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Studies on Cellular Accumulation of Satraplatin and Its Major Metabolite JM118 and Their Interactions with Glutathione

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Abstract: Before the active form of a Pt drug reaches its major pharmacological target in the cell nucleus, the Pt complex has to accumulate in cells, and during its transportation into cells and inside cells, it reacts with various biomolecules. Satraplatin is the first orally administered Pt drug under active clinical investigation. The major metabolite of this Pt^{IV} complex is its Pt^{II} analogue (JM118), which also has significant anticancer properties. Here we report the role of active transport in cellular entry of satraplatin and JM118 and interactions of these Pt complexes with glutathione. The results reveal that the organic cation transporters may play a more important role in the mechanism of cytotoxicity of JM118 than in the cytotoxicity of cisplatin. In contrast, satraplatin is a poor substrate of these transporters. In addition, satraplatin reacts with glutathione with the rate markedly lower than JM118 and cisplatin. Interestingly, satraplatin can be activated by glutathione allowing it to react with DNA, although to a much lower extent than in the case of another Pt^{IV} drug tetraplatin.

Keywords: Cisplatin; satraplatin; JM118; cellular accumulation; cimetidine; glutathione; DNA

Introduction

The *cis*-diamminedichloridoplatinum(II) (cisplatin) (Figure 1) and its direct analogues *cis*-diammine-[1,1-cyclobutanedicarboxylato]platinum(II) (carboplatin), 1*R*,2*R*-diaminocyclohexane oxalatoplatinum(II) (oxaliplatin) and *cis*-diammine glycolatoplatinum(II) (nedaplatin) are major clinically used chemotherapeutic agents that are applied alone or in combination in the treatment of several human tumors. All these platinum drugs are administered intravenously. Satraplatin [bis-acetato-ammine-dichlorido-cyclohexylamineplatinum(IV), JM216, Figure 1] is the first orally administered platinum drug under active clinical investigation, which shows promise in patients with prostate cancer.[1] The lipophilic character of satraplatin favors cellular accumulation of the drug[2] and increases its oral bioavailability. Satraplatin and its major metabolite, *cis*-ammine dichloro(cyclohexylamine)platinum(II) (JM118, Figure 1), have shown antitumor effects *in vitro*, *in vivo*, and in clinical trials.[3] Advantages of using satraplatin as an alternative platinum antitumor agent include the convenience of administration, milder toxicity profile, lack of cross-resistance with cisplatin, and activity in cancers that are nonresponsive to conventional platinum drugs used in the clinic.

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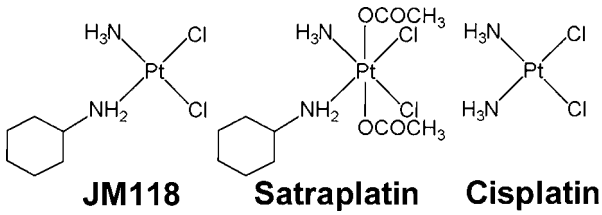

Figure 1. Structures of platinum complexes.

The generally accepted intracellular mechanism by which platinum complexes exert their antitumor effects involves the following initial phases: cellular entry, activation (typically aquation of Pt^{II} compounds and reduction of Pt^{IV} to Pt^{II} complexes), DNA binding, and the initial cellular responses to the DNA damage.[4−6] Pt^{IV} complexes, such as satraplatin, are inert and undergo ligand substitution reactions that are very slow compared with their Pt^{II} counterparts. Therefore, Pt^{IV} complexes must be activated *in vivo* by reduction to the kinetically more labile, and therefore reactive, Pt^{II} analogues that bind more rapidly to their major pharmacological target, DNA.

Unfortunately, Pt^{IV} complexes which demonstrated promising preclinical activity including satraplatin failed in the clinical trials hitherto performed.[7,8] In an effort to design improved Pt^{IV} antitumor agents, it is important to try to elucidate the biochemical and pharmacological factors that affect the cytotoxic properties of existing Pt^{IV} complexes. Satraplatin and its major biotransformation product JM118 differ from cisplatin and its clinically used analogues in that its nonleaving ligands are nonsymmetrical (an ammine and a cyclohexamine, compared with the two ammine groups of cisplatin, carboplatin or nedaplatin or the diaminocyclohexane group of oxaliplatin). These differences may affect some intracellular processes on the level of major pharmacological target of satraplatin and JM118, which is DNA.[9−14] However, before the active form of platinum drug reaches and binds to the DNA in the cell nucleus, thereby triggering responses from the tumor cells to the DNA damage, the platinum complex has to accumulate in cells and avoid detoxification by various biomolecules en route to the nucleus.[15] In the present study, we sought to extend previous work concerning the role of active transport in cellular entry of satraplatin and JM118, and although there is some evidence that GSH may not be the major target of cisplatin in cancer cells, the interactions of satraplatin and JM118 with glutathione (GSH), which is one of the most abundant nonprotein thiols in cells that can readily interact with Pt^{II} and Pt^{IV} drugs, should be studied.[16] The studies described in the present work also include JM118, the major metabolite of satraplatin, also since it is the Pt^{II} complex which confers the cytotoxic mechanism of action.[17]

Material and Methods

Chemicals. Satraplatin and JM118 were prepared as described.[18,19] Purity of satraplatin and JM118 was higher than 95% as established by combustion analysis carried out with a Hewlett-Packard 185 C, H, and N analyzer. Cisplatin and GSH were obtained from Sigma (Prague, Czech Republic) (purity of cisplatin was ~99.9% based on elemental and ICP trace analysis). The stock solutions of platinum

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compounds were prepared at a concentration of 5×10^{-4} M in 10 mM $NaClO_4$ and stored at 4 °C in the dark. Calf thymus (CT) DNA (42% G + C, mean molecular mass ca. 20 000 kDa) was prepared and characterized as described previously.[20,21]

Cellular Platinum Accumulation. Cellular uptake of satraplatin, JM118 and cisplatin was measured in human ovarian carcinoma cisplatin sensitive A2780 cells, cisplatin resistant A2780cisR (cisplatin resistant variant of A2780 cells) and colon carcinoma HCT116 cells. The cells were seeded in 60 mm tissue culture dishes (30 000 per cm^2). After overnight incubation, the cells were treated for 24 h at 37 °C with the platinum compound at a concentration of 10 μM or were cotreated with the platinum compound at a concentration of 10 μM and 1.5 mM cimetidine. The cell monolayers at the end of the incubation with the Pt complex were washed (twice) with ice-cold phosphate buffered saline (PBS), trypsinized and harvested into cold (4 °C) PBS. Cell suspensions were centrifuged, and the pellets were stored in PBS at −80 °C until assayed. Afterward, the pellets were digested with 12 M HCl and platinum content was determined by flameless atomic absorption spectrophotometry (FAAS). The results of cellular platinum uptake were corrected for adsorption effects.[22]

Cytotoxicity. Satraplatin, JM118 and cisplatin were dissolved in DMSO. The stock solutions (100 mM) of the Pt compounds in DMSO were diluted with the aqueous cell culture medium in several steps immediately after their dissolution in DMSO. The final concentration of DMSO in cell culture medium did not exceed 0.25%. The A2780, A2780cisR and HCT116 cell lines were grown in RPMI 1640 medium (GIBCO) supplemented with gentamycin (50 mg/mL, Serva) and 10% heat inactivated fetal bovine serum (GIBCO). The cells were cultured in a humidified incubator at 37 °C in a 5% CO_2 atmosphere and subcultured 2−3 times a week with an appropriate plating density. The cells were seeded in 96-well tissue cultured plates at a density of 10^4 cells/well. After overnight incubation (16 h), the cells were treated with the platinum compounds in the absence or presence of 1.5 mM cimetidine. After 72 h of incubation, 10 μL of a freshly diluted 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution (2.5 mg/mL) was added to each well and the plate was incubated at 37 °C in a humidified 5% CO_2 atmosphere for 4 h. At the end of the incubation period, the medium was removed and the formazan product was dissolved in 50 μL of DMSO. The cell viability was evaluated by measurement of the absorbance at 570 nm, using a Sunrise Tecan Schoeller absorbance reader. The IC_{50} values (defined as concentration of the drug inhibiting cell growth by 50%) were calculated from curves constructed by plotting cell survival (%) versus drug concentration (μM). All experiments were made in quadruplicate. The reading values were converted to the percentage of control (% cell survival).

Reactions of Satraplatin and JM118 with Glutathione. Reactions of GSH with satraplatin, JM118 and cisplatin were investigated by monitoring UV absorption at 260 nm of solutions containing the platinum complex and GSH exactly as described in the previous work;[23,24] the absorbance at 260 nm reflects the presence of platinum−sulfur and disulfide bonds. The kinetic data were fitted by nonlinear regression (GraphPad Prism) to one-phase or two-phase exponential association. The decision that fit to two-phase exponential association was more appropriate for each dependence was made by comparing the fits of two equations by using an *F* test (GrahPad Prism). The platinum compounds (from aged stock solutions equilibrated in 10 mM $NaClO_4$) were mixed with GSH at 37 °C in the medium of 4.6 mM NaCl plus 0.1 mM Tris-HCl buffer, pH 7.4, in the dark. Reactions were initiated by mixing the platinum complex with the buffer followed by immediate addition of GSH. The experiments were made in triplicate.

Glutathione-Mediated Platination of CT DNA in the Presence of Satraplatin in a Cell-Free Medium. Reactions with CT DNA were performed by incubating DNA (0.32 mg/mL) with satraplatin (15 μM) (the molar ratio of free platinum complex to nucleotides at the onset of incubation with DNA was 0.015) in 10 mM $NaClO_4$, pH ∼6 for 24 h at 37 °C. GSH was added so that its concentration in the reaction varied in the range of 0−10 mM. After 24 h the samples were divided into two parts. In the first part, Pt was directly assayed by FAAS. DNA in the other part was precipitated by ethanol, one half redissolved in 10 mM $NaClO_4$, and DNA concentration was determined by absorption spectrophotometry; the other half was dissolved in 0.1 M HCl, and Pt was assayed by FAAS. The percentage of Pt bound to DNA was calculated as the ratio of the concentration of Pt contained in the DNA sample after precipitation by ethanol ($Pt_{precipit}$) and that in the DNA sample before precipitation (Pt_{total}), % bound = $Pt_{precipit}$:Pt_{total}.

Other Physical Methods. Absorption spectra were measured with a Beckman 7400 DU spectrophotometer and quartz cells with a thermoelectrically controlled cell holder and a path length of 1 cm. The FAAS measurements were carried out on a Varian AA240Z Zeeman atomic absorption spectrometer equipped with a GTA 120 graphite tube atomizer; for FAAS analyses, DNA was dissolved in HCl (0.1 M) at 100 °C for 15 min.

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Results

The Effect of an OCT Inhibitor, Cimetidine, on Drug Accumulation and Sensitivity of JM118, Satraplatin and Cisplatin in Cancer Cell Lines. Transport across the plasma membrane of tumor cells represents the first step in the mechanism by which platinum complexes exert their anticancer effects.[25] It was traditionally accepted that antitumor platinum drugs enter cells by simple passive diffusion.[26] However, a large body of evidence was recently presented supporting the view that accumulation of platinum drugs in cells is affected by an active transport of the platinum drug mediated by various transporters, such as copper transporters (CTR1), although their importance in mediating the anticancer activity of these compounds is uncertain.[27] Recently, human organic cation transporters (OCTs) were identified as critical mediators of transport and toxicity of oxaliplatin and a monofunctional Pt^{II} complex, *cis*-diammine(pyridine)chloroplatinum(II), in human tissues.[6,28] The objective of this study was to obtain a deeper insight into the process by which satraplatin and JM118 are transported across the plasma membrane of human tumor cells and to see whether OCTs play a role in transporting these compounds across the cell membrane. We used in these experiments human ovarian carcinoma cell line A2780 and human colon tumor cells HCT116. We chose these tumor cell lines because they have been frequently used to test the cytotoxicity of a number of platinum complexes. Moreover, it has been shown that OCT1 but not OCT2 or OCT3 is expressed in these cells.[28,29] Interestingly, uptake of several antitumor Pt^{II} complexes is competitively inhibited by cimetidine, a known inhibitor of OCTs, including several colon cancer cell lines in which OCT1 but not OCT2 is expressed.[6,28]

In order to infer active transport mediated by OCTs, we measured accumulation of platinum in A2780 and HCT116 cells treated with satraplatin or JM118 in the presence and absence of the OCT inhibitor cimetidine (1.5 mM) in the same way as previously published[28] (Figure 2 and Table 1).

We find that the accumulation of platinum in absence of the OCT inhibitor was 17- or 7-fold higher when, respectively, the A2780 or HCT116 cells were treated with JM118 and 22- or 6-fold higher, respectively, when the A2780 or HCT116 cells were treated with satraplatin in comparison with the treatment of these cells with cisplatin (Table 1).

Cimetidine affected accumulation of platinum in A2780 and HCT116 cells treated with JM118, cisplatin and satraplatin differently (Table 1). The accumulation of platinum in the presence of this OCT inhibitor in A2780 cells treated with JM118 or cisplatin was reduced by 2.9- or 1.8-fold, respectively, and the accumulation of platinum in HCT116 cells treated with JM118 or cisplatin was reduced in a similar extent, by 2.7- or 1.9-fold, respectively. In contrast, the accumulation of platinum in A2780 and HCT116 cells treated with satraplatin was only slightly affected by the presence of cimetidine. Thus, the trend of the effect of cimetidine on the cellular accumulation of the drugs is JM118 > cisplatin $\gg$ satraplatin.

We also determined the sensitivities of the A2780 and HCT116 cells to JM118, satraplatin and for comparative purposes also to cisplatin in the presence or absence of cimetidine (1.5 mM). As shown in Table 2, the sensitivity of A2780 and HCT116 cells in absence of the OCT inhibitor to JM118 or satraplatin was higher than to cisplatin (to JM118 11- or 8-fold, respectively, and to satraplatin 2- or 3-fold, respectively) (Table 2). In the presence of cimetidine, sensitivity to JM118 and cisplatin was significantly decreased (IC_{50} values measured for A2780 cells increased 3.2- and 2.0-fold, respectively, and those measured for HCT116 cells increased 3.5- and 2.9-fold). In contrast, sensitivity of A2780 and HCT116 cells to satraplatin was affected by the presence of cimetidine only slightly ($\sim$1.2-fold). Thus, the trend, as far as the effect of cimetidine on sensitivity of A2780 and HCT116 cells to platinum complexes tested in the present work is concerned, was similar to that observed for the efficiency of cimetidine in reducing the accumulation of platinum in these cells, that is, JM118 > cisplatin $\gg$ satraplatin.

In order to examine how the effect of cimetidine on cellular accumulation and cytotoxicity of JM118 and cisplatin was affected by reaction of cimetidine with these Pt^{II} compounds, we analyzed this reaction by reversed phase HPLC (see Supporting Information). When the cells were cotreated with cimetidine and cisplatin or JM118 (Tables 1 and 2), the Pt^{II} compound and cimetidine were at concentrations of 10 μM and 1.5 mM, respectively. Therefore, we investigated reaction of 1.5 mM cimetidine (1.5 mM) with JM118 or cisplatin (10 μM) at 37 °C in H_2O. We found that the amount of the unreacted Pt^{II} complex in these reactions decreased and reached a minimum after ca. 6 h (Figure S1 in the Supporting Information). This minimum amount of unreacted Pt^{II} compounds in these reactions corresponded to 71 or 68% of the original amount of JM118 or cisplatin, respectively, present at onset of the reactions and remained unchanged at longer reaction times [24 h, i.e. also at the

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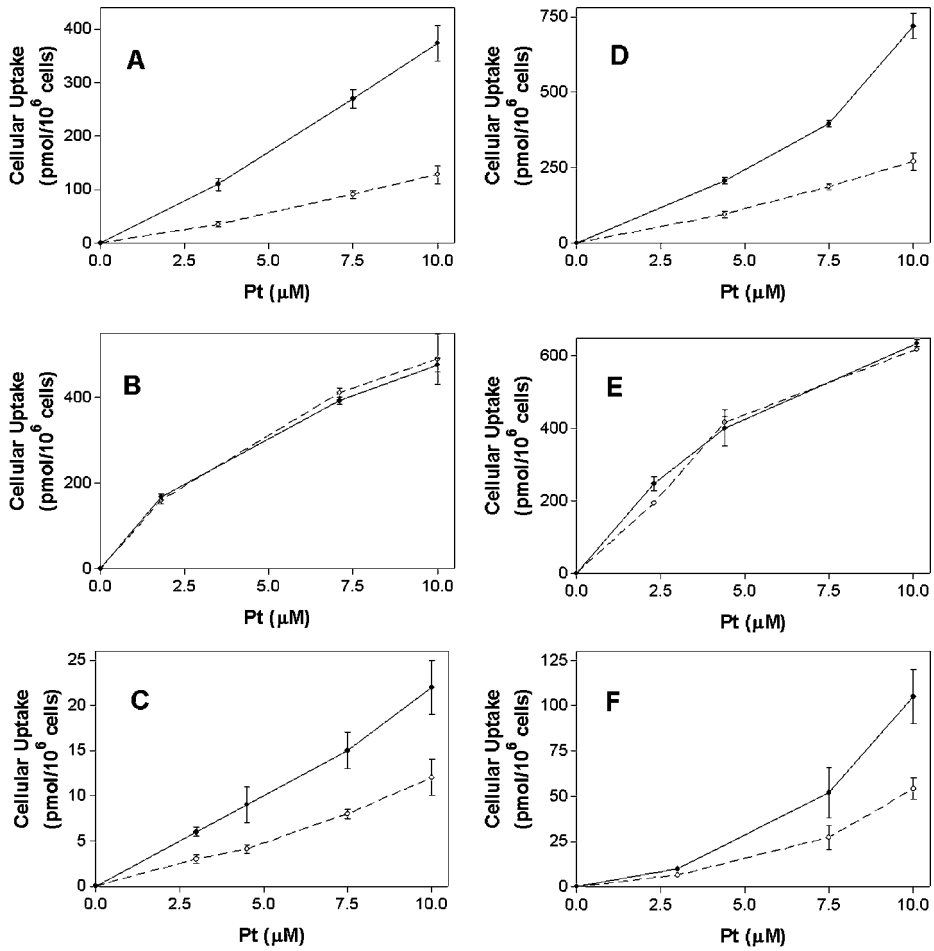

Figure 2. Cellular accumulation rates of platinum after 24 h exposure to JM118 (A, D), satraplatin (B, E), or cisplatin (C, F). Dependences of the cellular accumulation rates of platinum in A2780 (left panels) and HCT116 (right panels) cells after incubation with the platinum complex in the presence of 1.5 mM cimetidine (○) or in its absence absence (●) on the concentration of the platinum complex.

Table 1. Accumulation[a] of Platinum in A2780 and HCT116 Cells after 24 h Exposure to 10 μM JM118, Satraplatin or Cisplatin in the Presence and Absence of an OCT Inhibitor (Cimetidine)[b]

cell line	platinum compound	control	cimetidine treated	control/ cimetidine treated
A2780	JM118	373 ± 57	128 ± 29	2.9
	satraplatin	476 ± 16	411 ± 58	1.2
	cisplatin	22 ± 3	12 ± 2	1.8
HCT116	JM118	718 ± 42	270 ± 30	2.7
	satraplatin	635 ± 30	600 ± 5	1.1
	cisplatin	105 ± 12	54 ± 5	1.9

[a] The accumulation is expressed in pmol of Pt/10^6 cells; all the data are expressed as mean ± SD of six measurements, and each measurement was done in quadruplicate. [b] Cimetidine was at the concentration of 1.5 mM.

Table 2. Sensitivity of A2780 and HCT116 Cells (IC_{50} values[a]) after 24 h Exposure to JM118, Satraplatin or Cisplatin in the Presence and Absence of an OCT Inhibitor (Cimetidine)[b]

cell line	platinum compound	control	cimetidine treated[c]	cimetidine treated/ control
A2780	JM118	0.30 ± 0.04	0.97 ± 0.08	3.2
	satraplatin	1.8 ± 0.3	2.1 ± 0.5	1.2
	cisplatin	3.4 ± 0.4	6.7 ± 0.6	2.0
HCT116	JM118	2.2 ± 0.3	7.7 ± 0.9	3.5
	satraplatin	5.2 ± 0.9	6.4 ± 1.1	1.2
	cisplatin	17 ± 2	45 ± 3	2.6

[a] The IC_{50} values in μM represent concentration inhibiting cell growth by 50%; all the data are expressed as mean ± SD of six measurements, and each measurement was done in quadruplicate. [b] Cimetidine was at the concentration of 1.5 mM. [c] The cell viability measured in the presence of cimetidine was corrected for the effect of this inhibitor in absence of the platinum complex; cimetidine (1.5 mM) alone reduced the cell viability by ~6% on average.

times for which the cells were treated with the platinum complexes in the experiments when we investigated platinum accumulation and toxicity in cancer cell lines (Tables 1 and 2)]. In other words, these reactions of JM118 and cisplatin with cimetidine reached equilibrium after ca. 6 h when 29 and 32% of the original amount of JM118 and cisplatin, respectively, was contained in the complex with cimetidine.

In order to further support the thesis that OCTs play a significant role in accumulation and cytotoxicity of cisplatin and JM118, we also determined these pharmacological

parameters for these Pt^{II} compounds in A2780cisR cells (cisplatin-resistant variant of A2780 cells) (Tables S1 and S2 in the Supporting Information). It has been demonstrated[29] that expression of genes coding OCTs is reduced in the A2780cisR cells in comparison with parental A2780 cells. Thus, in agreement with the latter finding and with the assumption that OCTs play a role in the mechanism of accumulation and cytotoxicity of cisplatin and JM118, accumulation and cytotoxicity of these two Pt^{II} compounds were lower in A2780cisR cells than in parental A2780 cells. Consistent with this conclusion is also the observation that the presence of cimetidine when ovarian tumor cells were cotreated with the Pt^{II} compounds and this antiulcer agent reduced accumulation and cytotoxicity of cisplatin and JM118 in A2780cisR cells pronouncedly less than in A2780 cells (Tables 1 and 2 and Tables S1 and S2 in the Supporting Information).

Reactions of Satraplatin and JM118 with Glutathione. Pt^{II} compounds have a strong thermodynamic preference for binding to sulfur-donor ligands, such as thiolates;[30,31] hence, before Pt^{II} drugs reach the DNA in the nucleus of tumor cells, or even after they bind to DNA, they may still react with various compounds including sulfur-containing molecules.[15,32] These reactions are generally believed to play a role in mechanisms underlying tumor resistance to platinum compounds, their inactivation, and side effects. In addition, a distinct difference between Pt^{II} and Pt^{IV} drugs is that Pt^{II} is kinetically more reactive and more susceptible to deactivation.[33] Therefore, interest in the interactions of platinum antitumor drugs with sulfur-containing molecules of biological significance has recently markedly increased.[15]

In the present work, we investigated, using UV absorption spectrophotometry, irreversible binding of GSH with JM118 and satraplatin in comparison with cisplatin following exactly the procedure outlined by Dabrowiak et al.[23] JM118, satraplatin, and cisplatin at a concentration of 32 μM were incubated with 16 mM GSH (these concentrations of Pt complex and GSH and the ratio of thiol to drug (500:1) represent the physiologically relevant values[23]) at 37 °C in 4.6 mM NaCl plus 0.1 mM Tris-HCl buffer, pH 7.4. Figure 3 shows UV absorption I_d (at 260 nm) of the Pt complexes and GSH as a function of time, with the absorptions of GSH and Pt complex alone subtracted. To establish the rate of the initial reaction with respect to the Pt complex, each difference curve was fitted by nonlinear regression (GraphPad Prism) to the following equation $I_d = C + A_1 \exp(-b_1 t) + A_2 \exp(-b_2 t)$ (A_1, A_2, b_1, b_2 and C are constants, and t is the time of the reaction) and the initial slope (S_{in}) was calculated as $-(A_1 b_1 + A_2 b_2)$.[23] S_{in} values of 0.001256 min^{-1} and 0.001416 min^{-1} were calculated for reaction of GSH with JM118 and cisplatin, respectively. Thus, JM118 reacted with GSH somewhat more slowly than cisplatin. The absorbance at 260 nm reflects the presence of platinum−sulfur and disulfide bonds.[23] Interestingly, the absorbance at 260 nm determined for the reaction of GSH with JM118 was approximately 1.3-fold lower than that determined for the reaction of GSH with cisplatin (Figure 3).

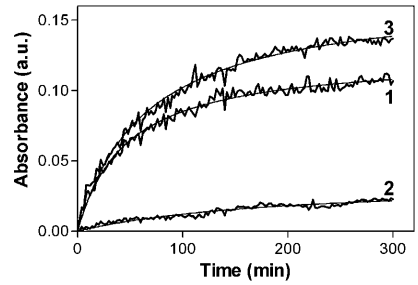

Figure 3. UV absorbance associated with the reaction of cisplatin, JM118 and satraplatin with GSH. Absorbance at 260 nm is shown as a function of time for a 300 min incubation at 37 °C of the platinum complexes at the concentration of 32 μM with 16 mM GSH in the medium of 4.6 mM NaCl plus 0.1 mM Tris-HCl buffer, pH 7.4, in the dark. The curves represent absorbances (260 mm) of a solution containing platinum complex plus GSH from which absorbances yielded by GSH and platinum complex alone were subtracted. Curves: 1, JM118; 2, satraplatin; 3, cisplatin.

Reaction of GSH with satraplatin was very slow or negligible, as judged by the change of absorption at 260 nm which is indicative of formation of Pt−S or S−S bonds. Thus, these results suggest that JM118, similarly to cisplatin, is inactivated by sulfur-containing compounds considerably more readily than its Pt^{IV} counterpart. The negligible absorption at 260 nm when satraplatin was incubated with a large excess of GSH suggests that GSH is not very effective at reducing satraplatin.

Glutathione-Mediated Platination of CT DNA in the Presence of Satraplatin in a Cell-Free Medium. Pt^{IV} complexes are far more inert than their Pt^{II} counterparts, and most do not react significantly with biomacromolecules in the bloodstream or in cells but are still highly effective anticancer agents.[34] Accordingly, satraplatin when incubated with CT DNA in a cell-free medium (10 mM $NaClO_4$) shows minimal reaction (Figure 4). For satraplatin to be cytotoxic in tumor cells requires a facile reduction either in the culture medium or within the cell, so that it is commonly accepted that Pt^{IV} drugs must be reduced to Pt^{II} to effect their action.

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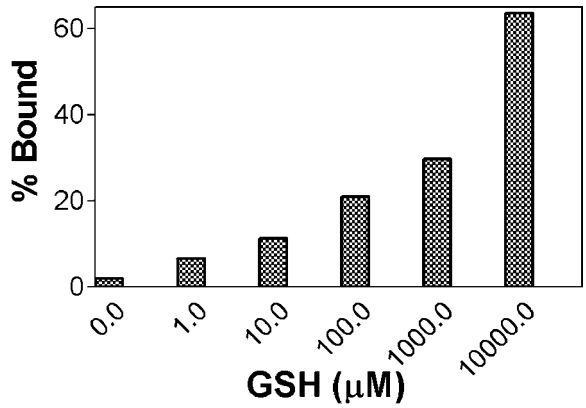

Figure 4. Effect of GSH on the reaction of satraplatin with CT DNA. DNA (0.32 mg mL^{-1}) and satraplatin (15 μM) were incubated in the presence of the indicated concentrations of glutathione for 24 h. Bound Pt was then analyzed by FAAS as described in the text.

That this reduction occurs for satraplatin was demonstrated, JM118 being the most abundant metabolite,[35] although there may be more than one reduction product.[36]

Although it seems likely that the reduction of the Pt^{IV} prodrugs is carried out by more than one reducing agent,[16] one reducing agent in the cell is tripeptide GSH.[17,33] Even when the role that GSH might play in cellular reduction of Pt^{IV} drugs is still under debate,[37] data on interaction of Pt^{IV} drugs, which are activated on reduction, with reduced form of GSH are of interest. Therefore, mixtures of CT DNA (1 mM, related to the phosphorus content), satraplatin (15 μM) and various concentrations of GSH (0−10 mM) were incubated in 10 mM $NaClO_4$ for 24 h at 37 °C. The amount of platinum bound to DNA increased with increasing concentration of GSH progressively over the whole range of its concentrations but was not complete even at the high ratio of GSH:satraplatin of 667 when only 64% of platinum was bound (Figure 4).

Discussion

Differences in the mechanism(s) controlling cellular accumulation of structurally different platinum antitumor drugs may contribute to understanding their different biological effects. In the present work, we observed that cellular accumulation of JM118 or cisplatin and concomitantly their toxicity in human ovarian carcinoma cells A2780 and human colon carcinoma HCT116 cells were significantly decreased by the known OCT inhibitor, cimetidine; the effect of cimetidine on the accumulation and cytotoxicity of cisplatin was considerably smaller (Tables 1 and 2).

It could be argued that the reduced cellular accumulation of JM118 and cisplatin (Table 1) or sensitivity of tumor cells to these Pt^{II} complexes owing to the addition of cimetidine (Table 2) might be a result of its extensive reaction with the Pt^{II} complexes and not a consequence of the inhibition of OCTs by this antiulcer agent. In such a case the addition of cimetidine should reduce the accumulation of the Pt^{II} complex in the paired A2780 and A2780cisR cells approximately to the same extent. However, cimetidine affected the accumulation of the Pt^{II} complex in paired A2780 and A2780cisR cells and sensitivity of these two cell lines to these Pt^{II} agents distinctly differently (Tables 1 and 2 and Table S1 and S2 in the Supporting Information). Hence, it is reasonable to suggest that the critical factor responsible for the differently reduced cellular accumulation of JM118 or cisplatin in A2780 and A2780cisR cells (Table 1 and Table S1 in the Supporting Information) or their sensitivity to these Pt^{II} compounds (Table 2 and Table S2 in the Supporting Information) owing to the addition of cimetidine was not only its reaction with JM118 or cisplatin. Expression of genes coding OCTs is considerably reduced in the A2780cisR cells in comparison with parental A2780 cells.[29] Hence, the results shown in Tables 1 and 2 and Tables S1 and S2 in the Supporting Information rather support the thesis that differently reduced cellular accumulation of JM118 or cisplatin in A2780 and A2780cisR cells (Tables 1 and S1) or their sensitivity to these Pt^{II} complexes (Table 2 and Table S2 in the Supporting Information) owing to the addition of cimetidine was to a considerable extent a consequence of the inhibition by this antiulcer agent of active transport mediated by the OCTs.

On the other hand, reactions of cimetidine with cisplatin or JM118 at the concentrations used in the experiments when the cells were cotreated with cimetidine and Pt^{II} complexes (Tables 1 and 2 and Tables S1 and S2 in the Supporting Information) in water reached equilibrium when only 29 and 32% of the original amount of JM118 and cisplatin, respectively, was contained in the complex with cimetidine (Figure S1 in the Supporting Information). Thus, the inhibition effect of cimetidine might be partially (∼30% at the most) also due to the direct reaction of the Pt^{II} compounds with cimetidine (see the section Results and Figure S1 in the Supporting Information). Nevertheless, it cannot be excluded that in the composite culture cell medium the yields of the reactions of JM118 or cisplatin with cimetidine might be lower than those in water since the components of the culture cell medium might compete for the Pt^{II} compounds with cimetidine yielding products which can accumulate in the cells and produce cytotoxic effects.

Our results also demonstrate that the effect of cimetidine on the accumulation and cytotoxicity of cisplatin was considerably smaller in comparison with the accumulation and cytotoxicity of JM118 (Tables 1 and 2). Thus, in aggregate, our results suggest that the OCTs may play a

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considerably more important role in the mechanism of cytotoxicity of JM118 than in the mechanism of cytotoxicity of cisplatin. The fact that JM118 is a relatively good substrate of OCTs is also consistent with the earlier observation[28] demonstrating a substantial increase of sensitivity to JM118 of Madin−Darby canine kidney cells as a consequence of their stable transfection with the human OCT cDNAs.

Previously published studies[6,28] show that the nature of the amine ligand bound to Pt^{II} is important for interaction with OCTs, with an organic component being required for effective interaction.[28] In addition, high lipophilicity is one of the key physicochemical properties of substrates of OCTs.[38] Thus, it is apparent that organic and lipophilic character of the cyclohexamine ligand seem to be major factor responsible for the enhanced potency of JM118 (in comparison with cisplatin) in interacting with the OCTs.

It should be also noted that JM118 retains activity in cells in which cisplatin resistance is due to the loss of copper transporter CTR1 very likely because CTR1 is involved in cisplatin accumulation, but not in JM118 accumulation.[39] On the other hand, JM118 does not retain its activity in cells in which resistance is due to enhanced expression of other copper transporters, namely, ATP7A or ATP7B, supporting the view that also other type(s) of active transport than those mediated by OCTs may play a role in accumulation of JM118 in certain types of tumor cells.

The observation that cellular accumulation of platinum in A2780 and HCT116 cells treated with satraplatin and concomitantly its toxicity in these cells were unaffected or were affected relatively slightly by cimetidine is consistent with the previously drawn conclusion and further supports the hypothesis that Pt^{IV} drugs only enter cells by passive diffusion which is radically enhanced by increased lipophilicity of these complexes.[26]

The results of the present work also demonstrate that JM118 reacts with GSH with a higher rate compared with cisplatin (Figure 3). Both clinical and preclinical studies have shown that cells with an elevated level of GSH (>10 mM) may be resistant to cisplatin and its analogues.[40−43] Further studies are warranted to dissect what part of the cytotoxicity of JM118 is attributable to its increased intracellular accumulation and is unfavorably affected by its faster reaction with GSH.

Results describing irreversible binding of GSH to JM118 and cisplatin showed that the absorbance at 260 nm determined for the reaction of GSH with JM118 was approximately 1.3-fold lower than that determined for the reaction of GSH with cisplatin (Figure 3). The absorbance at 260 nm reflects the presence of platinum−sulfur and disulfide bonds.[23,24] Hence, our results (Figure 3) can be interpreted to mean that, under conditions of our experiments, the major products of the reaction of this tripeptide with cisplatin contained a 1.3-fold higher amount of GSH per Pt atom than those afforded by the reaction of GSH with JM118. Cis-oriented compounds containing NH_3 non-leaving groups initially remain intact upon reaction with GSH, but eventually the NH_3 group trans to the sulfur atom might be lost.[15,44] Thus, the principal and initial product of the reaction of cisplatin or JM118 with GSH are the monosubstituted complexes *cis*-[PtCl(SG)$(NH_3)_2$] or *cis*-[PtCl(SG)(cyclohexylamine)(NH_3)], respectively.[45−47] The subsequent liberation of NH_3 or cyclohexylamine due to their trans labilization from cisplatin or JM118 moieties may result in some amount of species with a 1:2 Pt:GSH stoichiometry.

The 1.3-fold higher absorption at 260 nm observed for the reaction of GSH with cisplatin (Figure 3) compared with the reaction of GSH with JM118 may imply that the amount of products afforded by the reaction of GSH with cisplatin or JM118 initially containing only one sulfur-containing molecule which were slowly transformed to bis-sulfur or bridged Pt species (with the 1:2 Pt:GSH stoichiometry) was in the case of cisplatin by ca. 30% higher. Interestingly, for JM118, hydrolysis is faster for the chloride ligand trans to cyclohexylamine.[48] It might therefore be expected that the monosubstituted complex *cis*-[PtCl(SG)-(cyclohexylamine)(NH_3)] initially arising from reaction of JM118 with GSH would preferentially contain GS trans to

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the cyclohexylamine ligand. The differences in the trans effects of the different amines (NH_3, cyclohexylamine) are not very large. Thus, the more difficult binding of a second GSH to the Pt atom in *cis*-[PtCl(SG)(cyclohexylamine)(NH_3)] (in comparison with *cis*-[PtCl(SG)(NH_3)$_2$] initially arising from reaction of cisplatin with GSH) is consistent with the thesis that bulkier cyclohexylamine sterically blocks the Pt atom in *cis*-[PtCl(SG)(cyclohexylamine)(NH_3)] ligand more than a smaller NH_3 group in *cis*-[PtCl(SG)(NH_3)$_2$]. In aggregate, the steric properties of the amine ligands appear to be the dominating factor in determining the substitution kinetics of cisplatin and JM118 when bridged species (polymers) with the 1:2 Pt:GSH stoichiometry are formed.

The results of the present work also demonstrate that satraplatin reacts with GSH negligibly or at a rate substantially lower than JM118 and cisplatin (Figure 3). A very small increase in absorption at 260 nm was observed in the time scale of the experiments in which satraplatin was allowed to react with GSH. The absorption at 260 nm might be a consequence of the binding of GSH to satraplatin itself, but it cannot be ruled out that a small amount of satraplatin was first reduced by GSH yielding Pt^{II} products and oxidized glutathione (GSSG) which itself absorbs at 260 nm. Moreover, the *cis*-diam(m)inedichloridoplatinum(II) resulting from the reduction could further react with GSH to give complexes that also absorb at 260 nm (*vide supra*). The small or negligible reactivity of satraplatin with GSH observed in the present work is consistent with previous observation[49] that satraplatin was stable in solution with GSH for 2 h. Substitution reactions of Pt^{IV} complexes are generally slower than those of Pt^{II} complexes, and in particular Pt^{IV} complexes with axial acetato ligands (like in satraplatin) are expected to undergo extremely slow substitution reactions.[50,51] In addition, satraplatin and JM118 have increased lipophilicity relative to the approved cisplatin and its analogues (carboplatin, oxaliplatin and nedaplatin). These unique physicochemical properties could also affect GSH binding kinetics. In particular, the slower GSH interactions with satraplatin compared to cisplatin and JM118 can be explained by its higher oxidation state and axial acetato ligands, resulting in its inherent inertness.

The experiments focused on GSH-mediated activation of satraplatin in a cell-free medium confirmed that no platinum was bound to CT DNA in presence of satraplatin and absence of any reducing agent (Figure 4). The results of the present work also show that satraplatin can be activated by GSH in a manner that leads to its reaction with CT DNA (Figure 4). The amount of platinum bound to CT DNA increased with increasing concentration of GSH in the reaction mixture containing DNA and satraplatin. Similar experiments were performed earlier with another Pt^{IV} antitumor drug, tetraplatin.[52] The amount of platinum bound to CT DNA also increased with increasing concentration of GSH in the reaction mixture containing CT DNA and tetraplatin, but at approximately equimolar concentrations of GSH and tetraplatin, almost all platinum present in the reaction mixture was bound to DNA. This was in contrast with the reaction of satraplatin with CT DNA in presence of GSH when at approximately equimolar concentrations of GSH and satraplatin only ca. 12% of the total platinum present in the reaction mixture was bound to DNA; and the binding was not complete even at so high a ratio of concentrations of GSH and satraplatin as 667 (Figure 4). It has been proposed[52] that activation (presumably reduction to the Pt^{II} counterpart capable of binding to DNA) of tetraplatin was accomplished by low concentrations of GSH. However, decreased platination of CT DNA was observed at GSH concentrations higher than those stoichiometrically required to reduce tetraplatin present in the reaction due to subsequent complexing of the reduced Pt^{II} drug with excessive GSH.[52] The different efficiency of GSH to mediate platination of DNA in the presence of satraplatin and tetraplatin is consistent with the observations demonstrating that reduction of Pt^{IV} drugs occurs most readily when the axial ligands are chlorides and considerably less readily when they are carboxylates.[53] Hence, tetraplatin, with axial chloro groups and a more positive reduction potential, can be reduced by GSH relatively very rapidly[54] whereas reduction of satraplatin with axial acetato groups and a more negative reduction potential is considerably slowed.

In conclusion, we report here new information on the role of active transport in cellular entry of satraplatin and JM118 and interactions of these platinum complexes with GSH. The results may improve a basis for structure−pharmacological activity relationships that govern the anticancer drug potential of Pt^{IV}- or Pt^{II}-based antitumor agents. On the other hand, it should be noted that the results obtained using simplistic model systems should be still confirmed employing more sophisticated methods that allow examining the fate of platinum drugs in biological fluids.

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Supporting Information Available: Additional details as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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