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ORIGINAL ARTICLE

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Satraplatin activation by haemoglobin, cytochrome C and liver microsomes in vitro

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Abstract *Background*: Satraplatin is thought to require reduction to a reactive Pt(II) complex (JM118) before exerting chemotherapeutic activity. In this study, we investigated the role of heme proteins in this reductive activation of satraplatin. *Methods*: Satraplatin was incubated in solution with heme proteins and liver microsomes. The oxidation state of heme iron was monitored by visible absorption spectrometry. Satraplatin and JM118 were detected using a sensitive and specific HPLC-ICPMS assay. *Results*: Satraplatin was stable in solutions containing haemoglobin, cytochrome c, glutathione, liver microsomes or NADH alone. However, in solutions containing haemoglobin plus NADH, satraplatin disappeared with a half-life of 35.8 mins. Under these conditions, satraplatin was reduced to JM118 and haemoglobin was oxidised to methaemoglobin. The reaction between haemoglobin and satraplatin was inhibited by carbon monoxide or by cooling the reaction solution. Cytochrome c and liver microsomes also reduced satraplatin to JM118 in a manner that depended upon the presence of NADH and was inhibited by carbon monoxide. *Conclusion*: This study has identified a mechanism of satraplatin activation involving metal-containing redox proteins and the transfer of electrons to the Pt(IV) drug from protein-complexed metal ions. Heme proteins may act by this mechanism as reducing agents for the activation of satraplatin in vivo.

Keywords Platinum drugs · Satraplatin · Biotransformation · Heme proteins · JM118 · HPLC-ICPMS

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Abbreviations ICPMS: Inductively coupled plasma mass spectrometry · 95% CI: 95% confidence intervals · NADH: Nicotinamide adenine dinucleotide · HPLC: High performance liquid chromatography

Introduction

Satraplatin is a platinum-based anti-tumour drug currently in clinical trials. The drug has shown clinical activity in small-cell lung [9], ovarian [20] and prostate cancer [18]. In a randomised trial in hormone-refractory prostate cancer, satraplatin plus prednisone significantly prolonged disease progression-free survival compared to prednisone alone [31]. A definitive phase III trial in treatment-refractory prostate cancer, and other trials in other tumour types, are now underway. Satraplatin has several unique features compared with the existing clinical platinum-based drugs [17]. The drug is a quadrivalent platinum(IV) complex, whereas the existing clinical platinum drugs are square planar platinum(II) complexes. Satraplatin is given by mouth whereas cisplatin, carboplatin and oxaliplatin are all given by intravenous infusion. It is more lipophilic and more chemically stable than the other agents, properties that may be important for oral administration and bioavailability. Satraplatin has a single ammine and a single cyclohexylamine as stable ligands in contrast to the symmetrical diammine stable ligands of cisplatin and carboplatin. The asymmetrical stable ligands of satraplatin alter its DNA adduct profile [15] resulting in the potential for a different clinical spectrum of antitumour activity than with cisplatin and carboplatin.

Previous studies of the mechanism of action of platinum(IV) drugs suggest that the parent drugs must be reduced before reacting with DNA and exerting chemotherapeutic activity [2, 7]. The reduction of satraplatin results in the loss of its two trans acetato ligands and the formation of a platinum(II) complex known as JM118

[17]. This platinum species is thought to be a major active metabolite of satraplatin. JM118 is known to react with DNA at the same sites as cisplatin, but as a result of its asymmetrical stable ligands, two conformational isomers are formed [15]. Compared to cisplatin-DNA adducts, the lesions formed by JM118 are bulkier, more efficient at inhibiting trans-lesional DNA synthesis [32] and less easily recognised by DNA-mismatch repair [8] or high mobility group proteins [15, 33]. JM118 has been identified in the plasma of animals [11, 23] and patients [22, 24] treated with satraplatin. However, the mechanisms involved in the conversion of satraplatin to JM118 have not been studied in detail to date. It is not known what biological reducing agents are responsible for activating satraplatin in vivo or where in the body the drug is converted to JM118 or other active species.

Previously we had developed an analytical technique for detecting satraplatin, JM118 and other satraplatin metabolites using reverse phase HPLC with direct connection online to inductively coupled plasma mass spectrometry (ICPMS) for platinum detection. This analytical method is precise, accurate, sensitive and specific for determining these platinum complexes in methanol extracts of human plasma [10]. In a previous study, the method was used to investigate the stability of satraplatin in whole blood and other bio-fluids in vitro [3]. Satraplatin concentrations in methanol-deproteinised plasma declined with a half-life of 6.3 min during in vitro incubation in fresh whole human blood at 37°C. The presence of red blood cells appeared to be necessary for the rapid disappearance of satraplatin, its conversion into JM118 and irreversible binding of platinum in whole blood. This finding led us to consider what components of the red blood cell were reacting with satraplatin. Haemoglobin and glutathione are potential reducing agents that are abundant in red blood cells. In the current study, we incubated satraplatin together with haemoglobin and glutathione, and determined the activation of the drug by measuring the production of JM118 and the loss of the parent compound. In addition, satraplatin was incubated with liver microsomes and cytochrome c to further investigate the role of heme proteins in this reductive activation.

Materials and methods

Chemicals

Satraplatin and JM118 were kindly loaned by the Johnson Matthey Technology Centre (Sonning, Oxfordshire, UK). 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). Other chemicals including NADH, bovine haemoglobin, horse heart cytochrome c, sodium cyanide, potassium ferricyanide, *para*-hydroxymercuribenzoic acid (pHMB) and *N*-ethylmaleimide (NEM) were obtained from the Sigma Chemical Company (St Louis, MO, USA). Carbon monoxide (99.9%) was purchased from BOC gases (Auckland, New Zealand).

Incubation solutions

Commercial bovine haemoglobin, which contained approximately 90% methaemoglobin, was made up at 1 g/l in phosphate buffered saline (PBS) (pH 7.4). Haemoglobin was incubated with 10 mM NADH for 96 h at 4°C following the method of Stratton [30]. After 96 h, approximately 40% of the haemoglobin had been converted to the ferrous form, which made a final starting concentration of oxyhaemoglobin of 0.4 g/l. To remove excess NADH and NAD^+, haemoglobin was dialysed against large volumes of 0.2 M phosphate buffer, deionised water and PBS using a membrane with a 13 kDa molecular weight cut off. Horse heart cytochrome c was diluted in PBS (pH 7.4) to a final concentration of 1 g/l.

Human liver samples were obtained from patients undergoing resections for colorectal metastases in the liver. Studies with liver samples were approved by the Auckland Ethics Committee. Livers were stored at −70°C until use. Microsomes were prepared from liver samples by differential centrifugation using the method of Gill et al. [12]. Microsomal pellets were resuspended in phosphate buffer and stored in aliquots at −70°C. Microsomal protein concentration was measured using a bicinchoninic acid assay [29], with bovine serum albumin as the protein standard. For incubations with satraplatin, microsomes were diluted in PBS (pH 7.4) to a final protein concentration of 1 g/l, as commonly used for in vitro drug metabolism studies [13].

Drug incubations

Solutions of haemoglobin, cytochrome c or liver microsomes (1 g/l) were incubated in an atmosphere of 5% CO_2/95% air at 37°C for 10 min in the presence or absence of 10 mM NADH, before the addition of drug. The initial concentration of satraplatin was 2 μM, which is a pharmacologically relevant concentration of drug [20]. Drug incubations were carried out in six-well plates with a volume of 1 ml. Immediately after drug addition and at various time intervals, samples (100 μl) were taken from the incubations, added to an equal volume of ice cold methanol and placed immediately on ice. Solutions 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. Total platinum content of the solution was measured on the ICPMS after dilution of the samples in 0.1% nitric acid. The pH of solutions was monitored during the incubation with satraplatin, and at each time point was between 7.32 and 7.38. In experiments investigating the effect of inhibitors, proteins were pre-treated before the incubation by

exposure to a rapid stream of carbon monoxide for 10 min before incubation, a sulfhydryl group blocking agent, NEM (for 2 h at 45 μM), a cytochrome B5 reductase inhibitor, pHMB (for 30 min at 0.2 mM) and heat (for 10 min at 100°C), according to published methods [13] or in excess to achieve maximum inhibition.

HPLC-ICPMS

To detect satraplatin and its biotransformation products, methanol extracts of incubation solutions were analysed using an HPLC-ICPMS method as previously described [10]. Briefly, samples were injected onto a 4.6×150 mm Prodigy C_8 column (Phenomenex, Auckland, New Zealand) and eluted with a 0.85% phosphoric acid and methanol mobile phase (pH 2.5). The mobile phase ran on a step gradient, with 25% methanol for the first 10 min, then 40% methanol from 10 to 20 min. The HPLC eluate was introduced directly into an ICPMS for analysis using a 10 cm section of 0.25 mm diameter PEEK tubing. Platinum detection was undertaken using a Hewlett-Packard HP4500 ICPMS with a Babington (v-groove) nebuliser, and a Scott double-pass spray chamber maintained at 2°C. Platinum data was read by single ion monitoring at 194 and 195 amu. External standards prepared in the sample matrix were used to quantify all drug concentrations.

Visible absorption spectrometry of haemoglobin solutions

A Hewlett Packard 8452A diode array spectrophotometer was used. Methaemoglobin was measured as a percentage of total haemoglobin using the one wavelength method of Salvati and Tentori [27]. Briefly, the absorbance of the sample was measured at 630 nm before and after the addition of sodium cyanide. Complete conversion of the haemoglobin to methaemoglobin was then achieved by the addition potassium ferricyanide to the original sample. The change in absorbance at 630 nm of this solution before and after the addition of sodium cyanide was also measured. Methaemoglobin concentration, as a percentage of total haemoglobin was then calculated by dividing the change in absorbance of the solution by its change in absorbance after conversion to methaemoglobin. During incubations, changes in haemoglobin spectra were monitored over a range of wavelengths (470–670 nm). Changes in oxyhaemoglobin concentration were monitored at 542 and 576 nm and changes in methaemoglobin concentration were monitored at 500 and 630 nm.

Statistics

Concentration versus time data was analysed by non-linear regression using GraphPad® Software (California, USA). 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 concentration of drugs at two hours is shown as mean ± SEM for at least three experiments. Statistical significance of the differences between groups was determined using one way analysis of variance. P values of < 0.05 were considered significant.

Results

Haemoglobin

Visible absorption spectrometry of the starting solution of haemoglobin (15.5 μM in PBS pH 7.4) showed mainly methaemoglobin (Fig. 1a). After adding NADH (10 mM) and standing for 96 h at 4°C, absorption peaks appeared at 542 and 576 nm, which was consistent with conversion of methaemoglobin to oxyhaemoglobin (Fig. 1a). By the assay of Salvatti et al. [27], it was determined that approximately 40% of the methaemoglobin had been converted to oxyhaemoglobin at this time.

In the solution containing methaemoglobin alone, satraplatin (2 μM) was stable for at least 3 h (Fig. 1b). However, in the solution containing haemoglobin plus NADH, satraplatin disappeared with a half-life of 35.8 min (95% CI, 25.5–60.1 min), and during this time was converted to other platinum-containing species (Fig. 1c). The largest new platinum-containing peak appearing on platinum chromatograms had a retention time of 7.8 min and co-eluted with an authentic reference standard of JM118. The other platinum peaks did not co-elute with any known compounds. After two hours, the concentration of platinum remaining freely soluble in the haemoglobin plus NADH solution accounted for 49.5 ± 8.3% of the total drug because the remainder of the platinum had become irreversibly bound to haemoglobin. The platinum remaining free in the solution at two hours was either in the form of unchanged satraplatin (13.7 ± 3.4% of added drug), JM118 (25.7 ± 3.6% of added drug) or unknown platinum species (8.7 ± 1.1% of added drug). Visible absorption spectra of the solution containing haemoglobin plus NADH were obtained before and 2 h after the addition of satraplatin. At baseline there were absorption peaks at 500 and 630 nm, and at 542 and 576 nm, which were the absorbance maxima for methaemoglobin and oxyhaemoglobin respectively. After 2 h incubation with satraplatin, there was increased absorption at 500 and 630 nm and decreased absorbance at 542 and 576 nm compared to baseline (Fig. 1d). These changes in absorption spectra were consistent with oxyhaemoglobin being oxidized to methaemoglobin during its reaction with satraplatin.

Factors affecting the loss of satraplatin from solutions containing haemoglobin and NADH were investigated. The haemoglobin solution was dialyzed to remove the NADH and NAD^+, and was also pretreated

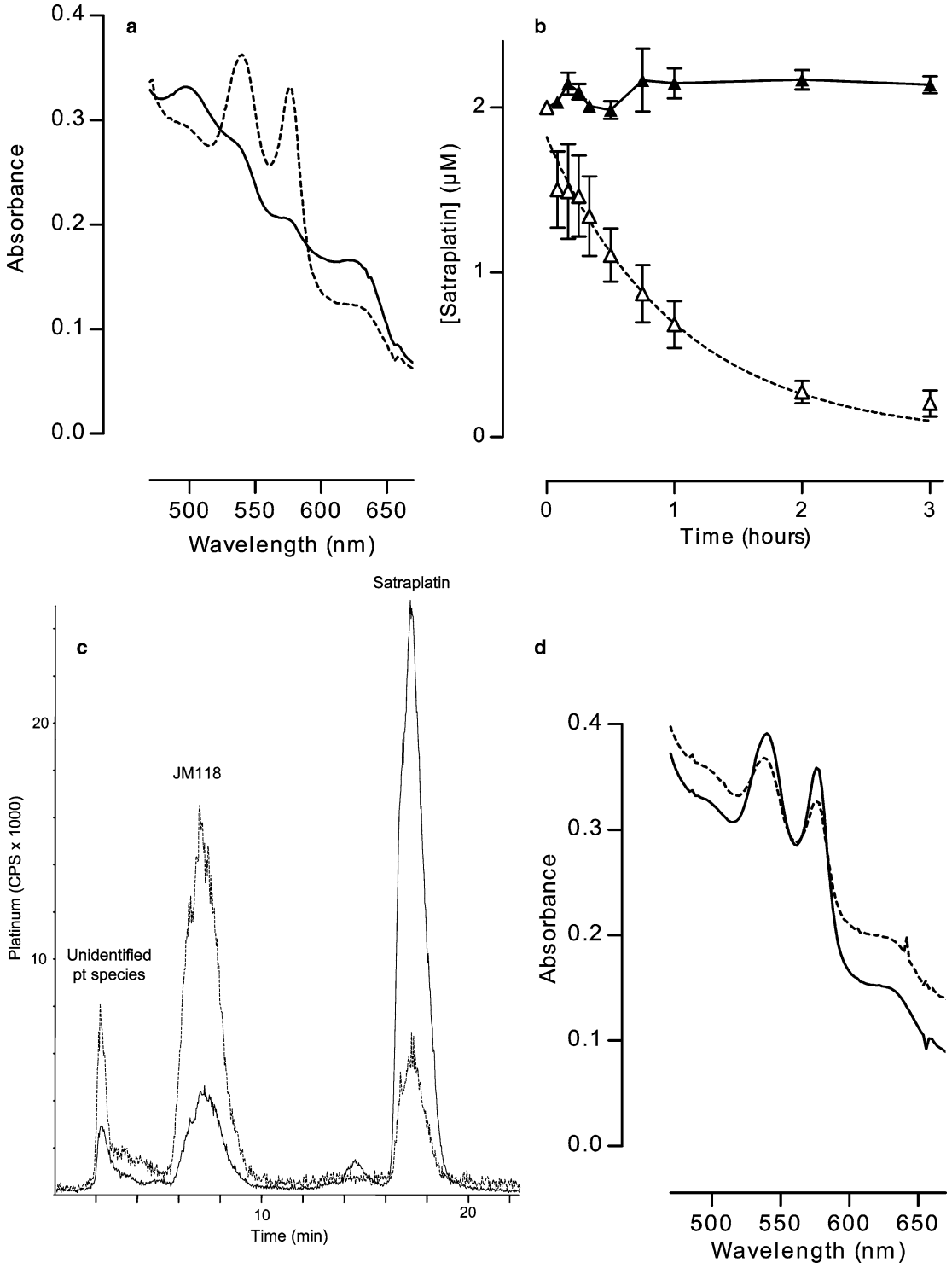

Fig. 1 **a** Visible absorption spectra (470–670 nm) of solutions containing haemoglobin alone (*solid line*) or haemoglobin plus NADH (*dashed line*) before addition of satraplatin. Note absorbance maxima for methaemoglobin (500 and 630 nm) and oxyhaemoglobin (542 and 576 nm). **b**. Stability of satraplatin in solutions containing haemoglobin alone (*solid line, filled triangles*) or haemoglobin plus NADH (*dashed line, open symbols*). Values shown are mean ± SE of four experiments. **c** Platinum chromatograms of solutions containing haemoglobin alone (*solid line*) or haemoglobin plus NADH (*dashed line*) 2 h after adding satraplatin and incubation at 37°C. **d** Visible absorption spectra (470–670 nm) of solutions containing haemoglobin plus NADH before (*solid line*) or after (*dashed line*) addition of satraplatin (2 μM) and 2 h incubation at 37°C. Note absorbance maxima for methaemoglobin (500 and 630 nm) and oxyhaemoglobin (542 and 576 nm)

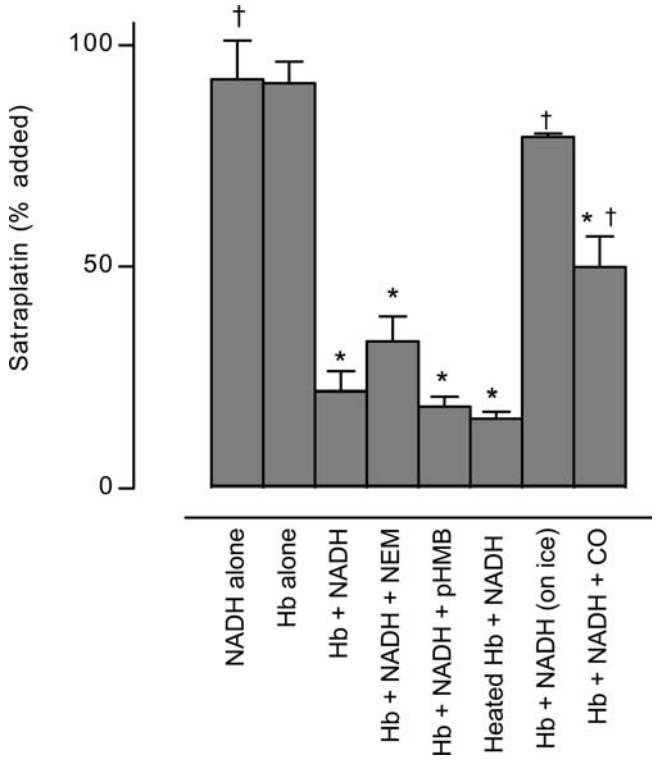

Fig. 2 Factors affecting loss of satraplatin from haemoglobin solutions. Satraplatin (2 μM) was incubated for 2 h at 37°C or on ice in solution with NADH alone, haemoglobin alone or haemoglobin plus NADH with or without pretreatment with NEM, pHBM, heat or carbon monoxide. Values shown are mean ± SE of at least three experiments; * $P < 0.01$ compared to "Hb alone"; † $P < 0.01$ compared to "Hb + NADH"

with a sulfhydryl group blocking agent, NEM (for 2 h at 45 μM), a cytochrome B5 reductase inhibitor, pHMB (for 30 min at 0.2 mM), heat (for 10 min at 100°C) and carbon monoxide (for 10 min in 99.99% CO) prior to adding satraplatin. Although, satraplatin had been stable when incubated with haemoglobin alone or NADH alone (Fig. 2), there was significant loss of the parent drug upon the addition of 10 mM NADH to the dialysed haemoglobin solution (recovery of added drug after 2 h was 21.9 ± 4.5%; $P < 0.01$). Pretreatment with NEM, pHMB or heat had no effect on the loss of satraplatin. However, the loss of satraplatin was significantly inhibited when the solution containing haemoglobin plus NADH was pretreated with carbon monoxide ($P < 0.01$), and when the drug incubation was performed on ice ($P < 0.01$).

Cytochrome c and glutathione

Satraplatin (2 μM) was also incubated with cytochrome c (1 g/l) and glutathione (2 mM) in PBS (pH 7.4) with or without NADH (10 mM). Satraplatin was stable in solution with cytochrome c alone. However, in solution with cytochrome c plus NADH, satraplatin concentrations decreased significantly over a two hour period ($P < 0.01$) (Fig. 3a). Loss of satraplatin in solutions containing cytochrome c plus NADH was associated with production of JM118 (Fig. 3b) and was inhibited by carbon monoxide ($P < 0.01$) (Fig. 3a). In contrast, satraplatin was stable in solution with glutathione with or without NADH. After 2 h, recovery of added satraplatin was 99.6 ± 3.7% from glutathione alone and 94.6 ± 9.9% from glutathione plus NADH.

Liver microsomes

Satraplatin (2 μM) was incubated in solutions containing 1 mg/ml of human liver microsomal protein pooled from four individual livers in PBS (pH 7.4) at 37°C, with or without NADH (10 mM). Satraplatin was stable during incubation with human liver microsomes alone for at least 2 h (Fig. 4a). However, in a solution containing liver microsomes plus NADH, satraplatin concentrations remaining after 2 h were significantly reduced compared to liver microsomes alones ($P < 0.01$). In the solution containing liver microsomes plus NADH, the loss of satraplatin was significantly inhibited by carbon monoxide ($P < 0.05$) (Fig. 4a). JM118 was formed during the incubation of satraplatin with liver microsomes plus NADH (Fig. 4b).

Discussion

The results of this study point to the involvement of iron atoms of the heme complexes of haemoglobin, cytochrome c and liver microsomal cytochrome P450 enzymes in the biotransformation of satraplatin. Evidence for the involvement of heme iron included the production of methaemoglobin, the inhibitory effect of carbon monoxide and the requirement for NADH for satraplatin biotransformation. Using visible absorption spectrometry, we showed that oxyhaemoglobin was oxidized to methaemoglobin during the incubation of satraplatin with haemoglobin and NADH. This finding can be rationalised by a mechanism involving the transfer of electrons to satraplatin from the ferrous irons on the heme complex of haemoglobin, resulting in the reduction of satraplatin concurrently with the oxidation of haemoglobin. Under these conditions, haemoglobin was oxidized at a faster rate than the reduction of methaemoglobin by NADH because the levels of methaemoglobin increased during the experiment.

It is well known that carbon monoxide binds to heme iron and that it inhibits heme enzymes [21]. In this study we showed that the pretreatment of haemoglobin, cytochrome c and liver microsomes with carbon monoxide significantly inhibited the biotransformation of satraplatin further implicating heme iron in the reaction mechanism. In addition, NADH may have interacted

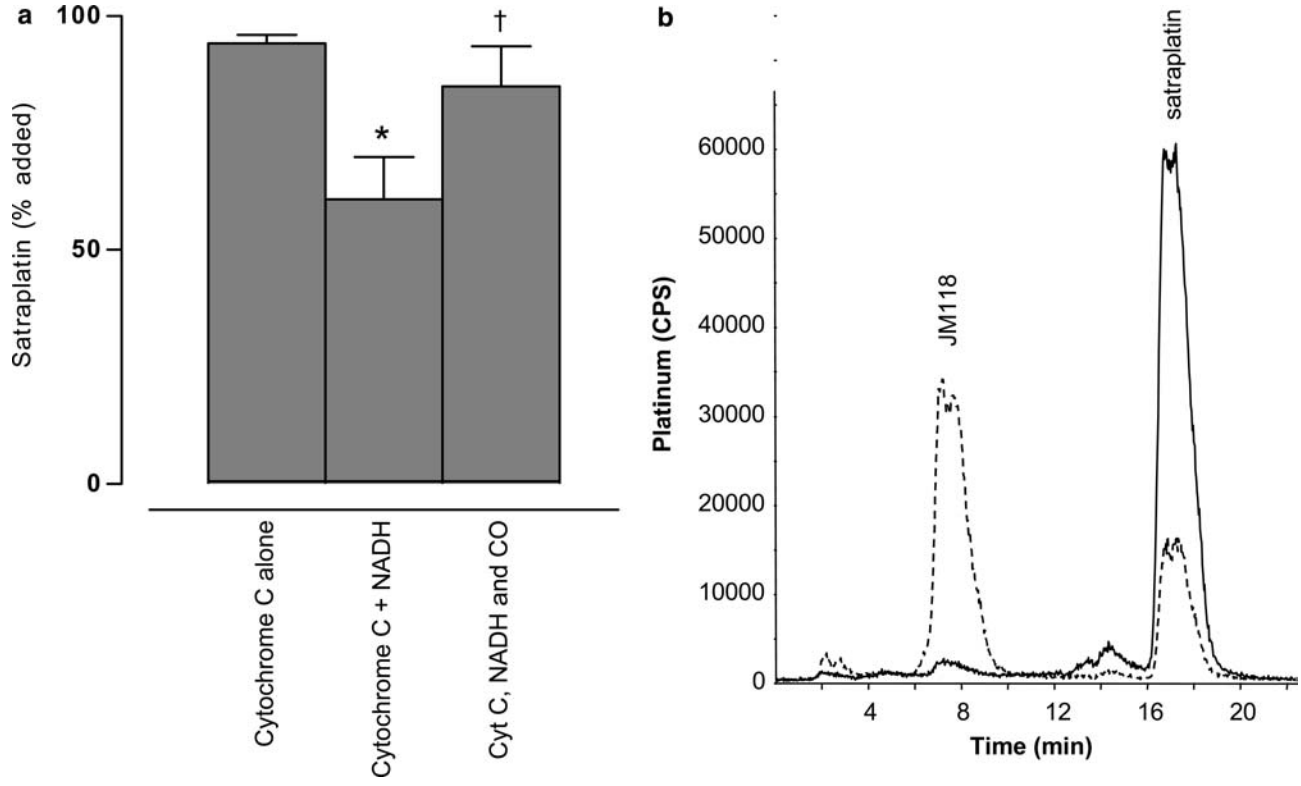

Fig. 3 **a** Stability of satraplatin in solutions containing cytochrome c with or without NADH and prior treatment with carbon monoxide. Satraplatin concentration was determined after 2 h incubation at 37°C. Values shown are mean ± SE of at least three experiments; * $P < 0.01$ compared to "Cytochrome c alone". † $P < 0.01$ compared to "Cytochrome c + NADH". **b** Platinum chromatograms of solutions containing cytochrome c with (*dashed line*) or without 10 mM NADH (*solid line*) 2 h after adding satraplatin and incubation at 37°C

indirectly with satraplatin via the heme proteins. It has been shown that NADH reduces methaemoglobin [30] by donating electrons to heme iron. This reduction of heme iron by NADH will have increased the availability of heme protein-complexed ferrous ions for electron transfer reactions with satraplatin. Furthermore, NADH is an allosteric effector of haemoglobin [4] that binds to the diphosphoglycerate binding site thereby promoting the formation of reduced unliganded haemoglobin, which was the form of haemoglobin that was reactive with satraplatin. A direct chemical reaction between satraplatin and NADH was unlikely because satraplatin was stable in NADH alone for at least three hours.

The reductive biotransformation of other platinum(IV) drugs is thought to involve sulfhydryl-containing amino acids, peptides and proteins, and reactions where two molecules of reduced sulfhydryl (RSH) react with a platinum(IV) complex producing an oxidized disulfide-bridged thiol (RSSR) and a platinum(II) analogue. For example, the reduction of tetraplatin to $PtCl_2$(DACH) by extracellular non-protein and protein sulfhydryls occurs very rapidly and is inhibited by the sulfhydryl blocking agent *N*-ethylmaleamide [5]. On a theoretical basis, satraplatin could have reacted in a similar way with the reduced sulfhydryl groups of the cysteine residues of haemoglobin [14], cytochrome c [26] and liver microsomal proteins. However, several findings in our study were inconsistent with a sulfhydryl-dependent mechanism. For instance, we showed that pretreatment of haemoglobin with *N*-ethylmaleamide had no effect on its reaction with satraplatin. In addition, satraplatin was stable for several hours when incubated together with physiological levels (2 mM) of reduced glutathione, a sulfhydryl-containing tripeptide, in the presence or absence of NADH. Furthermore, the finding that carbon monoxide inhibited the reactions of satraplatin with haemoglobin, cytochrome c and liver microsomes is difficult to rationalise on the basis of a sulfhydryl-mediated mechanism alone. The findings of this study can only allow conclusions to be made about presumed reactions occurring between the included components. Glutathione and other sulfhydryl-containing compounds could still have a significant role in the biotransformation of satraplatin in other contexts than those investigated. For example, glutathione conjugation reactions with platinum(II) metabolites, which were not investigated in the current study, may represent a major deactivation pathway for satraplatin [25].

Ascorbate has been used as a model reductant in two previous studies of the kinetics and mechanism of satraplatin reduction [6, 19]. However, the concentrations of ascorbate used in these published studies (5 mM) vastly exceeded levels found in red blood cells and blood

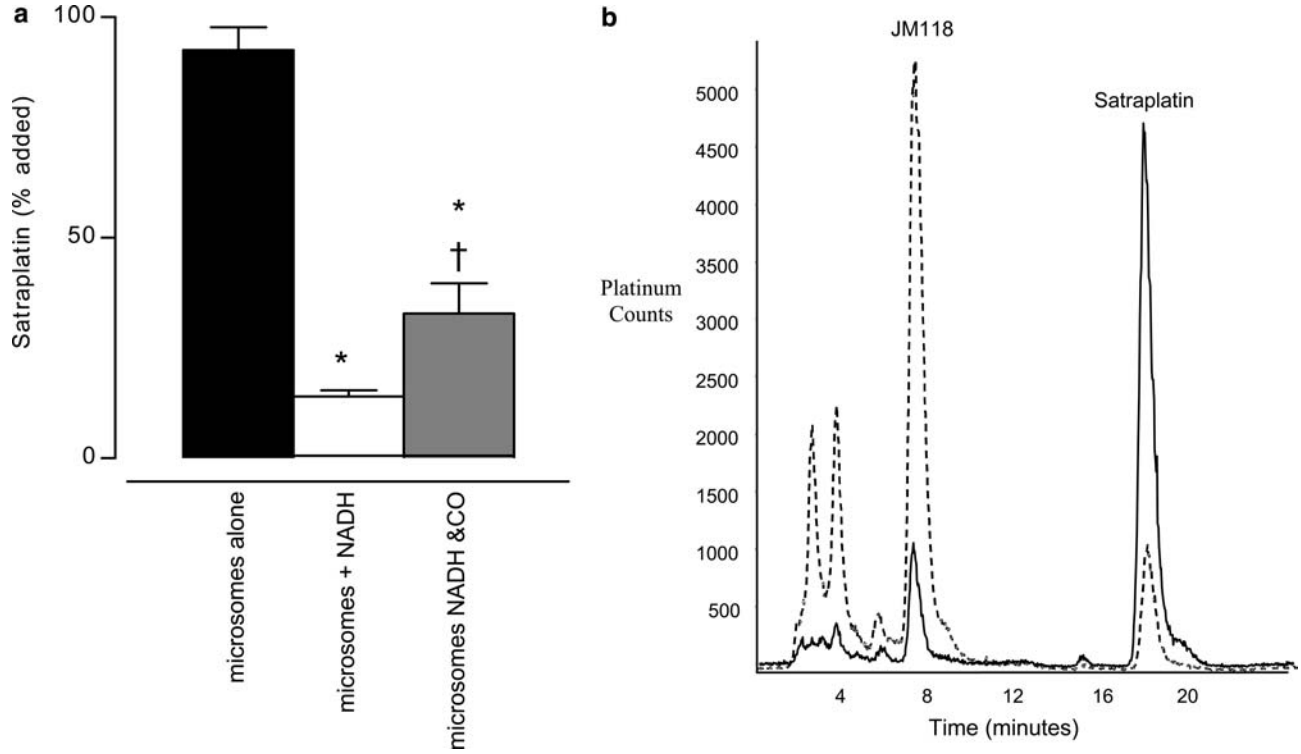

Fig. 4 **a** Stability of satraplatin in solutions containing human liver microsomes with or without NADH and prior treatment with carbon monoxide. Satraplatin concentration was determined after 2 h incubation at 37°C. Values shown are mean ± SE of at least three experiments; * $P < 0.01$ compared to "microsomes alone". † $P < 0.05$ compared to "microsomes + NADH". **b** Platinum chromatograms of solutions containing liver microsomes with (*dashed line*) or without 10 mM NADH (*solid line*) 20 min after adding satraplatin and incubation at 37°C

plasma under physiological conditions (0.025 mM) [28]. Considering its biological concentrations and the kinetics of its reaction with satraplatin, ascorbate is unlikely to contribute significantly to the overall satraplatin reducing capacity of whole blood and red blood cells. In contrast, haemoglobin is an abundant protein (> 2 mM) and maintained in a reduced state under physiological conditions in whole blood, which was the form of haemoglobin that was reactive with satraplatin. The concentration of haemoglobin used in the in vitro experiments presented here were much lower (0.015 mM) than those found in whole blood. Satraplatin disappeared with a half-life of approximately 30 min when incubated in these relatively dilute solutions of haemoglobin with NADH. Because of its physiological abundance and rapid reaction with satraplatin, haemoglobin could be a major reducing agent accounting for a significant portion of the satraplatin reducing capacity of whole blood and red blood cells.

Under clinical treatment conditions, satraplatin may become activated within the blood stream by reacting with haemoglobin. Within the red blood cells, satraplatin could accept electrons from haemoglobin and undergo reductive biotransformation into reactive platinum species such as JM118, which would then react with the red blood cells and plasma proteins or be transported in an activated form to the tumour. We also investigated the biotransformation of satraplatin by other heme proteins, cytochrome c and liver microsomes. We showed that cytochrome c could reduce satraplatin into JM118 in a reaction that was inhibited by carbon monoxide and depended upon the presence of an electron donor NADH. In addition, we found that liver microsomal preparations can reduce satraplatin to JM118. The ability to utilise NADH, coupled with the inhibition by carbon monoxide, indicates that the reaction between satraplatin and liver microsomal preparations may involve a cytochrome b5 reductase-cytochrome P450 mechanism [16] which could explain the reported inhibition of these enzymes by satraplatin [1]. These findings suggest a lack of specificity for satraplatin reduction among different heme proteins and that satraplatin may be activated within the body by various tissues expressing heme proteins, such as the red blood cell and liver.

In conclusion, this study has identified a mechanism of satraplatin activation involving metal-containing redox proteins and the transfer of electrons to the Pt(IV) drug from protein-complexed metal ions. Heme proteins may act by this mechanism as reducing agents for the activation of satraplatin in vivo.

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