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Studies on the human metabolism of iproplatin

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Summary. We have previously shown that a significant portion of the total platinum in the plasma of patients receiving iproplatin is protein-bound [17]. We have also identified *cis*-dichloro-*bis*-isopropylamine platinum(II) (CIP) as a major metabolite of iproplatin [19]. To understand the nature of the bound platinum, we carried out in vitro comparative protein-binding studies for iproplatin and CIP. These studies indicate that when CIP is incubated in plasma, protein binding occurs, with a 2.7-h half-life for the disappearance of CIP; the parent complex does not bind and is stable in plasma for at least 48 h. The time dependence of protein binding with CIP suggests the formation of other chemical species from CIP that may be responsible for the observed protein binding. The results indicate that in patients receiving the drug, the reduction of iproplatin to CIP must take place intracellularly and that CIP or its protein-binding derivatives must efflux from the cells into the plasma. Efflux studies carried out to explore this possibility with cells in the whole blood showed that iproplatin was taken up into cells, but the efflux of protein-binding iproplatin metabolites did not occur. To understand further the nature of the metabolites of iproplatin, we carried out ^{195}Pt-NMR (nuclear magnetic resonance) studies with urine from two patients who received a high dose of iproplatin (500 mg/m^2). The predominant signals from the ^{195}Pt-NMR corresponded to the divalent platinum complexes and not to quadrivalent complexes, indicating that the iproplatin metabolites in urine are divalent in nature.

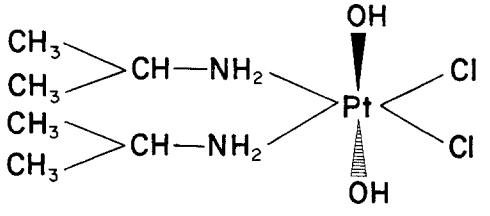
Introduction

Iproplatin [CHIP; *cis*-dichloro-*trans*-dihydroxy-bis-isopropylamine platinum(IV)] (Fig. 1) is unique among the platinum (Pt) complexes that have entered clinical trial because it is a complex of quadrivalent Pt, whereas the others are divalent. The quadrivalent complexes are octahedral, whereas the divalent complexes have a square planar structure. In the octahedral configuration, two axial ligands project at a 90° angle above and below the square plane. In the case of iproplatin, these axial ligands are the two hydroxy groups. We have previously identified *cis*-dichloro-*bis*-isopropylamine Pt(II) (CIP) (Fig. 1) by mass spectroscopy as one of the urinary metabolites of iproplatin in patients receiving the drug [19]. Free CIP was also shown to be present in the plasma, which demonstrated that the quadrivalent complex is reduced to the divalent form in vivo.

In patients receiving iproplatin, 85%–95% of the Pt in the plasma is protein-bound [17]. However, iproplatin does not bind to plasma proteins, indicating that the protein-bound materials must be a metabolite of the drug [15, 16, 23]. To develop a more complete picture of the reduction of iproplatin in vivo, we carried out studies of the plasma protein binding of CIP and ^{195}Pt-NMR (nuclear magnetic resonance) studies of urine from patients receiving iproplatin. The results of these studies suggest that much of the urinary Pt exists in the divalent form and that much of the plasma Pt is also likely to be divalent.

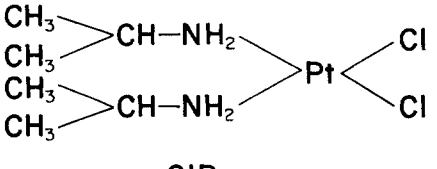
Materials and methods

Analytical grade iproplatin and CIP were obtained from Bristol-Myers Co. [^{14}C]-Iproplatin (sp. act., 24.6 mCi/mmol) was synthesized from 2-[^{14}C]-aminopropane



Iproplatin

(CHIP, Cis-Dichloro-Trans-Dihydroxy-Bis-Isopropylamine Platinum IV)



CIP

(Cis-Dichloro-Bis-Isopropylamine Platinum II)

Fig. 1. Structures of iproplatin and its major metabolite *cis*-dichloro-*bis*-isopropylamine Pt II (CIP)

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(sp. act., 12.3 mCi/mmol; New England Nuclear, Boston, Mass) and K_2PtCl_4 at Roswell Park Memorial Institute by a proprietary procedure supplied by Johnson Matthey Inc. (Specialties Division, 456 Devon Park Drive, Wayne, PA 19087). The procedure was tested by preparing a batch of unlabelled iproplatin on the same scale that was to be used to prepare the [^{14}C]-labelled material; 36 mg iproplatin was obtained from 184 mg K_2PtCl_4 and 49 mg 2-aminopropane as starting materials (21% yield). The synthesized iproplatin had the same retention time in a reverse-phase high-performance liquid chromatographic (HPLC) system, the same ^{1}H-NMR and IR spectra as the analytical iproplatin provided by Bristol-Myers Co. This procedure was then used to synthesize [^{14}C]-iproplatin. The synthesized [^{14}C]-iproplatin was 99% pure as determined by HPLC.

Protein binding studies. [^{14}C]-Iproplatin or CIP (JM6, Bristol-Myers Co.) was incubated at 37° C in human plasma at a final concentration of 5, 10, or 20 μg/ml. Aliquots of plasma (1 ml) were ultrafiltered using the Centrifree micro-partition system (Danvers, Mass). The radioactivity in plasma before and after ultrafiltration (UF) and in the iproplatin peak after separation by HPLC was determined by liquid scintillation counting (Searle Mark III, Des Plaines, Ill). Automatic background subtraction was programmed into the instrument during radioactivity counts. An external standard method was used for quench correction. For protein-binding studies with CIP, the CIP in plasma ultrafiltrate (PUF) was quantitated by HPLC separation and UV absorption at 214 nm. In time-course experiments, percentage-of-recovery calculations were made using the zero time samples, which were the same as control CIP solutions prepared in saline under identical conditions and similarly ultrafiltered.

HPLC separations. The HPLC system consisted of a Waters Associates (Milford, Mass) M6000A pump, an automatic sample injector WISP, and a M441 UV detector, interfaced with a Varian 402 data system. In protein-binding and efflux studies, the measurements of iproplatin and CIP in PUF were made by an isocratic reverse-phase separation carried out on a μBondapak phenyl radial compression cartridge, with 3% methanol as the mobile phase, at a flow rate of 1.5 ml/min [18]. Detection was accomplished by UV absorption at 214 nm or by radioactivity monitoring of the fractions eluting off the column, whichever was appropriate.

Iproplatin and metabolites in the urine of patients receiving the drug (the same urine samples on which NMR studies were carried out) were separated in a 60-min, water-to-methanol gradient using a low-pressure gradient system (Autochrom OPGS, Milford, Mass) [17]. A 10-μl sample of urine was injected directly onto a μBondapak C18 column with a flow rate of 1.5 ml/min, 1-min fractions eluting off the column were collected, and Pt was determined by flameless atomic absorption spectrophotometry (FAAS) (Instrumentation Laboratories, Wilmington, Mass).

Efflux of [^{14}C]-iproplatin metabolites from blood cells. [^{14}C]-Iproplatin was added to fresh whole blood collected in heparinized tubes, to a final concentration of 10 μg/ml. The solution was incubated at 37° C for 7 h and 2-ml samples were taken at specific times. Plasma was obtained by centrifugation and PUF, by centrifugal UF of plasma using an Amicon Centrifree micropartition system. The radioactivity in the plasma and PUF were compared during the 7-h period.

NMR studies. Urine from two patients receiving iproplatin at 500 mg/m^2 in a phase-II, high-dose iproplatin study was analyzed by ^{195}Pt-NMR. The urine samples for NMR studies were selected on the basis of the Pt concentration. Urine was collected in 4-h pools from the time the drug infusion started and was assayed for Pt by FAAS. Only one sample from each patient contained the required minimal concentration of Pt (0.4–0.5 mg/ml) for the NMR studies.

The ^{195}Pt-NMR spectra were obtained in water at room temperature using a Bruker WM360wb NMR spectrometer operating at 77.2 MHz for the ^{195}Pt-NMR frequency and equipped with a selective ^{195}Pt-probe. The chemical shift is expressed with respect to external $PtCl_6^{2-}$ (0 ppm). The sample from patient 1 was lyophilized and, later, redissolved in water and centrifuged prior to NMR analysis; the sample from patient 2 was directly analyzed.

Results

Plasma protein-binding studies

The time course of the recovery of CIP in PUF after incubation of the former in plasma at 37° C is shown in Fig. 2. The percentage of recovery of CIP from plasma was identical for concentrations of 5, 10, and 20 μg/ml; at 7 h incubation only 22% of CIP was recovered in PUF. The estimated half-life for the disappearance of CIP in plasma is 2.7 h, with a rate constant of 0.257 h^{-1}. Incubation of CIP in plasma at 4° C resulted in a complete recovery of CIP in PUF at all times measured.

When [^{14}C]-iproplatin was incubated in plasma up to 48 h at 37° C, all radioactivity in the plasma was recovered in the UF and all of the recovered radioactivity was in the

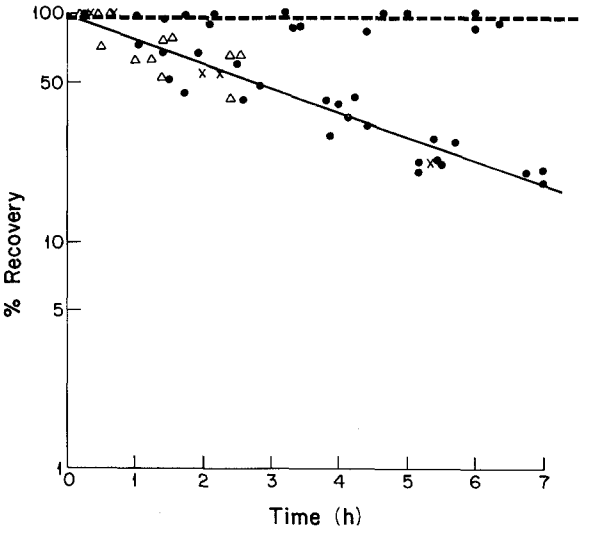

Fig. 2. Recovery of unchanged CIP in the ultrafiltrate after incubation of CIP in human plasma at 37° C (– △ –, 5 μg/ml; –X–, 10 μg/ml; – ● –, 20 μg/ml). The *dotted line* represents incubation at 4° C. Combined data from two separate experiments is presented

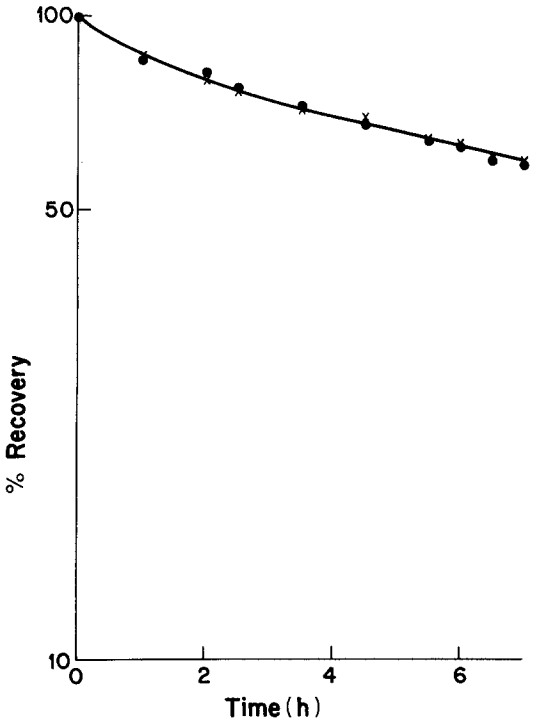

Fig. 3. Uptake of [^{14}C]-iproplatin by whole blood cells. $-X-$, radioactivity in plasma prior to ultrafiltration; $-\bullet-$, radioactivity in PUF. Data presented represents the percentage of recovery of radioactivity in plasma and PUF when [^{14}C]-iproplatin is incubated in whole blood. Each data point shown represents the average of two separate measurements

form of iproplatin as determined by HPLC (data not shown).

Efflux of [^{14}C]-iproplatin metabolites from blood cells

Since iproplatin is stable in plasma and does not bind to plasma proteins, protein-bound Pt species in the plasma of patients receiving this drug [17] must be metabolites that form elsewhere and reenter the plasma. To test whether an efflux of iproplatin metabolites that bind to proteins may occur from blood cells, [^{14}C]-iproplatin was incubated in whole blood at 37° C for 7 h and samples were periodically analyzed for radioactivity in plasma and PUF. Figure 3 shows that the radioactivity in plasma decreases with time to approximately 60% at 7 h, suggesting that the drug is taken up by the cells. The radioactivity in plasma and PUF was the same during this period, indicating that an efflux of iproplatin metabolites that bind to proteins had not occurred. An HPLC analysis of the PUF during this period revealed the presence of iproplatin only, indicating that an efflux of other (non-protein-binding) radioactive species did not occur (data not shown).

HPLC profiles of urinary metabolites

With reverse-phase gradient HPLC, in conjunction with FAAS, CIP and two major metabolite peaks more polar than CIP were detected in urine from patients receiving iproplatin (Fig. 4). The retention times for iproplatin and CIP under the conditions of separation are 9 and 17 min, respectively. The major Pt-containing peak in the urine of patient 1 eluted close to the solvent front. In patient 2, the CIP peak was large and another Pt-containing peak larger than that of CIP eluted immediately before the CIP peak.

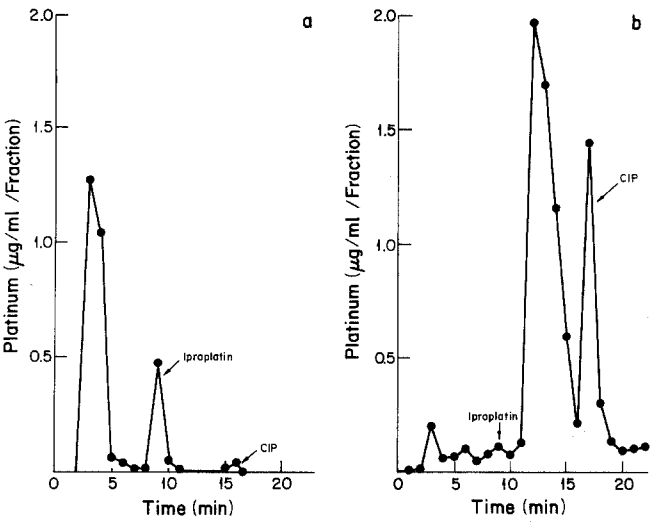

Fig. 4. HPLC separation of platinum-containing compounds in the urine of **a** patient 1 and **b** patient 2

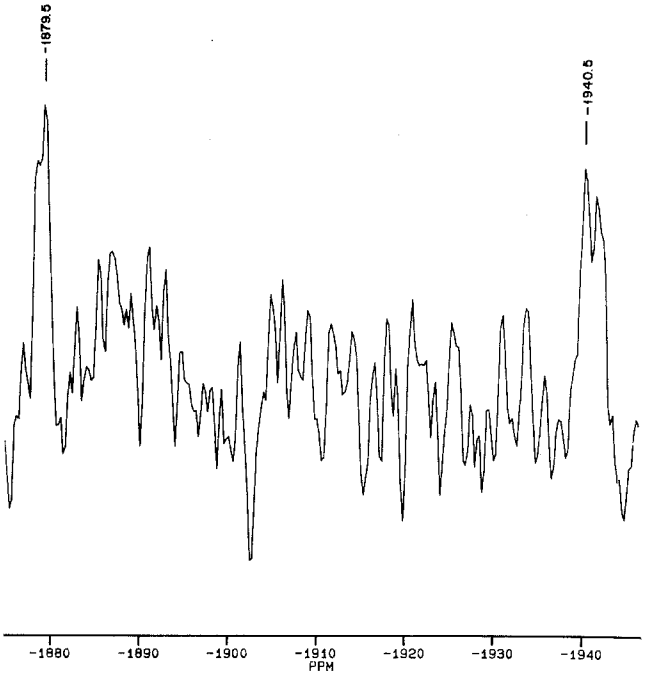

Fig. 5. ^{195}Pt-NMR spectrum of the urine from patient 1

NMR studies

Urine from patient 1 showed two weak resonances at $-1{,}880$ and $-1{,}941$ ppm (chemical shifts are expressed with respect to external hexachloroplatinate) (Fig. 5). The two weak resonances shown in the spectrum correspond to divalent Pt(II) species. There were no other signals in the CIP or iproplatin region (around 930 ppm). The ^{195}Pt-NMR of urine from patient 2 showed one signal at $-2{,}244$ ppm, corresponding to CIP, and another at $-2{,}102$, again indicating the presence of a monochloro CIP species (Figs. 6, 7).

Discussion

To understand the mechanism of action of quadrivalent Pt complexes, it is important to determine whether quadrivalent Pt complexes are reduced to the divalent form in vivo,

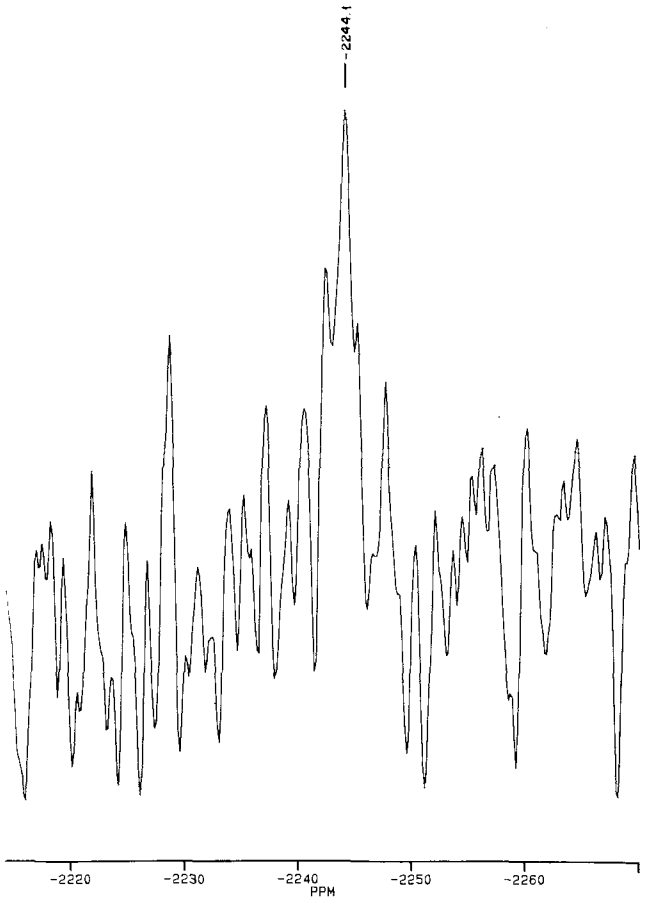

Fig. 6. ^{195}Pt-NMR spectrum of the urine from patient 2

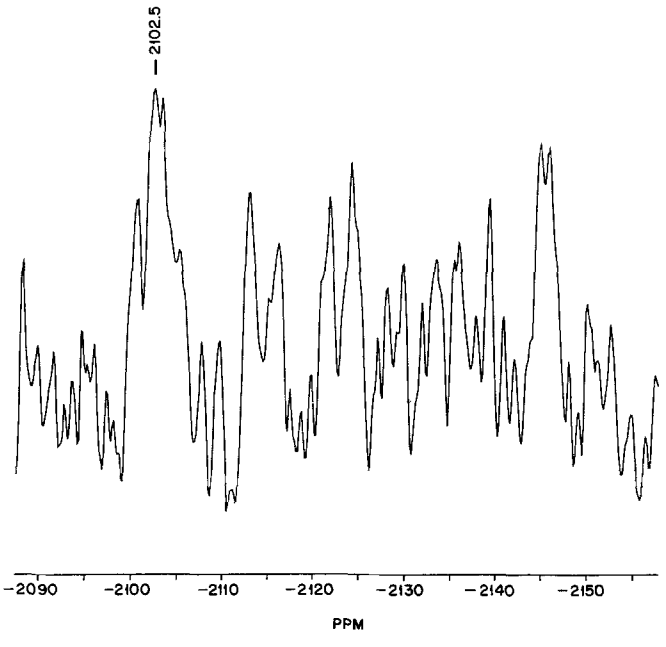

Fig. 7. ^{195}Pt-NMR spectrum of the urine from patient 2, scan from another region of the spectrum

and, if so, whether the divalent form may be the species that interacts with the DNA [4, 5, 7, 9, 10, 21, 22, 24]. The present study addresses the first question. The data obtained indicate that divalent Pt species form the bulk of Pt found in the urine of patients receiving the drug and that the same may be true for the Pt in plasma. The evidence for these conclusions comes from two different types of studies: (1) NMR studies, which suggest that the urine samples of patients receiving iproplatin show ^{195}Pt-NMR signals only for the divalent Pt species and not for the quadrivalent species; and (2) plasma protein-binding studies, which indicate that incubation of the divalent species, CIP, in plasma results in protein binding, whereas incubation of the parent drug, iproplatin, does not.

Since Pt(IV) is known to be kinetically inert [8], the second finding suggests that the protein-bound Pt found in patients receiving the drug must result from CIP, not iproplatin. Protein binding should occur rapidly, but we observed a reaction half-life of 2.7 h; this may be an indication that the actual protein-binding species may not be CIP itself but one or more species derived from CIP.

In the case of cisplatin, the aquated as well as unchanged forms have been postulated to be the protein-interactive species [20]. From the chemistry of cisplatin and other divalent Pt complexes [15, 20, 23] it seems likely that the aquated form of CIP may be one such species that may interact with proteins. If, in fact, the aquated CIP or some other CIP derivative is the protein-bound species in the plasma of patients receiving iproplatin, the amount of protein-bound Pt that appears with time in the plasma of these patients may serve as an indication of the rate of reduction of iproplatin to its divalent counterpart.

The pharmacokinetics of this drug, studied during the phase I clinical trial, indicate that although unchanged iproplatin could be measured in plasma for 6–12 h after drug administration, plasma protein binding of Pt could also be measured very early; often, 15 min into the infusion of iproplatin, approximately 20%–50% of the total Pt in the sample occurs in the form of protein-bound Pt, suggesting rapid in vivo reduction [17]. The urine samples studied with NMR were obtained from the patients immediately following the drug infusion; again, the presence of the predominantly divalent species indicates that the in vivo rate of reduction for this complex is high.

The demonstrated stability of iproplatin in plasma in our in vitro experiments suggests that reduction must take place in cells and that the reduction products must efflux into the plasma compartment and bind to proteins. Since iproplatin is given to patients intravenously, one obvious possibility is that the drug may be taken up by the blood cells, after which CIP or a derivative effluxes into the plasma to bind to proteins. The experiments carried out to test this possibility indicate that although iproplatin is taken up by the blood cells, protein-binding species do not efflux from the cells. Whether these results indicate that the protein-binding species are not formed in the blood cells or that they are formed but do not efflux is not known at this time. The finding suggests that other tissues or organs may possibly be involved in this process.

The ^{195}Pt-NMR studies of the urine samples indicate the presence of CIP and three other divalent Pt(II) species, with chemical shifts ranging from $-1{,}880$ to $-2{,}103$ ppm. Two of these chemicals shifts, ($-1{,}880$ and $-1{,}941$ ppm) occurred in the urine of patient 1, the HPLC profile of which showed a predominant polar peak close to the solvent front, in agreement with our previous studies [18, 19]. Since we used a reverse-phase HPLC system, all charged species eluted close to the solvent front; thus, it is not surprising to find more than one Pt species corresponding to

this peak. The resonance at −1,880 ppm (patient 1) may represent the divalent Pt(II) species diaquodiisopropylamine $Pt(II)^{2+}$. The chemical shift falls within the range one would expect for similar types of complexes [12]. The other resonance at −1,941 ppm may be due to monoaquomonochloro diisopropylamine $Pt(II)^{+}$. This chemical shift is also consistent with those reported in the literature for similar divalent Pt species [3].

Surprisingly, HPLC revealed a Pt peak immediately preceding the CIP peak in the urine of patient 2. This peak was not observed in our previous studies [18, 19]. Whether this might represent a unique species formed in this patient due to unknown factors, such as other medications and food interactions, is not known at this time. However, from the ^{195}Pt-NMR studies, this peak appears to represent a monochloro form of CIP with a chemical shift of −2,103. Although the exact identification of this chemical species is not possible at this time from the ^{195}Pt-NMR studies, the studies did enable us to conclude that the divalent form is predominant.

Other studies with iproplatin and other quadrivalent complexes have indicated that the divalent species may be DNA-interactive [4, 5]. Our finding that the divalent species were predominant after drug administration may be an indication that these species are responsible for the observed antitumor activity of this complex in vivo [1, 2, 6, 11, 13, 14].

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