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## Modulation of the cellular pharmacology of JM118, the major metabolite of satraplatin, by copper influx and efflux transporters

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**Abstract** Satraplatin is an orally bioavailable platinum analog that has activity in prostate cancer. JM118 is the most abundant species found in the plasma following the oral ingestion of satraplatin and has anti-tumor activity in vitro against cell lines that are resistant to cisplatin (DDP). The goal of the current study was to determine whether the activity of JM118 in some DDP-resistant cells can be explained by differences in the cellular pharmacology of the two drugs. The effect of each of the Cu transporters CTR1, ATP7A and ATP7B on sensitivity to the growth inhibitory effect of JM118 and its cellular pharmacology was examined to identify the characteristics of JM118 that distinguish it from DDP. These studies were performed using wild type and CTR1<sup>-/-</sup> homozygous knockout mouse embryo cells, and human Me32a Menkes disease fibroblasts that do not express either ATP7A or ATP7B plus sublines molecularly engineered to express either ATP7A (MeMNC cells) or ATP7B (MeWNC cells). Knockout of the Cu influx transporter CTR1 in murine embryo cells increased their resistance to DDP and reduced its cellular accumulation but had no effect on sensitivity to JM118 or its uptake. In the case of DDP, forced expression of either of the two Cu efflux transporters, ATP7A or ATP7B, in Me32a cells rendered them resistant to DDP, increased whole cell accumulation of Pt but reduced the amount of Pt in DNA. In the case of JM118, forced expression of either ATP7A or ATP7B rendered Me32a cells resistant, increased not only whole cell Pt accumulation but also increased rather than decreased the amount of Pt in DNA. These results demonstrate that both ATP7A and ATP7B mediate resistance to JM118 as well as DDP and suggest that

they sequester both DDP and JM118 into vesicular compartments within the cell resulting in enhanced whole cell accumulation and reduced cytotoxicity. We conclude that there are two important differences between DDP and JM118 with respect to the effect of Cu transporters on their cellular pharmacology. First, whereas CTR1 is involved in DDP accumulation it does not play a role in the uptake of JM118. Second, ATP7A and ATP7B, while they both mediate resistance, have opposite effects on the accumulation of Pt in DNA following exposure to the two drugs. ATP7A and ATP7B appear to be able to modulate the toxicity of the Pt that accumulates in DNA following exposure to JM118. These results suggest that JM118 will retain activity in cells in which DDP resistance is due to the loss of CTR1, but not in cells in which resistance is due to enhanced expression of ATP7A or ATP7B.

**Keywords** ATP7A · ATP7B · CTR1 · Cisplatin · Satraplatin · JM118

**Abbreviations** DDP: Cisplatin · ECFP: Enhanced cyan fluorescent protein · EYFP: Enhanced yellow fluorescent protein · ICP-MS: Inductively coupled plasma mass spectroscopy · PBS: Phosphate buffered saline · TGN: *Trans*-Golgi network

### Introduction

Acquired resistance to DDP appears to result from the cellular engagement of numerous and different defense mechanisms (reviewed in [21]). However, almost all cells selected for acquired resistance to DDP have defects in drug accumulation [1, 10, 15, 16, 23, 24, 26, 27]. Recently it has been discovered that the transporters that maintain Cu homeostasis also regulate the cellular pharmacology of many platinum drugs [5–8, 11, 14, 18, 19]. CTR1 is the main Cu influx transporter at the plasma membrane and has been shown to play a role in

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DDP uptake [5, 6, 14]. ATP7A and ATP7B are intracellular Cu transporters that sequester Cu from the cytoplasm into the TGN for subsequent export from the cell. ATP7A is expressed in many tissues whereas the expression of ATP7B is limited to the liver. Both ATP7A and ATP7B modulate the cellular pharmacology of DDP; forced expression of these proteins renders cells resistant and reduction of their expression renders them hypersensitive to DDP [11, 18].

Satraplatin (JM216, Fig. 1) is the first orally available platinum chemotherapeutic drug (reviewed in [9]). It has potent *in vitro* growth inhibitory properties and an attractive pharmacokinetic profile [25]. Satraplatin has demonstrated activity in patients with advanced prostate cancer in a randomized Phase II trial [22] and is currently being investigated in the same patient population in a Phase III trial. Following oral administration of satraplatin no native drug is found in the plasma ultrafiltrate. However, at least six metabolites have been identified among which JM118 is the most abundant; JM118 represented 31% of the ultrafiltrable platinum during the first 6 h after drug administration [17]. JM383 accounted for <5% of the ultrafiltrable platinum and other metabolites were even less abundant. JM118 and JM383 have similar potency [17] and JM118 has been reported to be two- to sixfold more potent than satraplatin against human tumor cell lines tested *in vitro* [3]. JM118, whose structure is shown in Fig. 1, was selected for the current study on the basis of the fact that it is the most abundant cytotoxic metabolite found in plasma.

In an effort to identify the specific characteristics of JM118 that distinguish it from DDP, we have examined the effects of altering the expression of CTR1, ATP7A and ATP7B on the cellular pharmacology and cytotoxicity of JM118 relative to their effects on DDP. Studies of the effect of altering CTR1 expression were performed using mouse embryonic fibroblasts in which both alleles of CTR1 were knocked out [13]. Studies of the effect of ATP7A and ATP7B were performed using the human fibroblast cell line Me32a, established from a patient with Menkes disease, that does not express either ATP7A or ATP7B, and sublines transfected to express

either human ATP7A or murine ATP7B [12]. We report here that, whereas CTR1 is important to the cellular accumulation and toxicity of DDP, it is not important for JM118. Increasing the level of expression of ATP7A and ATP7B in the Menkes cells rendered them resistant to the cytotoxic effects of both DDP and JM118. This change in sensitivity was accompanied by increased whole cell accumulation of platinum consistent with enhanced vesicular sequestration that limits drug access to critical targets. However, ATP7A and ATP7B were found to differentially affect the accumulation of Pt in DNA following exposure to DDP and JM118.

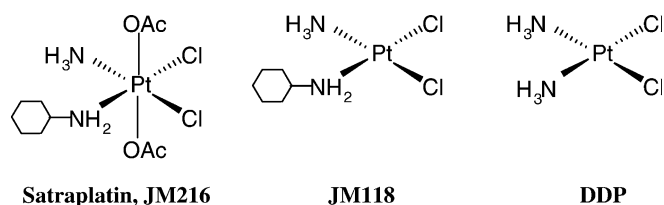
## Materials and methods

### Drugs

Cisplatin was generously provided by Bristol-Myers Squibb (Princeton, NJ, USA) and JM118 by GPC Biotech (Munich, Germany). DDP was stored as a 3.3-mM stock solution in 0.9% NaCl in the dark at room temperature and JM118 was stored as a lyophilized powder in the dark at  $-20^{\circ}$ . DDP was diluted directly into media. JM118 was resuspended in 1:1 vol/vol ethanol:water 1:1 to form a stock solution of 1.3 mM before being diluted directly into media.

### Cell lines

CTR1<sup>+/+</sup> and CTR1<sup>-/-</sup> mouse embryonic cells were generously provided by Dr. Dennis J. Thiele (Duke University); their development and characterization has been reported elsewhere [13]. The cells were maintained in DMEM medium supplemented with 20% fetal bovine serum, 1X MEM non-essential amino acids, 1 mM MEM sodium pyruvate, 50 mg/l uridine, 55  $\mu$ M 2-mercaptoethanol and 100 U/ml penicillin/streptomycin at 37°C in a humidified incubator containing 5% CO<sub>2</sub>. The ATP7A- and ATP7B-non-expressing fibroblast cell line Me32a, the isogenic ATP7A-transfected cell line MeMNK and the isogenic ATP7B-transfected cell line MeWND, described elsewhere [12], were provided by Michael J. Petris, Ph.D. (University of Missouri). For fluorescent growth assay studies the Me32a cells were transfected with a vector expressing enhanced yellow fluorescent protein, and the MeMNK and MeWND cells were transfected with a vector expressing enhanced cyan fluorescent protein. Single cell clones that expressed these fluorescent proteins at high levels were recovered by flow cytometry and used in subsequent experiments. These cell lines were maintained at 37°C in a humidified incubator containing 5% CO<sub>2</sub> in alpha-MEM medium supplemented with 10% fetal bovine serum. MeMNK and MeWND cells and fluorescent protein-transfected Me32a, MeMNK and MeWND sublines were grown in the presence of 500  $\mu$ g/ml G418.



**Fig. 1** Structure of satraplatin (JM216), its main metabolite JM118, and DDP

## Measurement of drug sensitivity

Me32a-EYFP, MeMNK-ECFP and MeWND-ECFP cells were separately plated in 96-well plates at a density of 5,000 cells/well in a volume of 200  $\mu$ l and grown overnight in alpha-MEM medium without phenol red. Fluorescence intensity was measured immediately after the addition of 50  $\mu$ l of JM118 or control drug from a 5 $\times$  stock solution to each well as well as after 72 h using a Safire microplate reader. Excitation/emission wavelengths were set at 434/476 and 518/535 for ECFP and EYFP, respectively. Because Me32a-EYFP, MeMNK-ECFP and MeWND-ECFP cells grew at different rates, the growth rate in the presence of drug was expressed as a fraction of that in the absence of drug for each concentration tested. Each experiment was performed with quadruplicate wells and each experiment was performed a total of three independent times.

For growth rate inhibition assays, CTR1<sup>-/-</sup> and CTR1<sup>+/+</sup> mouse embryonic cells were plated in 6-well plates at a density of 1,000 cells/well in 1 ml of medium and grown overnight. The following day, 1 ml of drug from a 2 $\times$  stock solution was added to each well. Following 48 h of exposure, each well was gently washed with PBS and trypsinized with 500  $\mu$ l of 0.25% trypsin. Once the cells were detached from the well, 500  $\mu$ l of medium was added to each well, and the samples were centrifuged in Eppendorf tubes at 13,000 rpm for 5 min. Cell pellets were resuspended in 100  $\mu$ l of medium. The number of surviving cells was determined by cell counting using a hemocytometer and growth inhibition was determined relative to the total number of surviving cells in the untreated samples. Each experiment was performed with triplicate wells and each experiment was performed two independent times.

## DDP whole cell uptake

Cells were grown to 80% confluency in six-well plates. The cells were incubated in fresh medium containing 2  $\mu$ M DDP or JM118 for 24 h or 90 min. The cells were then washed twice with cold PBS and lysed by the addition of 215  $\mu$ l 70% nitric acid to each well. The cells were then collected and dissolved at 65°C overnight, after which the samples were diluted with water/0.1% Triton-X to a final concentration of 5% acid. Each experiment was performed with triplicate wells and each experiment was performed at least three independent times. Pt measurements were made by ICP-MS using a Thermo Finnigan ICP-MS (model Element2) at the Analytical Facility at the Scripps Institute of Oceanography and normalized to protein levels or DNA amounts for each cell line. Indium was added to each sample at 1 ppb as a control for flow variation.

## Pt content of DNA

Cells were grown to 80% confluency in 145 mm plates. The cells were incubated in fresh medium containing 2  $\mu$ M DDP or JM118 for 24 h. The cells were then washed twice with PBS, scraped and pelleted. Genomic DNA was harvested using DNAzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Following quantification by spectroscopy, 215  $\mu$ l 70% nitric acid was added to the DNA and the samples were dissolved at 65°C for 2 hrs after which the samples were diluted with water/0.1% Triton-X to a final concentration of 5% acid. Each experiment was performed with triplicate plates and each experiment was performed three independent times. Pt measurements were made by ICP-MS.

## Statistics

Comparisons were made using Student's *t* test with the assumption of unequal variance and by determining the 95% confidence interval with an alpha of 0.05.

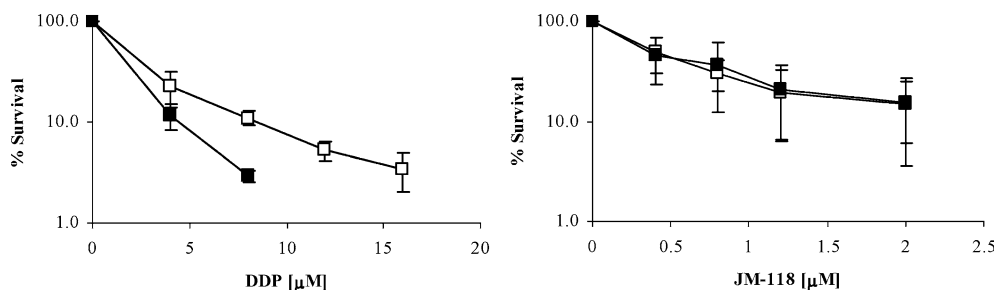
## Results

### Sensitivity to the growth inhibitory effects of DDP and JM118

The mouse embryonic cells and the human fibroblasts used in these studies do not form well-defined colonies when cultured on plastic, therefore sensitivity to the cytotoxic effect of DDP and JM118 was determined using a growth rate inhibition assay. The growth rate was determined over a period of 48–72 h during which the cells were continuously exposed to the drug. In the case of the CTR1<sup>+/+</sup> and CTR1<sup>-/-</sup> mouse embryonic cells this was accomplished by counting cells in a hemocytometer after 48 h of exposure. In the case of the human fibroblasts this was accomplished by engineering them to express either EYFP or ECFP and quantifying cell number by measuring the intensity of the fluorescent signal as a function of time up to 72 h of drug exposure. The engineered ATP7A and ATP7B-deficient subline was designated Me32a-EYFP, the ATP7A-expressing subline MeMNK-ECFP and the ATP7B-expressing subline MeWND-ECFP.

Figure 2 shows that the mouse embryonic cells that lack CTR1 expression were 2.1  $\pm$  0.08 (SEM)-fold ( $P < 0.01$ ) more resistant to the growth inhibitory effects of DDP than wild type cells, confirming previous results obtained both with these cell lines [6] and in *S. cerevisiae* in which CTR1 was deleted [14]. However, the CTR1<sup>-/-</sup> cells showed no difference in sensitivity to the growth inhibitory effects of JM118. Thus, the effect of the loss of CTR1 clearly distinguishes between these two compounds.

Figure 3 shows that the ATP7A-expressing MeMNK cells were significantly more resistant to both DDP and JM118, the Me32a cells. Linear regression was used to estimate the slope of the curve of the logarithm of sur-



**Fig. 2** Sensitivity of  $CTR1^{+/+}$  cells (filled square) and  $CTR1^{-/-}$  cells (open square) to a 48 h exposure to increasing concentrations of DDP (left panels) or JM118 (right panels). Each point represents the mean of two independent experiments performed with triplicate cultures for each drug concentration. Vertical bars,  $\pm$  SEM; where bars are missing SEM was less than the size of the symbol

vival as a function of drug concentration for each experiment individually. The average ratio of the slope of the concentration–growth rate curve was  $1.8 \pm 0.3$  (SEM) for DDP and  $1.9 \pm 0.3$  (SEM) for JM118; the 95% confidence interval was 1.4–2.2 for DDP and 1.6–2.2 for JM118. Similarly, the ATP7B-expressing MeWND cells were significantly more resistant to DDP and JM118 than the Me32a cells. The average ratio of the slopes of the concentration–survival curves was  $1.6 \pm 0.3$  (SEM) for DDP and  $1.8 \pm 0.2$  (SEM) for JM118; the 95% confidence interval was 1.2–2.0 for DDP and 1.5–2.0 for JM118. Thus, increasing the expression of either of the Cu efflux transporters rendered the cells more resistant to both DDP and JM118 and the magnitude of the effect was similar for both compounds.

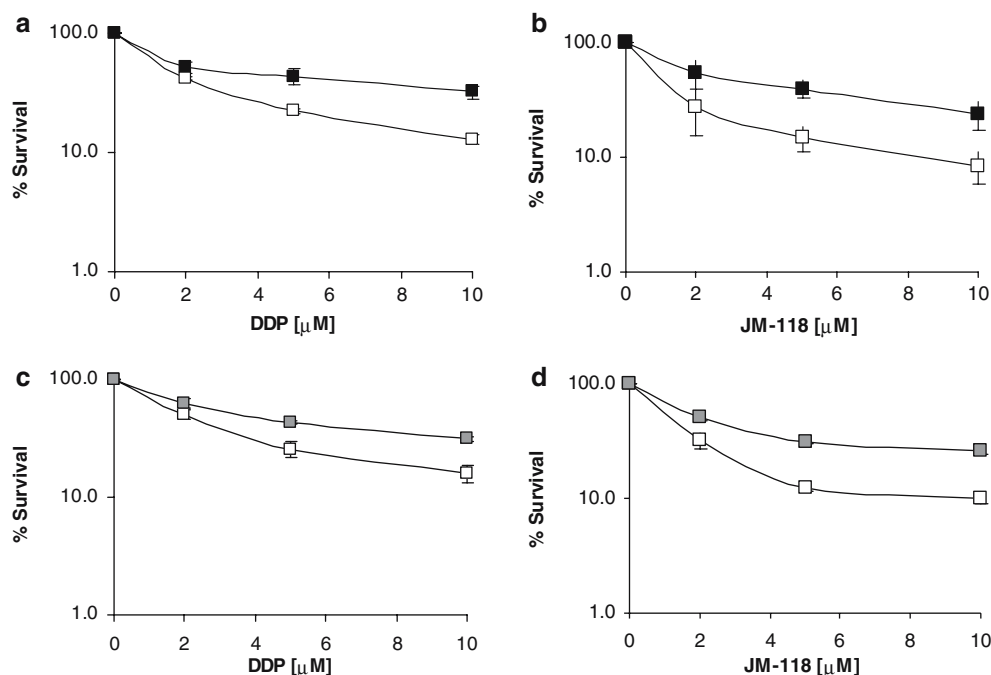
#### Whole cell drug accumulation.

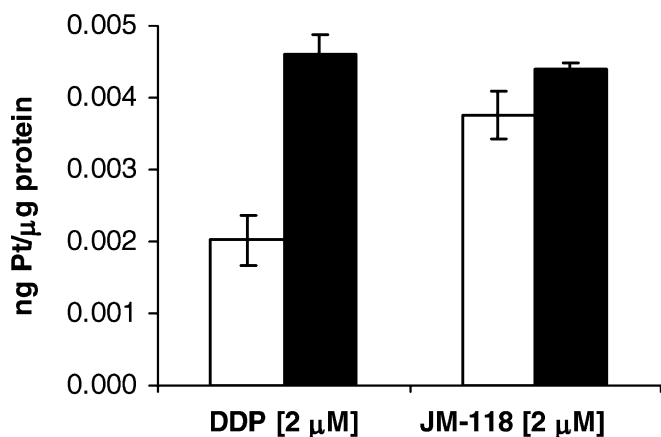
To determine whether the changes in sensitivity to the growth inhibitory effects of DDP and JM118 were

accompanied by differences in whole cell Pt accumulation, the  $CTR1^{+/+}$  and  $CTR1^{-/-}$  cells were exposed to 2  $\mu$ M DDP or JM118 for 90 min, and the Me32a, MeMNK and MeWND cells were exposed to 2  $\mu$ M DDP for 24 h. The whole cell Pt content was then quantified by ICP-MS following the digestion of all cellular constituents in nitric acid. The goal in these studies was to use a clinically relevant concentration of DDP and JM118. Due to differences in uptake between mouse and human cells, a longer duration of exposure was needed to allow the cells to accumulate an accurately quantifiable amount of Pt for the human cells.

The data presented in Fig. 4 show that, while the loss of  $CTR1$  significantly decreased DDP uptake, this loss had no effect on the accumulation of JM118. Following exposure to DDP the  $CTR1^{-/-}$  cells accumulated only 44% of the amount of Pt as the  $CTR1^{+/+}$  cells following a 90 min exposure to 2  $\mu$ M DDP ( $P < 0.02$ ). In contrast, there was virtually no difference in whole cell uptake of Pt following a 90 min exposure to 2  $\mu$ M JM118. These results indicate that while  $CTR1$  plays a

**Fig. 3** Sensitivity of Me32a cells (open square) and MeMNK cells (filled square) (a and b) and Me32a cells (open square) and MeWND cells (shaded square) (c and d) following 72 h exposure to increasing concentrations of DDP (left panels) or JM118 (right panels). Each point represents the mean of three independent experiments performed with quadruplicate cultures for each drug concentration. Vertical bars,  $\pm$  SEM; where bars are missing SEM was less than the size of the symbol

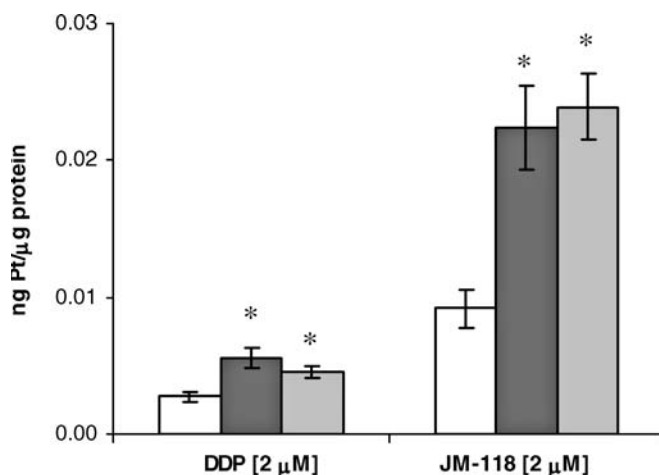




**Fig. 4** Whole cell Pt accumulation in CTR1<sup>-/-</sup> cells (open square) and CTR1<sup>+/+</sup> cells (filled square) following 90-min exposure to 2 μM DDP (left) or JM118 (right). Each bar represents the mean of three independent experiments performed with triplicate cultures for each drug. Vertical bars, ± SEM. \**P* < 0.02

role in the uptake of DDP, it does not play an important role in the cellular accumulation of JM118.

Similar experiments were carried out with the Me32a, MeMNK and MeWND cells. The data presented in Fig. 5 show that increased ATP7A or ATP7B expression was associated with increased uptake of both DDP and JM118. In Me32a cells, the average accumulation of Pt in the cells following a 24 h exposure to 2 μM drug was 0.003 ± 0.0004 (SEM) ng Pt/μg protein for DDP and 0.009 ± 0.001 (SEM) ng Pt/μg protein for JM118. In the ATP7A-expressing MeMNK cells, the average accumulation of Pt in the cells was 0.006 ± 0.0007 ng Pt/μg protein for DDP (*P* < 0.03) and 0.020 ± 0.003 ng Pt/μg protein for JM118 (*P* < 0.02). In the MeWND cells, the average accumulation of Pt was 0.005 ± 0.0005 ng Pt/μg protein for DDP (*P* < 0.03) and 0.02 ± 0.002 ng Pt/μg



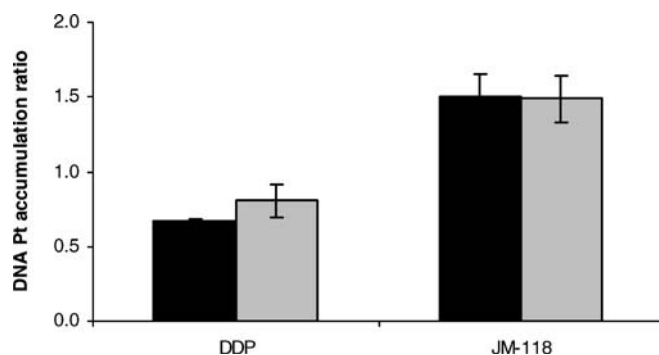
**Fig. 5** Whole cell Pt accumulation in Me32a (open square), MeMNK (filled square) and MeWND cells (shaded square) following 24 h exposure to 2 μM DDP (left) or JM118 (right). Each bar represents the mean of four independent experiments performed with triplicate cultures for each drug. Vertical bars, ± SEM. \**P* < 0.02 or smaller

protein for JM118 (*P* < 0.01). Thus, forced expression of ATP7A resulted in a twofold increase in the accumulation of DDP and a 2.2-fold increase in JM118 uptake. Forced expression of ATP7B resulted in a 1.6-fold increase in the accumulation of DDP and a 2.2-fold increase in the uptake of JM118. Thus, despite the fact that the MeMNK and MeWND cells were significantly less sensitive to the growth inhibitory effects of both DDP and JM118, they accumulated substantially more intracellular Pt. This suggests the possibility that, in the Menkes cell line model, ATP7A and ATP7B serve to sequester these Pt drugs intracellularly rather than exporting them from the cell, thus effectively detoxifying the drugs while at the same time increasing total cell accumulation.

#### Pt content of DNA

The Pt drugs are believed to injure cells primarily through the formation of DNA adducts. The extent of adduct formation, as reflected by the amount of Pt in DNA, was measured in Me32a, MeMNK and MeWND cells following a 24 h exposure to DDP or JM118. Studies were not done in the CTR1<sup>+/+</sup> and CTR1<sup>-/-</sup> cells since expression of CTR1 had no effect on JM118 accumulation. Total genomic DNA was isolated and Pt levels were quantified by ICP-MS and compared between cell lines. The results of these studies, presented in Fig. 6, show that increased ATP7A or ATP7B expression was associated with a decrease in DNA Pt content following exposure to DDP. The Pt content of DNA in the MeMNK cells following a 24 h exposure to 2 μM DDP was 0.7 ± 0.01 (SEM) of that in the Me32a cells (95% CI, 0.64–0.70) and the Pt content of the DNA in the MeWND cells was 0.8 ± 0.1 times of that in the Me32a cells. In contrast, in the case of JM118, forced expression of either ATP7A or ATP7B resulted in a significant increase in DNA Pt content. The Pt content of DNA following a 24 h exposure to 2 μM JM118 was 1.5 ± 0.1 (SEM) times higher in the MeMNK cells (95% CI 1.2–1.7) and 1.5 ± 0.2 (SEM) times higher in the MeWND cells (95% CI 1.2–1.8) than in the Me32a cells. Thus, the effect of forced expression of ATP7A or ATP7B produced opposite effects on the accumulation of Pt in the DNA of these cells for DDP and JM118. While the reduction in DNA Pt content following exposure to DDP was anticipated based on the fact that the cells became resistant when ATP7A or ATP7B was expressed, the increased DNA Pt content following exposure to JM118 did not fit the paradigm of a direct link between drug sensitivity and the extent of adduct formation in DNA.

To further examine the relationship between resistance and the accumulation of Pt in DNA further, the degree of growth inhibition per unit Pt in DNA was calculated for DDP and JM118. The data presented in Table 1 show that in the Me32a cells the percent inhibition of growth per unit Pt in DNA was similar for DDP and JM118. In the case of DDP, forced expression



**Fig. 6** Ratio of Pt accumulation in DNA in MeMNC cells (filled square) and MeWND cells (shaded square) to that in Me32a cells following 24 h exposure to 2  $\mu$ M DDP or JM118. Each bar represents the mean ratio determined from three independent experiments each performed with triplicate cultures for each drug. Vertical bars  $\pm$  SEM

of either ATP7A or ATP7B did not change this relationship very much. However, in the case of JM118, forced expression of either ATP7A or ATP7B reduced the growth inhibitory effect of a given amount of Pt in DNA by more than 50%. Thus, these two Cu exports appear to be influencing the relative toxicity of the adducts formed in DNA.

## Discussion

The Cu transporters CTR1, ATP7A and ATP7B have previously been shown to modulate the cellular pharmacology of DDP and several of its analogs [6–8, 11, 14, 18, 19]. Given the apparent activity of satraplatin in prostate cancer, a disease that is not responsive to any of the other Pt-containing drugs, it was of interest to determine whether the Cu transporters also influence the cellular pharmacology of JM118, the major metabolite of the drug found in plasma following oral administration of satraplatin [9]. The results reported here indicate that, while some of the transporters do affect sensitivity and drug accumulation, there are substantial differences in the way they influence JM118 as compared to DDP. It should be noted again that while JM118 is the most abundant satraplatin metabolite, others such as JM383 are also found in plasma and are known to be cytotoxic. Thus, the effect of the Cu transporters on the cellular pharmacology of JM118 may well not fully describe effects on the overall clinical behavior of satraplatin.

**Table 1** Relative growth inhibition per amount of Pt present in DNA

	DDP			JM118		
	Percent inhibition of growth <sup>a</sup>	ng Pt/ $\mu$ g DNA <sup>b</sup>	Percent inhibition of growth/Pt in DNA	Percent inhibition of growth <sup>a</sup>	ng Pt/ $\mu$ g DNA <sup>b</sup>	Percent inhibition of growth/Pt in DNA
Me32a	58.0	0.011	5,272	72.8	0.014	5,200
MeMNC	48.3	0.007	6,900	45.5	0.021	2,167
Me32a	49.2	0.007	7,029	68.0	0.010	6,800
MeWND	37.4	0.006	6,233	48.8	0.015	3,253

<sup>a</sup>Percent inhibition of growth rate produced by exposure to 2  $\mu$ M drug for 72 h determined by fluorescent growth inhibition assay

<sup>b</sup>ng Pt/ $\mu$ M DNA following a 24 h exposure to 2  $\mu$ M drug determined by ICP-MS

The CTR1<sup>+/+</sup> and CTR1<sup>-/-</sup> mouse embryo fibroblasts provide a powerful model with which to assess the role of CTR1 in drug influx due to the fact that these cells differ only in the expression of CTR1 and that they permit comparison of wild type cells expressing normal levels of CTR1 to cells that express none. It was previously reported that CTR1 knockout cells were approximately eightfold more resistant to DDP and accumulated only 30% as much Pt as CTR1-proficient cells [6]. The results of the current study confirm this observation, although the magnitude of the resistance in the CTR1<sup>-/-</sup> cells was only 2.1-fold despite the use of the same type of assay that measured inhibition of growth rate. Additionally, these results demonstrate that loss of CTR1 function does not result in resistance to JM118 and does not alter the extent of JM118 accumulation in the whole cell. The lack of involvement of CTR1 in JM118 accumulation clearly distinguishes JM118 from DDP. It is interesting to note that DDP and JM118 differ in structure only by the presence of the cyclohexyl ring attached to one of the two amino groups. The details of how CTR1 transports DDP into the cell remain unknown, but this change in structure apparently eliminates JM118 as a substrate for this influx transporter. The fact that, like DDP, JM118 is a highly polar molecule suggests that its entrance into the cell is mediated by other yet-to-be-identified transporters or mechanisms. This is consistent with the prior observation that JM118 can overcome resistance to DDP in cells in which DDP resistance is associated with reduced DDP uptake [20].

As was the case for DDP, Menkes cells engineered to express ATP7A or ATP7B were found to be significantly more resistant to the growth inhibitory effects of JM118. Thus, although JM118 differs in structure enough that it is not a substrate for CTR1, it nevertheless appears to be a substrate for some function of both ATP7A and ATP7B and this function serves to either detoxify the drug or limit its access to critical cellular targets. Even though the magnitude of the resistance is not large, in the case of DDP even small changes in sensitivity are sufficient to account for the clinical failure of treatment [2] and thus these two exporters are likely to be important determinants of satraplatin activity in vivo.

Although ATP7A and ATP7B function as Cu exporters, and forced expression of these two transporters in Me32a cells does in fact reduce Cu accumulation [12], such forced expression paradoxically resulted

in an increase rather than a decrease in whole cell Pt content following exposure to either DDP or JM118. This finding confirms the result of an earlier study on the effect of ATP7A and ATP7B that demonstrated increased whole cell accumulation of not just DDP but also carboplatin and oxaliplatin [18]. The fact that vesicles isolated from both the MeMNK and MeWND cells were found to contain more Pt than similar vesicles isolated from Me32a cells [18] suggests that, as for Cu, these transporters function to sequester DDP and JM118 into vesicular structures. In the case of Cu, such sequestration is associated with efficient export from the cell via the vesicle secretory pathway. In the case of the Pt-containing drugs, sequestration appears to dominate over export such that the net effect is greater whole cell accumulation of drug.

Enhanced vesicular sequestration provides a plausible explanation for why forced expression of ATP7A and ATP7B is associated with reduced accumulation of Pt in DNA despite increased whole cell accumulation when cells are exposed to DDP. Presumably enhanced sequestration limits access of the drug to the nucleus and thus renders the cell more resistant to the cytotoxic effect of the drug. However, this paradigm does not hold for JM118. In the case of JM118, while forced expression of either ATP7A or ATP7B rendered the cells more resistant and enhanced whole cell accumulation, it also increased the amount of JM118 reaching the DNA. The basis for the lack of correspondence between change in drug sensitivity and change in DNA Pt content is not currently understood. There are several possible explanations for this paradoxical result. One is that nuclear DNA is not the most critical target for JM118, and while enhanced sequestration does a good job of limiting access of the drug to a key unknown non-DNA target it does not limit, and in fact encourages, access to nuclear DNA. This concept is supported by earlier studies on other cell lines showing no correlation between the extent of JM118 DNA adduct formation and cytotoxicity [3]. Another possibility is that ATP7A and ATP7B alter the relative abundance of JM118 adducts formed in nuclear versus mitochondrial DNA, or the distribution of adducts between critical and non-critical parts of the genome. This hypothesis is supported by the observation that the relative level of cell growth inhibition per unit Pt in DNA in JM118-treated MeMNK and MeWND cells was less than in Me32a cells, while the relative level of cell kill per Pt in DNA in DDP-treated MeMNK and MeWND cells was similar to that in the Me32a cells. It would be of interest to further analyze the types of adducts formed by JM118 in cells expressing ATP7A or ATP7B. While it is currently unclear how cells are rendered resistant to JM118 while accumulating relatively higher levels of platinum in their DNA, this is one cellular pharmacological characteristic of JM118 that distinguishes it from DDP.

The Me32a cells express neither ATP7A nor ATP7B whereas the MeMNK and MeWND sublines express

very large amounts of the two proteins, respectively, thereby making this cell system an excellent model for characterizing the effects of ATP7A and ATP7B. However, Me32a cells are human fibroblasts, and the vesicle export system in which ATP7A and ATP7B operate may be quite different in these cells than in a fully malignant epithelial cell. The polarity of epithelial cells is one factor likely to be of importance; previous reports have suggested that the function of ATP7A and ATP7B is dependent on cellular polarity that is lacking in fibroblasts [4]. Additionally, the ATP7B protein expressed in this cell line is of murine origin, and it is currently unknown whether the phenotype associated with the expression of the murine protein differs from that associated with the expression of the human protein. Thus, caution is required in extrapolating the effects of ATP7A and ATP7B in the fibroblast system to fully malignant epithelial human tumors. To further assess the potential clinical importance of ATP7A and ATP7B as determinants of sensitivity to satraplatin it would be desirable to study their effect in fully malignant epithelial cells.

JM118 retains activity in some types of DDP-resistant cells [9]. The results of the current study suggest that JM118 is likely to retain activity in cells in which DDP resistance is due to the loss of CTR1, but not in cells in which resistance is due to enhanced expression of ATP7A or ATP7B. It remains to be determined whether loss of CTR1 is in fact a common mechanism of DDP-resistance, and it is likely that other factors also play a role in the lack of full cross-resistance between JM118 and DDP.

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