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Binding properties of an orally active platinum anti-tumor drug JM216 with metallothionein *in vivo*

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Abstract

The abilities of the orally active platinum anti-tumor drug JM216 [*trans*-bis-acetato-*cis*-dichloroammine (cyclohexylamine) platinum (IV)] to induce the biosynthesis of metallothionein (MT) were investigated in rabbits given oral administrations or injections s. c. It is revealed that oral administration of JM216 can induce the MT synthesis in the liver but not in the kidney. The hepatic MT contained 7.11 ± 0.11 Zn and only little Pt or Cu. Injections of JM216 to rabbits can greatly elevate the MT levels in the liver, but increase the renal MT levels only slightly. The MT content as well as Pt concentration in the liver was much higher than that in the kidney. The metal stoichiometry in the purified renal MT was determined to be 4.41 ± 0.04 Zn, 0.36 ± 0.11 Pt and 2.50 ± 0.18 Cu per mole protein. The hepatic MT was still characterized as Zn_7MT. Both the treatment with oral administration and injections s.c. cannot lead to the increase of Pt content in the kidney. The oxidation state of platinum in the MT from the kidney was determined to be +2 by X-ray photoelectron spectroscopy. As compared with zinc compounds, JM216 was a very poor stimulator for MT biosynthesis *in vivo*. Pre-injections with $Zn(NO_3)_2$ significantly enhanced the MT levels as well as the Pt concentration compared with that resulting from injections with JM216 alone. Based on the experimental data, the role of MT in relation to its involvement in the metabolism and the mechanism of detoxification of Pt(IV) complexes are discussed.

Abbreviations: MT – metallothionein; JM216 – *trans*-bis-acetato-*cis*-dichloroammine (cyclohexylamine) platinum (IV); p.o. – oral administration; s.c. – subcutaneous; ICP – inductively coupled argon plasma-atomic emission.

Introduction

Cisplatin [*cis*-dichlorodiammineplatinum(II) (CDDP)] and carboplatin[*cis*-diammine (1,1-cyclobutane-di--carboxylato)platinum(II)] are widely used for the treatment of a number of human malignancies (Loehrer *et al.* 1984; Neijt *et al.* 1991; Ozols *et al.* 1991; Wong *et al.* 1999). However, besides the side effects such as dose dependent nephrotoxicity, neurotoxicity and severe emesis, another disadvantage of these drugs is the lack of oral activity. To facilitate patient comfort and convenient drug chemotherapy, a new oral Pt(IV) drug (JM216) was introduced into the clinic trials by Giandomenico *et al.* (1991). Phase I clinical and pharmacokinetic studies by McKeage *et al.* (1997) have demonstrated that JM216 exhibited lack of ototoxicity, neurotoxicity and neophrotoxicity. This drug also has superior *in vitro* and *in vivo* activity compared to CDDP or carboplatin against human cervical, small cell lung and ovarian cell lines (Kelland *et al.* 1993). JM216 is currently undergoing phase II clinical trials in the USA, Japan and Europe (McKeage *et al.* 1995). Incubation of JM216 with human plasma could result in different biotransformation products including mono- and dihydroxo-Pt(IV) complexes and the dichloroplat-

inum(II) complex cis-[$PtCl_2(NH_3)$(cyclohexylamine)] (Raynaud *et al.* 1996). Moreover, binding of the Pt(IV) complex to DNA fragments seems to be successful only in the presence of a reducing agent (Talman *et al.* 1997). The intracellular protein metallothionein (MT) has a strong affinity for heavy metal ions because of its large number of sulhydryls groups. It is well known that MT can play an important role in the detoxification of cadmium and mercury (Cherian & Goyer 1978) and may also reduce the nephrotoxicity of CDDP (Naganuma *et al.* 1987; Sharma *et al.* 1983). However, little attention has been paid to the interaction of Pt(IV) complexes with this cysteine-containing protein *in vivo* or *in vitro* until recently (Zhong *et al.* 1997; Nielson *et al.* 1985). In order to elucidate the role of MT in the metabolism of Pt(IV) complexes and in the mechanism of the detoxification of Pt(IV) compounds, we have carried out *in vivo* studies on the interaction of JM216 with MT in present work.

Materials and methods

Chemicals

JM216 was synthesized from K_2PtCl_4 by multistep procedures as previously described (Talman *et al.* 1997; Giandomenico *et al.* 1995), and analyzed 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, 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.

Animal experiments

Four groups of gray male rabbits (body weight about 3 kg) were used for the experiments and the doses are listed in Table 1. In the first group, JM216 was suspended in archis oil and given to rabbits by oral gavage (injection volume: 10 ml kg^{-1}). In the second group, the rabbits were injected s.c. with solutions of JM216 for ten days. In the third group, rabbits were first given $Zn(NO_3)_2$ injection to elevate the MT level in the tissues, then from the 7th day, the rabbits were injected simultaneously with JM216 and $Zn(NO_3)_2$. Rabbits in a parallel experiment were used as control and injected with solution of saline for successive 10 days.

Isolation and purification of MT (Comeau *et al.* 1992)

The rabbits were sacrificed under anesthesia 24 h after the last oral administration or injection. The liver and kidney tissues were homogenized separately with a mixture of 0.01 M Tris-HCl buffer solution pH 8.60, anhydrous alcohol, and chloroform (v/v 1.00: 1.03: 0.08), then centrifuged at 10 000 $\times$ *g*, for 30 min at 4 °C to remove the precipitate. Three folds of volume anhydrous ethanol were added slowly with stirring per volume of supernatant. After overnight incubation at −20 °C, the precipitate was collected by centrifugation at 10 000 $\times$ *g*, 4 °C for 20 min and dried at room temperature. The dried precipitate was dissolved in 0.01 M Tris-HCl buffer solution, pH 8.60, and centrifuged under the same condition. The resultant supernatant was fractionated on the Sephadex G-50 column (2.6 $\times$ 90 cm), pre-equilibrated with 0.01 M Tris-HCl buffer solution, pH 8.60, at a flow rate of 1 ml/min, using the same buffer as the eluant and eluted at the same flow rate, monitoring absorbance at 254 nm. The effluent was collected in 5 ml fractions. The MT-enriched fractions were further purified on a DEAE-Cellulose DE-52 column (1.6 $\times$ 40 cm). A continuous linear gradient of 0.01–0.18 M Tris-HCl buffer solution, pH 8.60, was used to elute the two isoforms of the protein, MT1 and MT2. The concentrated MT was desalted on Sephadex G-25 (2.6 $\times$ 60 cm), pre-equilibrated with water, using water as eluant. The fractions of MT were then pooled and lyophilized. MT was characterized by its amino acid composition. The apparent molecular weight was determined on the Sephadex G-50 column by calibration with several standard proteins: bovine superoxide dismutase (MW 33 000 Da), cytochrome C (MW 14 000 Da) and rabbit liver Zn_7MT (MW 6500 Da).

Protein concentration was determined by the absorption of apo-metallothionein at 220 nm (ε_{220} = 48 200 mol^{-1} cm^{-1}) after acidification (Good & Vasak 1986). Metal contents were determined by pumping samples into ICP spectrophotometer and recording emission counts at the following lines: S: 181.987 nm, Zn: 213.856 nm, Pt: 224.552 nm and Cu: 324.754 nm. Each sample was run side by side with a blank containing all reagents except the proteins. The metal composition was obtained according to the ratio of metal to sulfur, as all mammalian metallothioneins have identical S content (21 mol of S/mol of protein) (Bongers *et al.* 1988).

Table 1. Treatment to rabbits with $Zn(NO_3)_2$ and JM216. Doses are expressed in mg (metal)/kg (body wt)/day

No.	Dose	1	2	3	4	5	6	7	8	9	10
1	Control	–	–	–	–	–	–	–	–	–	–
2	p.o. JM216	13	13	13	16	20	–	–	–	–	–
3	s.c. JM216	0.15	0.15	0.35	0.75	1.5	3.5	3.5	5.0	5.5	7.0
4	$Zn(NO_3)_2$+	10	10	20	20	40	40	40	40	40	40
	s.c. JM216	–	–	–	–	–	–	5.4	7.2	7.2	10.8

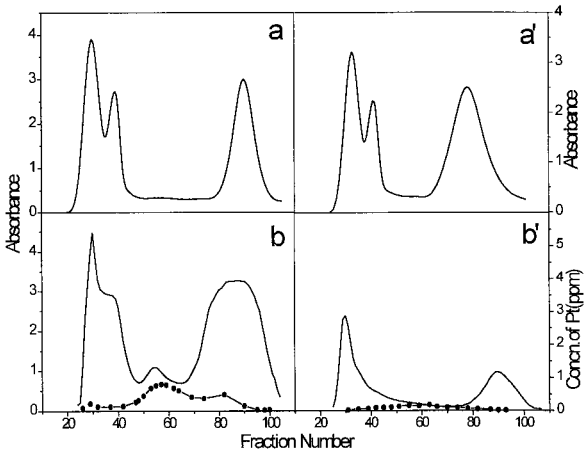

Figure 1. Elution profile of liver and kidney tissues following p.o.JM216 into rabbits. Tissues extracts from liver (L) and kidney (K) homogenates were fractionated by ethanol-chloroform precipitation and the solubilized precipitate was fractionated on a Sephadex G-50 column, 2.6 × 90 cm pre-equilibrated with Tris-HCI, 0.01 mol/l, pH 8.6; 5.0 ml. aliquots of fraction were collected and monitored by measuring absorption at 254nm (—) and the concentration of Pt (-·-·-·-). Tissues and treatments: (a), L (a′), K, control (injection with saline solution), (b), L, (b'), K, JM216 (oral administration).

X-ray photoelectron spectroscopic measurement

Platino-MT isolated from kidney with JM216 injection was used to determine the oxidation state of platinum by measuring the X-ray photoelectron spectrum (XPS) performed on an ESCALAB MKII electron spectrometer using the Al-Kα radiation (1486.6eV) as the X-ray excitation source. The C(1s) line from oil contamination binding energy was used as internal standard for calibrating the spectra. The oxidation state of sulphur was determined by the same method.

Results and discussion

Abilities of JM216 to induce MT biosynthesis in the liver and kidney

After five times oral administration of JM216 to rabbits, the hepatic and renal cytosols were fractionated on the sephadex G-50 column. Their elution profiles recorded by monitoring the effluent absorbance at 254 nm, were shown in Figure 1b and 1b′, respectively. Compared with the control (Figure 1a, 1a′), the elution profile of liver extracts had a new peak ranging from fractions 50 to 65, the elution volume corresponding to that of metallothionein. Pt concentration profile also showed the peak in the same the region. After further purification by DE-52 and Sephadex G-25 chromatograph, the hepatic MT fractions were identified as Zn_7MT and binding 7.11 ± 0.11 and little Pt (0.05 ± 0.01), or Cu (0.21 ± 0.03) per mole protein. With the aid of molecular weight markers to calibrate the gel column, sizes of the hepatic Zn_7MT were evaluated to be approximately 6500 Da. The elution profile of the renal extracts exhibited the similar pattern to that of the control in which no MT fractions appeared. The metal distribution pattern also showed that only little Pt was accumulated in the soluble fractions of the kidney. So it is suggested that oral administration of JM216 can only induce the biosynthesis of MT in the liver but not in the kidney and Pt was mainly presented in the hepatic MT fractions.

After ten days injections s.c. of JM216 into rabbits, the gel-elution profile of the hepatic cytosol (Figure 2a) displayed the MT peak from fractions 50 to 70. The peak intensity of the MT fractions was higher than that in Figure 1b. The elution profile of the renal extracts (Figure 2a′) exhibited a weak peak ranging from 50 to 70 fractions, which did not appear on the profiles of the controls and that of the oral administration, characterized as MT. Both of the metal distribution patterns showed that Pt was mainly in the MT frac-

Table 2. 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	–
Methionine	–	–	162.8
Cysteine	–	–	163.2
Platino-MT	72.9	75.8	162.5
Pt(II)-MT	73.0	76.4	163.2
Platino-MT[b]	72.3	75.7	162.6

[a]Binding energies are accurate to ± 0.2 eV.
[b]The Platino-MT was isolated from liver with iproplatin injection (Zhong *et al.* 1997).

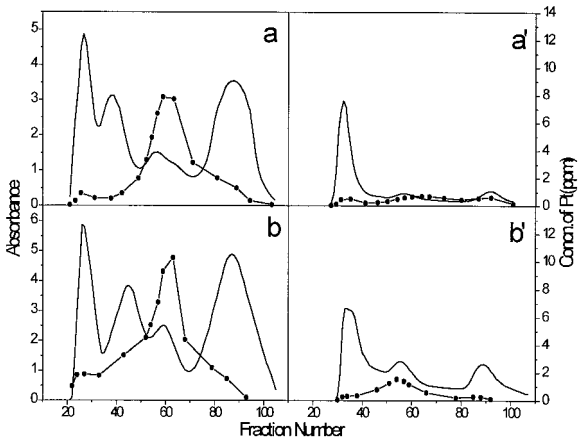

Figure 2. Distribution of Pt in liver and kidney tissues following injections with JM216 or co-administration with $Zn(NO_3)_2$ solution. Tissues extracted from liver (L) and kidney (K) homogenate were treated as mentioned in Figure 1. (a), L, (a′), K, injection with JM216; (b), L, (b′), K, $Zn(NO_3)_2$ and JM216 co-administration. Concentration of Pt(·-·-·-) and absorption at 254 nm (—).

tions. Pt concentrations in the hepatic fractions were considerably higher than those in the renal ones, suggesting that Pt preferentially accumulated in the liver rather than in the kidney. The proportions of the cytosolic Pt associated with MT in the liver and kidney were similar. The metal stoichiometry in the purified hepatic and renal MT fractions were determined to be 6.57 ± 0.73 Zn, 0.32 ± 0.05 Pt, 0.50 ± 0.16 Cu and 4.41 ± 0.04 Zn, 0.36 ± 0.01Pt, 2.50 ± 0.18 Cu, per mole protein, respectively. It could be concluded that injections of JM216 can greatly elevate the hepatic MT levels but only increase the MT levels slightly in the kidney. Neither the oral administration nor injections of JM216 to rabbits can increase the Pt content in the kidney. This may be the reason why this drug has lower nephrotoxicity.

Influence of pretreatment with $Zn(NO_3)_2$ on the distribution of Pt in rabbit liver and kidney tissues

It had been established that Zn^{2+} is an efficient inducer for the biosynthesis of MT (Suzuki *et al.* 1990). In order to elucidate the influence of pre-existing MT on the distribution of Pt in the liver and the kidney, $Zn(NO_3)_2$ was used to elevate metallothionein levels before the injection of JM216 into rabbits.

The chromatographic patterns of hepatic and renal cytosols (Figures 2b, 2b′) after co-administration of $Zn(NO_3)_2$ and JM216 exhibited the intense peak characteristic for MT from fractions 50 to70, showing that pretreatment with $Zn(NO_3)_2$prior to JM216 injections elevated the MT levels in both liver and kidney significantly. The metal distribution patterns also revealed that the increased MT biosynthesis by Zn could correspondingly increase the binding of Pt to MT. Almost all the Pt was still bound to the MT fractions. The MT levels as well as Pt concentration in the liver was much higher than that in the kidney. This was similar to that of treatment with only JM216 injections. After further purification, both of the hepatic and renal fractions were identified as Zn_7MT. These results demonstrated that JM216 was a poorer inducer than Zn compounds and Pt ions could be accumulated by pre-existing MT *in vivo*. This implied that participation of MT, elevated by Zn(II) compounds, could protect the tissues from Pt damage.

Oxidation state of platinum in platino-MT from the liver and kidney

The sample of Pt-MT from the kidney with JM216 injection was studied by X-ray photoelectron spectrum (XPS). The binding energies for 4f(7/2) and 4f(5/2) levels of Pt in the platino-MT from the kidney were 72.9 and 75.8 eV. Compared to the values in K_2PtCl_6 (75.7 and 79.0 eV) (Katrib 1980), the binding energies were 3–4 eV lower, but were similar to those found in K_2PtCl_4 (73.2 and 76.4 eV) and to those of Pt (II)-MT formed in the reaction of K_2PtCl_4 with Zn_7MT *in vitro* (73.0 and 76.4 eV) (Table 2). They were also similar to our previously reported values for platino-MT (72.3 and 75.7 eV) from liver after treatment with iproplatin injections (Talman *et al.* 1997). Therefore, it could be concluded that the oxidation state of platinum in Pt-MT isolated from the kidney was +2. These data suggested that the Pt(IV) complexes were reduced in

the metabolism of compounds *in vivo*, and the potential reductants could be MT, glutothionein, cysteine or other reducing molecules or proteins (Blatter *et al.* 1984). These results also implied that the active form of Pt(IV) compounds were their Pt(II) counterparts. Table 2 showed that the binding energy for the 2p level of sulfur in platino-MT from the kidney was 162.6 eV, which was similar to the value found for cysteine (163.2 eV) and methionine (162.8 eV), but different from that in R-SS-R (about 165 eV) (Baker & Befferidge 1972). Thus, there were no CyS-SCy disulfides in the platino-MT induced by JM216, and the two metal-thiolate clusters, M_3S_9 and M_4S_{11} (M = Cu, Zn, Cd) also existed in the induced Cu, Pt, Zn-MTs.

Conclusion

Based on the experiments, it is evident that oral administrations of JM216 only have the ability to induce the synthesis of MT in the liver but not in the kidney. Injections of JM216 to rabbits can elevate the MT contents greatly in the liver, but only increase the renal MT levels slightly. The metal distribution patterns showed that Pt was mainly in the MT fractions. MT levels as well as Pt concentration in the liver was much higher than that in the kidney. The higher content of Pt in the MT could protect the tissues from the toxicity of the Pt compounds. Neither the oral administration nor injections of JM216 to rabbits can increase the Pt content in the kidney. This may be the reason why JM216 lacks nephrotoxicity. The oxidation state of bound platinum is +2 in platino-MT in the absence of CyS-Scy containing disulfides, suggesting that Pt(IV) compounds are reduced to Pt(II) complexes *in vivo*. Pretreatment with Zn elevates the MT levels and Pt content significantly in both the liver and kidney and Pt can be accumulated by the pre-existing MT. These findings suggest that MT may play an important role in the metabolism and the detoxification of Pt compounds *in vivo*.

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