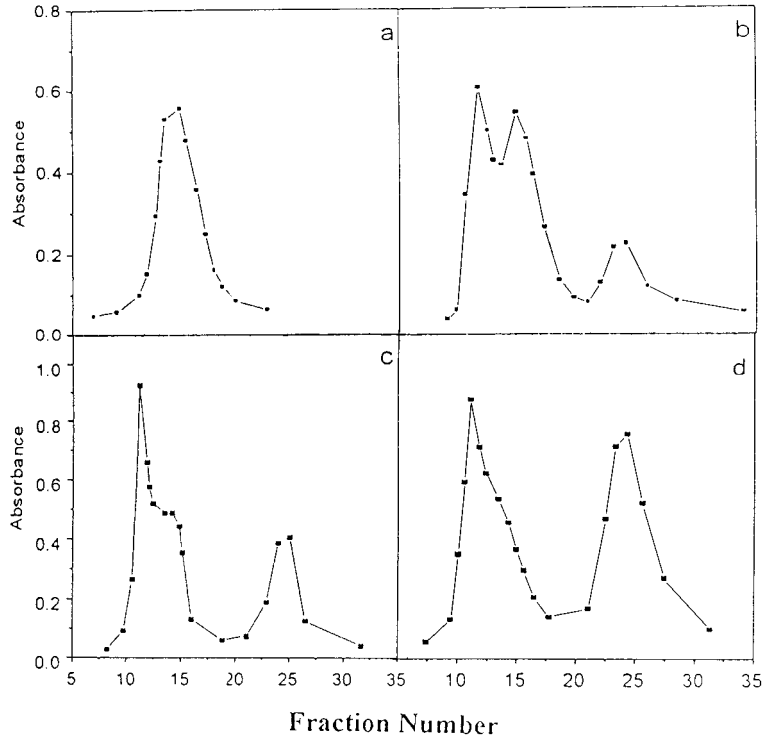

Fig. 2. Elution Profiles of reactions between Zn_7MT-II (2.0×10^{-4} mol.l^{-1}) and JM216 in 3.6×10^{-3} mol.l^{-1}, pH 7.40, potassium phosphate solution after standing at room temperature. Sephadex G-50 column (1.1×55 cm), eluted with the same buffer solution. Fractions of 2 ml were collected. (a) 20 μM native Zn_7MT-II; The reaction molar ratios of JM216 to MT were (b) 2:1; (c) 4:1; (d) 7:1.

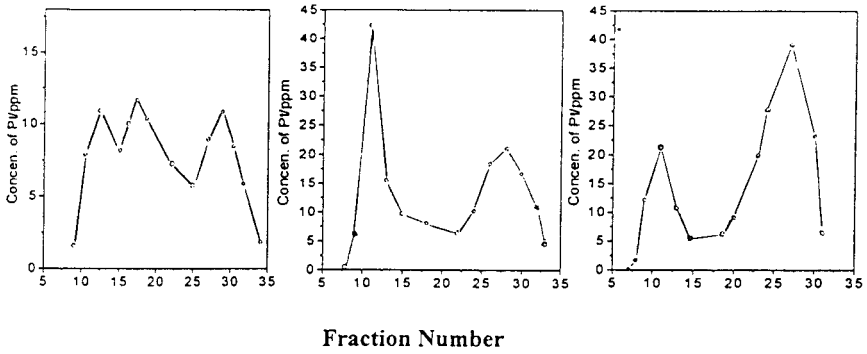

Fig. 3. Metal ion distribution patterns from gel-filtration of Pt (○) for the reaction molar ratios of JM216 to Zn_7MT-II being (a) 2:1; (b) 4:1; (c) 7:1. The reactions were mentioned in the experiment.

Table 2. Binding Stoichiometry of Metal Ions in MTs

Reaction Ratio	Reaction Time	Monomeric Products (g atom metal/mol protein)		Dimeric Products (g atom metal/monomeric unit)	
(MT:JM216)	(h)	Zn	Pt	Zn	Pt
Zn_7MT-II	–	7.10 ± 0.10	–	–	–
2:1	3	6.05 ± 0.03	1.13 ± 0.23	5.10 ± 0.14	1.39 ± 0.43
4:1	3	6.63 ± 0.14	1.01 ± 0.42	4.80 ± 0.06	2.97 ± 0.27
7:1	3	–	–	3.42 ± 0.08	4.42 ± 0.61

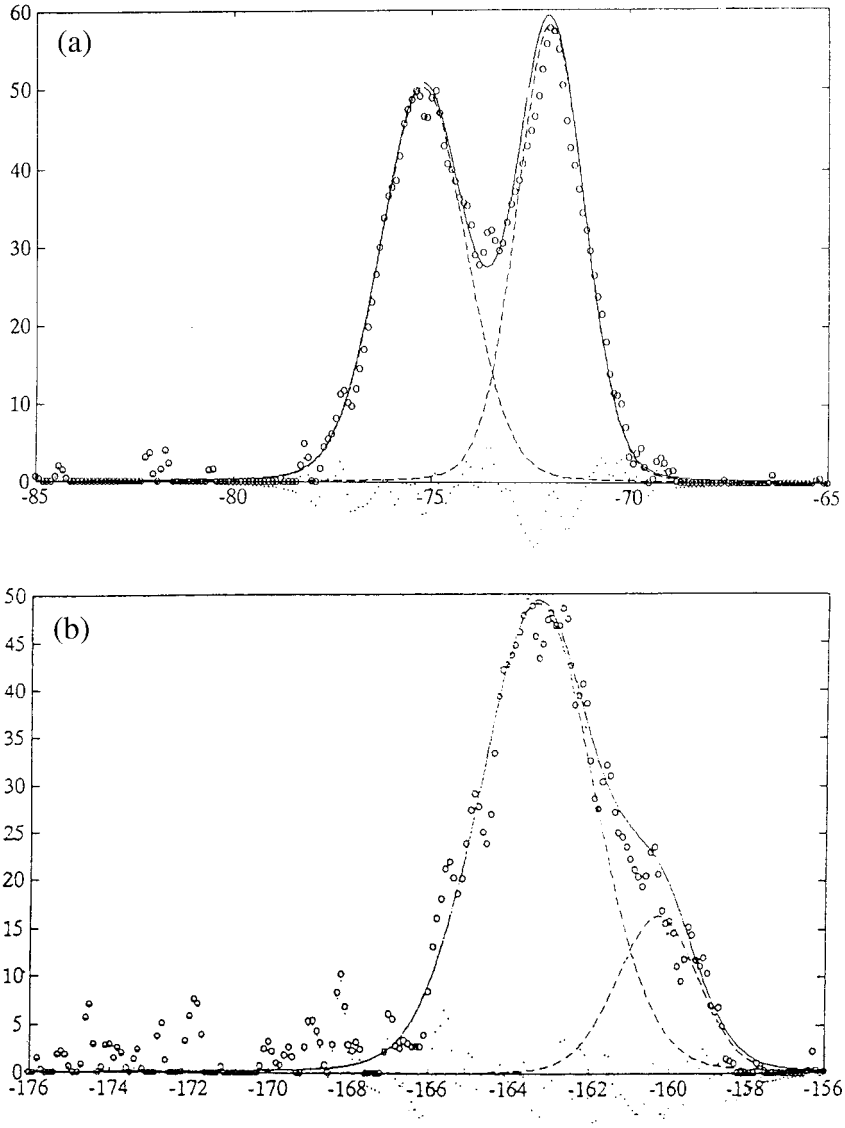

Fig. 4. X-Ray photoelectron spectra for 4f levels of platinum and 2p level of sulfur in the dimeric product separated from the reaction mixture of native Zn_7MT-II and JM216 in molar ratio of Pt/MT = 7:1. (a), Pt and (b), S.

ter the precipitate was removed by centrifugation, the elution profile was shown in Figure 2c. We found that the peak for the monomeric MT form in the absorption elution profile almost disappeared. The peak intensity for the dimeric MT was increasing further. The metal ion distribution pattern (Figure 3c) also showed that only two peaks existed, which were corresponding to the dimeric product and small molecular component, respectively. The ICP measurement indicated that the dimeric contained 3.42 ± 0.08 Zn and 4.42 ± 0.61 Pt per monomeric unit.

Oxidation state of platinum and sulfur in reaction products

X-Ray photoelectron spectroscopy (XPS) was used to determine the oxidation states of platinum and sulfur by comparing the binding energies in the products. Figure 4 shows the XPS spectrum of the dimeric and precipitate products from Zn_7MT-II with 7 molar ratio of JM216. After calibrating the spectra by using the C(1s) line from oil contamination (binding energy 285 eV) as an internal standard, the binding energy for 4f(7/2) and 4f(5/2) levels of platinum in the dimeric

product was 72.4 eV and 75.6 eV, respectively (Table 1), which just resembled those in K_2PtCl_4 (73.2 and 76.4 eV) (Katrib *et al.* 1980) and those in Pt-MT from Zn_7MT-II with K_2PtCl_4 (73.0, 76.4 eV). They were also similar to those from Zn_7MT-II with K_2PtCl_6 (72.6 and 75.9 eV) (Zhong *et al.* 1997), but were $2 \sim 3$ eV less than those in K_2PtCl_6 (75.6 and 79.0 eV). The similar results were found in the dimeric products from Zn_7MT-II with 2 and 4 molar ratios of JM216 too (data not shown). From Table 1, it can also be found that the binding energy for the 2p level of S in the dimeric products of native Zn_7MT-II with 7 molar ratio of JM216 was 162.4 eV. It was similar to that in cysteine (163.2 eV) and in methionine (162.8 eV), but different from that in RSSR (about 164.2 eV) (Baker *et al.* 1972). These results suggested that the oxidation state of platinum in the MT products from Zn_7MT-II with JM216 was $+2$ and there were no CyS-SCy disulfides in the dimeric products. While the binding energy for the 2p level of sulfur in the precipitate product was different. The values were 162.7 eV and 164.4 eV, the former is similar to that in cysteine and the latter is similar to the value in cystine (164.2 eV), respectively. It was suggested that the composition of the precipitate product was very complicated and there were some CyS-SCy disulfides existed in the precipitate. Therefore, it can be concluded that the reaction between Zn_7MT-II and JM216 involved a redox reaction. Some of the sulfhydryl of MT can be oxidized to CyS-SCy disulfides by JM216, which existed in the precipitate products. Pt(IV) was reduced to its Pt(II) counterparts. Then the reduced Pt(II) could replace the metal ions (such as Zn or Cd) in the native MT. Meanwhile Pt(II) can also lead to the nonoxidative dimerization of intermolecular MT-Pt-MT linkages.

From the UV-visible, chromatography and XPS measurement, it can be suggested that the reaction of Zn_7MT-II with JM216 comprised a redox and a substitution reaction. The reaction of native Zn_7MT-II with JM216 generated precipitate product with intra or intermolecular CyS-SCy disulfides linkages and the monomeric and dimeric products in which contained no CyS-SCy disulfides. The oxidation state of platinum in the products was $+2$. With the molar ratio of JM216 to MT increasing, more sulfhydryl of MT will be oxidized. This result leads to a decrease in the apparent yield of MT monomers and a concomitant buildup of MT precipitate products. In addition, more metal ions in native MT will be replaced by the reduced Pt(II).

The mechanism of the reaction between JM216 and Zn_7MT-II including a redox and a substitution reaction indicates that Pt(IV) complexes antitumor drugs can be reduced to Pt(II) compounds by sulfhydryl-containing protein (such as MT) or other reducing agents (such as cysteine and GSH) *in vivo*. Therefore, it is reasonable to hypothesize that MT may play important roles in the metabolism and reducing cytotoxicity of platinum-containing drugs. Although the detailed mechanism still remains unclear, one point can be confirmed that Pt(IV) based anticancer drugs may endow with anti-tumor activities through their reduced Pt(II) products.

Acknowledgement

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