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ORIGINAL ARTICLE

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Rapid biotransformation of satraplatin by human red blood cells in vitro

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Abstract *Purpose*: Satraplatin is an orally administered platinum complex that has demonstrated clinical activity and manageable toxicity in phase II trials. The presence of several different platinum-containing species and very little intact parent drug in the systemic circulation indicates extensive biotransformation of satraplatin in vivo. To investigate the basis for the biotransformation of satraplatin, studies were carried out into the stability of the drug in whole blood and various other biological fluids in vitro. *Methods*: Concentrations of satraplatin and platinum-containing biotransformation products in incubation fluids were measured using high-performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICPMS). The fate of satraplatin-derived platinum in whole blood in vitro was determined by analysis of blood fractions for platinum by ICPMS. *Results*: In fresh human whole blood in vitro, satraplatin concentrations fell very rapidly, resulting in a half-life for the disappearance of the drug of only 6.3 min (95% CI, 5.9 to 6.7 min). After the addition of drug to red blood cells that had been prepared from whole blood and suspended in 0.9% NaCl, satraplatin also disappeared very rapidly. Satraplatin was much more stable in fresh human plasma ($t_{1/2}$ 5.3 h) and fully supplemented cell culture medium ($t_{1/2}$ 22 h). Two new platinum-containing species appeared on HPLC-ICPMS platinum chromatograms of methanol extracts of plasma after the addition of the drug to whole blood. Their identities were assigned as the platinum(II) complex known as JM118 and a platinated protein with similar electrophoretic mobility to that of serum albumin. During the incubation of satraplatin in blood, platinum associated with red blood cells at an accumulation half-life of 9.5 min (95% CI, 7.1 to 14.2 min). At equilibrium, 62% of the added platinum was associated with red blood cells in a form that was not exchangeable in methanol or 0.9% NaCl. *Conclusions*: The rapid disappearance of satraplatin from human blood in vitro depends upon the presence of red blood cells. Generation of JM118 and irreversibly bound membrane- and protein-associated platinum indicates that satraplatin undergoes rapid biotransformation in whole blood.

Keywords Satraplatin · Biotransformation · Red blood cells · Drug stability · ICPMS

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Introduction

Satraplatin, a third-generation platinum complex, has demonstrated antitumour activity and manageable toxicity in phase II clinical trials [10]. Also known as JM216, satraplatin was the first platinum-based drug developed for oral administration in an attempt to exploit the practical advantages of oral as compared to intravenous therapy. Responses to oral satraplatin have been documented in patients with measurable hormone-refractory prostate cancer [17] and small-cell lung cancer in phase II clinical trials [4]. Oral satraplatin has been associated with gastrointestinal and haematological toxicity that has been manageable or controllable with simple supportive measures. Since oral therapy with satraplatin has been shown to be feasible and promising, its clinical development will continue with the impending commencement of phase III trials [16].

Like other platinum-based drugs, satraplatin is thought to exert its biological activity via reactive biotransformation products that bind to DNA causing the inhibition of DNA replication, cell cycle arrest and induction of programmed cell death [2, 3, 7, 8]. An inherently inert ammine amine platinum(IV) dicarboxylate complex, satraplatin is thought to require reduction to a platinum(II) species before reaction with

its biological target [10]. In support of this mechanism, several different platinum-containing species derived from satraplatin have been detected in the systemic circulation of experimental animals [6, 19] and human subjects [18, 20]. The presence of very little intact parent drug in the bloodstream during experimental and clinical trials has provided further evidence for the extensive biotransformation of satraplatin in vivo.

To investigate the basis for the in vivo biotransformation of satraplatin, a high-performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICPMS) technique was developed in our laboratory to detect satraplatin separately from its biotransformation products in human plasma [5]. This validated technique was used to investigate the possible role of the biotransformation of satraplatin within the bloodstream in the drug biotransformation in vivo. Satraplatin was found to have a very short half-life in human blood in vitro due to its biotransformation by red blood cells.

Materials and methods

Drugs

Satraplatin [JM216, bis-acetato-ammine-dichloro-cyclohexylamine platinum(IV)], JM118 [ammine-dichloro-cyclohexylamine platinum(II)], JM518 [bis-acetato-ammine-hydroxy-chloro-cyclohexylamine platinum(IV)] and JM383 [bis-acetato-ammine-dihydroxy-cyclohexylamine platinum (IV)] were kindly donated by the Johnson Matthey Technology Centre (Sonning, UK). A satraplatin stock solution was made up in 0.9% NaCl at 200 μ*M* before each experiment.

Chemicals

Ortho-phosphoric acid (85% v/v) and nitric acid (90% v/v) were purchased from Riedel-de Haën (Seezle, Germany). Sodium chloride (0.9% w/v) was purchased from Baxter Healthcare (Old Toongabbie, Australia). All solutions for SDS-PAGE were purchased from Bio-Rad Laboratories (Hercules, Calif.). Other materials were obtained from the Sigma Chemical Company (St Louis, Mo.) or Life Technologies (Auckland, New Zealand) unless otherwise indicated. Freshly collected heparinized human blood was used whole or centrifuged at 2000 *g* for 10 min at 4°C to prepare plasma and red blood cells. Red blood cells were reconstituted in 0.9% NaCl to a haematocrit of 0.45. Dulbecco's modified Eagle's medium (DMEM) was supplemented with 20% fetal bovine serum and 2% penicillin-streptomycin-glutamine.

HPLC-ICPMS

Ultrafiltrate and methanol extracts of plasma were analysed for satraplatin and its biotransformation products using an HPLC-ICPMS method as previously described [5]. Briefly, samples were injected into an HPLC system consisting of a 4.6×150 mm Prodigy C_8 column (Phenomenex, Auckland, New Zealand) and a 0.85% phosphoric acid mobile phase (pH 2.5). The mobile phase was run on a step gradient with 25% methanol for the first 10 min, then 40% methanol from 10 to 20 min. Fractions were collected at 1-min intervals and diluted with 1% nitric acid for analysis on the ICPMS. Platinum analysis was undertaken using a Hewlett-Packard HP4500 ICPMS with a Babington (v-groove) nebulizer, and a Scott double-pass spray chamber maintained at 2°C. Platinum data were read by single ion monitoring at 194 and 195 amu. External standards prepared in the sample matrix were used to quantify both platinum and satraplatin.

Drug incubations

Drug incubations were carried out in six-well plates. Blood, medium, plasma red blood cells, and 0.9% NaCl or water were placed in separate wells and incubated in an atmosphere of 5% CO_2/95% air at 37°C for 10 min before the addition of satraplatin. Immediately after the addition of satraplatin and at various times thereafter, samples were taken from the incubations and placed immediately on ice. Plasma was prepared by centrifugation at 2000 g and 4°C for 5 min. To prepare methanol extracts, plasma and other incubation fluids were added to an equal volume of ice-cold methanol (100 to 300 μl). Methanol extracts were left at –20°C for at least 18 h before centrifugation to remove precipitated proteins and analysis of the supernatant on the HPLC-ICPMS system. Satraplatin has previously been shown to be stable under these conditions [5]. To obtain plasma ultrafiltrate, aliquots of plasma were diluted to 1 ml with 0.9% NaCl and filtered through a Centristart I membrane with a 10-kDa molecular weight cut-off (Sartorius, Goettingen, Germany) by centrifugation at 2000 *g* for 15 min at 4°C. To separate the red blood cell membrane and cytosol, the red blood cells were washed three times in 0.9% NaCl, resuspended in 1 ml 0.9% NaCl, then sonicated on ice for 2×20 s and centrifuged at 13,000 *g* at 4°C for 10 min. Wash solutions were analysed for the presence of platinum, confirming that platinum was not being lost through this process. Red blood cell membrane fractions and cytosol were resuspended in 1 ml 0.9% NaCl. Total blood, plasma, plasma ultrafiltrate, red blood cells, cytosol and membrane preparations were assayed for platinum on the ICPMS by adding 100 μl of each sample to 2.4 ml MilliQ water.

SDS-PAGE

A SDS polyacrylamide gel was made up using 11% acrylamide. A 20-μl aliquot of a broad molecular weight marker was loaded in lane 1 together with samples diluted 1:3 with sample buffer in neighbouring lanes. The gel was run at 200 V for approximately 1 h in electrophoresis buffer, then fixed and stained with Coomassie blue. Molecular weights of the bands on the gel were estimated using the molecular weight marker. Following this, the bands were cut out and dissolved in 70% nitric acid overnight. Samples were then heated at 90°C for 2 h before being diluted in water and the levels of platinum in each sample determined by ICPMS.

Statistics

Concentration versus time data were analysed by non-linear regression using GraphPad Software (San Diego, Calif.). The half-life of satraplatin was defined as 0.69/k where k is the rate constant of the one-phase exponential decay of satraplatin. The statistical significance of differences between groups was determined from 95% confidence intervals.

Results

Stability of satraplatin

To determine the stability of satraplatin during in vitro incubation in various biological fluids, samples of incubation fluid were analysed for satraplatin at different times by HPLC-ICPMS (Fig. 1A). In fresh human whole blood, satraplatin concentrations fell very rapidly

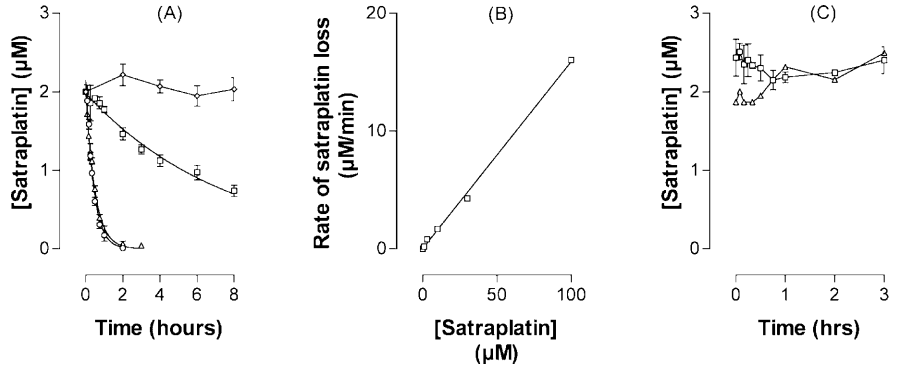

Fig. 1. **A** Time-course of satraplatin concentration during incubation at 37°C in cell culture medium (*diamonds*), fresh human plasma (*squares*), fresh human whole blood and (*circles*), human red blood cells resuspended in 0.9% NaCl (*triangles*). **B** Relationship between satraplatin concentration and rate of satraplatin loss in fresh human whole blood. **C** Time course of satraplatin concentration during incubation at 37°C in 0.9% NaCl (*squares*) and water (*triangles*)

resulting in a half-life for the disappearance of the drug of only 6.3 min (95% confidence interval (CI), 5.9 to 6.7 min). In fresh human plasma, satraplatin was more stable and had a half-life of 5.3 h (95% CI, 4.7 to 6.2 h). In fully supplemented DMEM, satraplatin had a half-life of 22 h (95% CI, 19 to 28 h).

To determine the possible role of red blood cells in mediating the very rapid disappearance of satraplatin in whole blood, red blood cells were prepared from whole blood and resuspended in 0.9% NaCl. After addition of the drug, satraplatin disappeared very rapidly in the presence of red blood cells with similar kinetics to those shown with whole blood ($t_{1/2}$ 13 min; 95%CI, 11 to 17 min; Fig. 1A). The rate of loss of satraplatin from whole blood was linearly related to satraplatin concentrations ranging from 1 to 100 μ*M*, with a rate constant of 0.1587 min^{-1} (95% CI, 0.1490 to 0.1683 min^{-1}; Fig. 1B). There was no loss of satraplatin from drug solutions made up in water or 0.9% NaCl at 37°C for at least 3 h (Fig. 1C), indicating that chloride ligand exchange reactions did not appear to be involved in the mechanism of drug loss. Together, these results indicated that red blood cells might be responsible for the rapid disappearance of satraplatin from whole blood, via a mechanism that was unsaturable at pharmacologically relevant concentrations.

Satraplatin biotransformation in whole blood in vitro

To determine whether other platinum-containing species were formed from satraplatin in whole blood in vitro, methanol extracts of plasma were prepared 15 s, 5 min and 30 min after the addition of satraplatin to blood. HPLC-ICPMS chromatograms of methanol extracts of plasma taken at these times are shown in Fig. 2. Studies with authentic standards showed that the chromatographic conditions separated satraplatin (peak d, fraction 18) from three of its biotransformation products, JM118 (peak b, fraction 8), JM383 (peak a, fraction 4) and JM518 (peak c, fraction 15; Fig. 2A). The stock solution of satraplatin contained only the parent compound before being added to the blood (Fig. 2B). Two new platinum-containing peaks appeared 15 s after the addition of satraplatin to whole blood in fractions 2 (peak e) and 8 (peak b) of the chromatogram (Fig. 2C). After 5 min all the platinum-containing peaks had reduced in size compared with the 15-s time-point

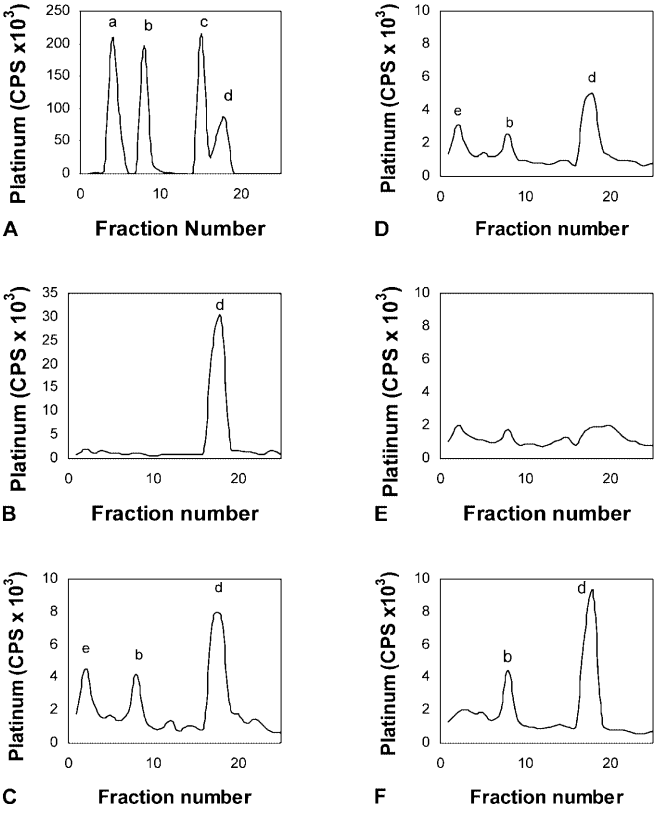

Fig. 2A–F. Platinum chromatograms of: **A** authentic standards; **B** satraplatin stock solution; **C–E** methanol extracts of plasma taken at 15 s (**C**), 5 min (**D**) and 30 min (**E**) after the addition of satraplatin to fresh whole human blood; **F** ultrafiltrate of plasma methanol extract taken 15 s after the addition of satraplatin to fresh human whole blood (*peak a, fraction 4* JM383; *peak b, fraction 8* JM118), *peak c, fraction 15* JM518; *peak d, fraction 18* satraplatin; *peak e, fraction 2* unknown)

(Fig. 2D). After 30 min, all the platinum-containing peaks had disappeared from the chromatogram (Fig. 2E). Although new platinum-containing species appeared in methanol extracts of plasma, their formation did not account for the complete loss of satraplatin that occurred within minutes of the addition of the drug to whole blood.

Characterization of biotransformation products

One of the new platinum-containing peaks that appeared on HPLC-ICPMS chromatograms during the incubation of satraplatin in whole blood coeluted with the authentic standard of JM118 (Fig. 2A, C). The second new platinum-containing peak (peak e) did not coelute with any of the authentic standards and was therefore an unknown metabolite. To characterize the unknown metabolite, plasma methanol extracts containing this species were subjected to ultrafiltration using a membrane with a 10-kDa cut-off. Chromatograms of the ultrafiltrate did not contain peak e, indicating that the unknown platinum-containing species had a molecular weight greater than 10 kDa (Fig. 2F). Further analysis was carried out by SDS-PAGE and ICPMS and showed the presence of platinated proteins in methanol extracts of plasma (Fig. 3). Platinum was associated with a protein band that had a molecular weight of approximately 60 kDa, which is similar to the molecular weight of albumin (Fig. 3). The new platinum-containing species appearing on chromatograms of methanol extracts of plasma were therefore identified as the platinum(II) species known as JM118 and a platinated protein with a similar electrophoretic mobility as serum albumin.

Fate of satraplatin in blood

To account for the rapid loss of satraplatin, the platinum content of blood fractions was analysed after the addition of the drug to fresh human whole blood (Fig. 4). After the addition of drug to whole blood, equilibrium levels of platinum were reached in various blood fractions within 30 min. Satraplatin-derived platinum became rapidly associated with red blood cells, at an accumulation half-life of 9.5 min (95% CI, 7.1 to 14.2 min). At equilibrium, 62% of the total platinum in the blood was associated with the red blood cells, and 38% was associated with the plasma (Fig. 4A). Of the total amount of platinum associated with the plasma, 71% was bound to the plasma proteins while only 29% was found in ultrafiltrates or methanol extracts (Fig. 4B). Of the total amount of platinum associated with red blood cells, the majority was associated with the red blood cell membrane (Fig. 4C). Thus, most of the platinum derived from satraplatin became associated with the red blood cell membrane and plasma proteins within minutes of adding the drug to fresh human whole blood.

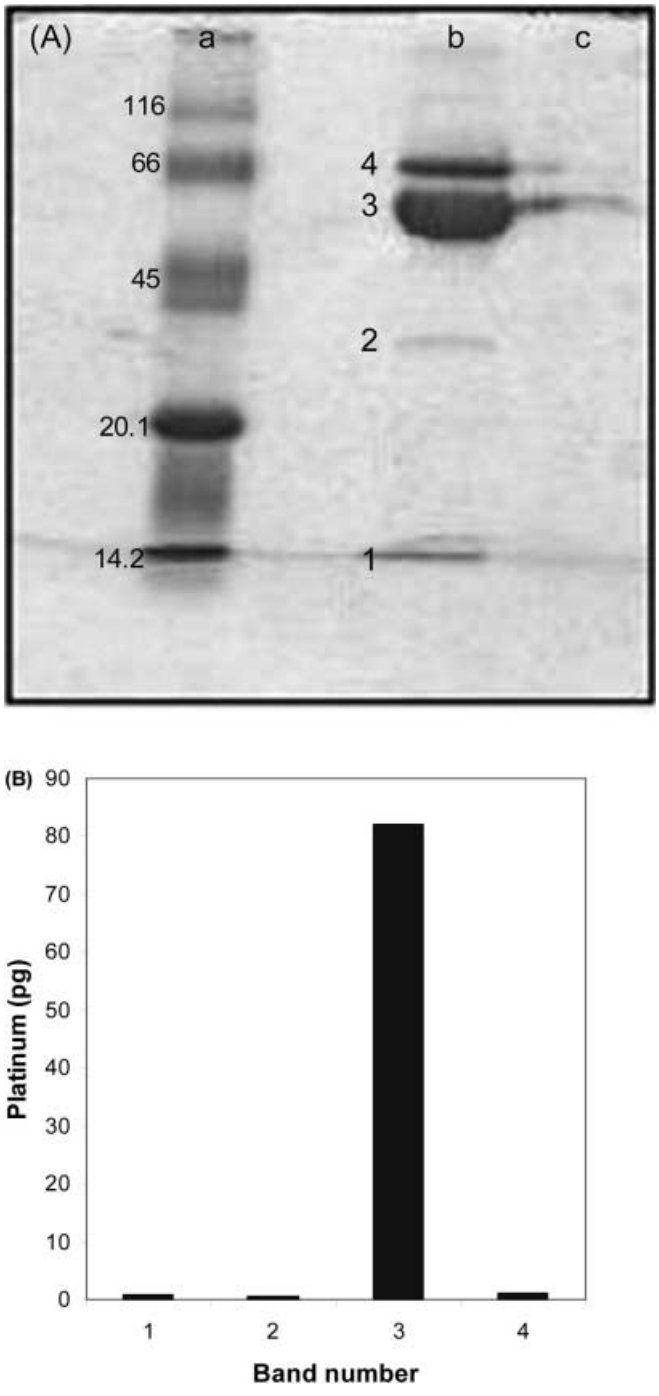

Fig. 3. **A** SDS gel containing molecular weight markers (*lane a*), a methanol extract of plasma taken 15 s after the addition of satraplatin to fresh human whole blood (*lane b*) and ultrafiltrate of the same methanol extract shown in lane b (*lane c*). **B** Platinum content of protein bands (*band 1, 2, 3, 4*) as determined by ICPMS

To investigate the nature of the platinum associated with the red blood cell membrane, satraplatin was incubated with resuspended red blood cells for 30 min at 30 μM. After the red blood cells had been resuspended in drug-free 0.9% NaCl, no platinum appeared in the extracellular fluid for 3 h. The red blood cell membrane was then isolated by sonication and centrifugation and

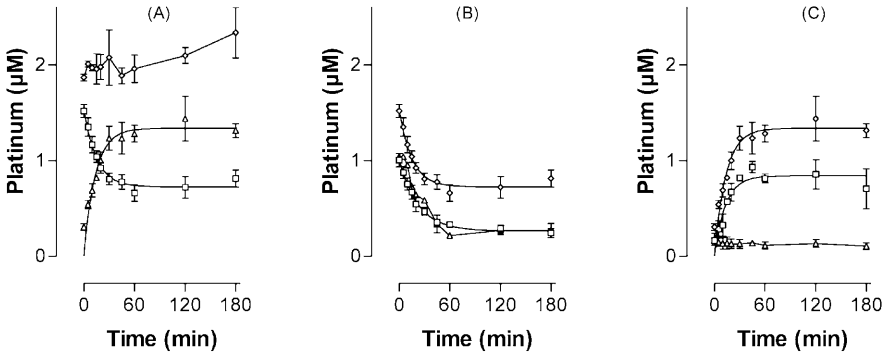

Fig. 4A–C. Time-course of platinum concentration in blood fractions after the addition of satraplatin to fresh human whole blood. **A** Total blood (*diamonds*), plasma (*squares*), red blood cells (*triangles*). **B** Total plasma (*diamonds*), plasma ultrafiltrate (*squares*), plasma methanol extract (*triangles*). **C** Total red blood cells (*diamonds*), red blood cell membrane (*squares*), red blood cell cytosol (*triangles*)

added to an equal volume of methanol. Supernatants of the methanol extracts of red blood cell membranes were analysed for satraplatin and its biotransformation products by HPLC-ICPMS. Platinum chromatograms demonstrated the absence of any methanol-extractable platinum-containing compounds associated with the red blood cell membrane. Thus, the platinum derived from satraplatin that became associated with the red blood cell membrane was in a form that was not exchangeable with methanol or 0.9% NaCl.

Discussion

The results of the experiments described in this paper provide evidence for the very rapid loss of satraplatin from human blood. For example, within 30 min of the addition of satraplatin to fresh human whole blood in vitro, there was no parent compound detectable in the plasma as determined by HPLC-ICPMS. Also, the half-life of satraplatin in fresh human whole blood was only 6.3 min at drug concentrations ranging from 1 to 100 μ*M*, as indicated by the slope of the linear relationship between the rate of satraplatin loss and drug concentration. These findings are consistent with those of other studies demonstrating the presence of very little intact parent compound in the systemic circulation of experimental animals [6, 19] and human subjects [18, 20]. Loss of satraplatin within the bloodstream is therefore a possible explanation for as to why very little parent drug has been found in the systemic circulation during experimental and clinical trials.

The results obtained also provide evidence for the rapid disappearance of satraplatin in whole blood depending on the presence of red blood cells. For example, in whole blood, satraplatin-derived platinum became associated with red blood cells with similar kinetics (accumulation half-life 9.5 min) compared with the loss of free satraplatin from the plasma (disappearance half-life 6.3 min). The amount of platinum associated with the red blood cells accounted for most of the satraplatin lost after the addition of the drug to fresh whole human blood. When red blood cells were purified from whole blood and suspended in 0.9% NaCl, concentrations of satraplatin declined after the addition of the drug with rapid kinetics that were similar to those for the disappearance of the drug in whole blood. In the absence of red blood cells, satraplatin was much more stable, as demonstrated in this study in fresh human blood plasma and cell culture medium, and in other studies of cultured human ovarian carcinoma cells [21]. Taken together, these findings suggest that red blood cells may cause the rapid disappearance of satraplatin from whole blood.

Evidence was also obtained to support the loss of satraplatin occurring by a mechanism involving the chemical transformation of the drug, rather than by the reversible binding of the parent drug molecule by blood components. For example, the formation of new chemical species from satraplatin in whole blood was demonstrated by the appearance on HPLC-ICPMS chromatograms of JM118 and a platinated protein that had similar electrophoretic mobility to serum albumin. It appears that the methanol deproteination of plasma was not completely effective, as some serum proteins remained in the supernatant. Previous studies have also demonstrated the presence of proteins in solvent extracts of plasma [9, 13]. Although satraplatin was soluble in aqueous solution and methanol, the red blood cell membrane-associated platinum was not exchangeable in methanol or 0.9% NaCl. Similarly, the plasma protein-associated platinum was not exchangeable in methanol. Together, these results suggest that satraplatin undergoes biotransformation in whole blood resulting in the generation of new forms of platinum, including JM118 and platinum that becomes irreversibly bound to plasma proteins and the membranes of red blood cells.

Questions arise about the nature of the biotransformation pathway that is responsible for the loss of satraplatin from the bloodstream. Reduction of the parent platinum(IV) compound to JM118, and the reaction of this platinum(II) species with DNA, has been proposed as a possible mechanism of action of satraplatin [19, 20]. The appearance of JM118 in the plasma after the addition of satraplatin to fresh human whole blood and in the systemic circulation of experimental animals [6, 19] and human subjects [18, 20], suggests that this platinum(II) species may be an intermediate in the biotransformation pathway. To account for the rapid loss of satraplatin, JM118 would have to be removed from the plasma almost as rapidly as being formed from satraplatin, since free JM118 appeared in the plasma only at low levels and for a short time. However,

previous studies of authentic standards have shown that JM118 has a long life-time in the systemic circulation compared with satraplatin and relatively slow reaction kinetics with plasma proteins in vitro [19]. The presence of large amounts of free cyclohexylamine ligand in the urine and plasma ultrafiltrate of dogs and rats given oral ^{14}C-labelled satraplatin [6] suggests that removal of the cyclohexylamine group from the platinum atom of satraplatin may also occur during the biotransformation. Overall, it is presently unclear whether the red blood cell- and plasma protein-associated platinum, generated during the in vitro incubation of satraplatin in whole blood, originates directly from the parent drug, JM118, or some other platinum-containing decomposition product.

Questions also arise about the mechanism of the red blood cell-dependent biotransformation of satraplatin in human blood. Unlike other dichloroplatinum drugs [2], the replacement of chloride ligands by water in the intracellular environment is unlikely to be a critical step in the red blood cell-mediated biotransformation of satraplatin because the drug was shown to be very stable in water. Redox reactions between haemoglobin and satraplatin could be a possible mechanism of drug biotransformation by red blood cells, since other platinum-based drugs have enhanced metabolic activation in the presence of haemoglobin [23, 24]. Ferrous haemoglobin could be an abundant source of reducing equivalents for satraplatin because of its high concentration and maintenance in the reduced ferrous oxidation state under physiological conditions. The glutathione content of red blood cells could also play a role in the biotransformation since reduction and substitution reactions between satraplatin and glutathione have been documented previously [12], and the biotransformation of satraplatin by other cell types is dependent on glutathione levels [21].

Whatever the mechanism, red blood cell-mediated biotransformation of satraplatin could have implications for the clinical pharmacokinetic behaviour of the drug. For example, high levels of non-exchangeable platinum might be expected to accumulate in red blood cells during clinical treatment with satraplatin. Although several clinical pharmacokinetic studies of satraplatin have been carried out [1, 11, 14, 15, 22], determinations of the platinum content of red blood cells from patients during treatment with satraplatin have not been reported to date. Satraplatin-derived platinum has been demonstrated in these studies to become rapidly and extensively associated with plasma proteins during clinical treatment, as indicated by averaged plasma-free fractions (ratio of plasma ultrafiltrate and total platinum AUC) of approximately 10% [1, 11, 14, 15, 22]. Protein-reactive forms of platinum generated within the bloodstream during clinical treatment with satraplatin may arise because of the biotransformation of the drug by red blood cells.

In conclusion, the disappearance of satraplatin from whole blood in vitro with a half-life of only 6.3 min appears to depend on the presence of red blood cells. Generation of red blood cell membrane- and plasma protein-associated platinum, along with the transient appearance of JM118, provided evidence that the disappearance of satraplatin is due to biotransformation in the bloodstream. The presence of very little intact parent compounds in the systemic circulation, as found in experimental [6, 19] and clinical trials [18, 20], may be due to the rapid biotransformation of satraplatin by red blood cells.

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