[Cancer Biology & Therapy 5:8, 943-949, August 2006]; ©2006 Landes Bioscience

Research Paper

Impaired Cisplatin Influx in an A2780 Mutant Cell Line

Evidence for a Putative, Cis-Configuration-Specific, Platinum Influx Transporter

Jozien Helleman[1,†,*]
Herman Burger[1,†]
Irene H.L. Hamelers[2]
Antonius W. M. Boersma[1]
Anton I.P.M. de Kroon[2]
Gerrit Stoter[1]
Kees Nooter[1,*]

[1]Department of Medical Oncology; Erasmus MC/Daniel den Hoed Cancer Center; Rotterdam, The Netherlands

[2]Department Biochemistry of Membranes; Bijvoet Institute and Institute of Biomembranes; Utrecht University; Utrecht, The Netherlands

[†]These authors contributed equally to this work.

*Correspondence to: Kees Nooter; Erasmus MC; Department of Medical Oncology; Josephine Nefkens Institute; Room Be422; P.O. Box 1738; 3000 DR The Netherlands; Tel.: +31104088357; Fax: +31104088363; Email: k.nooter@erasmusmc.nl/Herman Burger; Erasmus MC; Department of Medical Oncology; Josephine Nefkens Institute; Room Be422; P.O. Box 1738; 3000 DR The Netherlands; Tel.: +31104088357; Fax: +31104088363; Email: h.burger@erasmusmc.nl

Received 03/10/06; Accepted 05/02/06

Previously published online as a *Cancer Biology & Therapy* E-publication: http://www.landesbioscience.com/journals/cbt/abstract.php?id=2876

KEY WORDS

resistance, cisplatin, carboplatin, oxaliplatin, tetraplatin, nanocapsules, platinum influx transporter, impaired accumulation

ACKNOWLEDGEMENTS

We gratefully express our thanks to Desirée van Boven-van Zomeren and Rutger Staffhorst for the excellent technical assistance with the AAS measurements and preparation of nanocapsules, and Walter Loos for statistical analysis.

ABSTRACT

The effectiveness of platinum drugs in the treatment of cancer is hindered by intrinsic and acquired resistance. The cause of clinical resistance to platinum compounds is still unknown. In an attempt to identify new cellular mechanisms of cisplatin resistance, a one-step cisplatin-selection procedure was used to generate resistant sublines of the platinum sensitive A2780 ovarian cancer cell line. In the present study we selected an A2780 subline, A2780-Pt, that has a significantly reduced ability to accumulate cisplatin (36% of the parent A2780 cell line) and consequently shows a clear cisplatin-resistant phenotype (resistance factor, i.e., RF: 8.6). The A2780-Pt cell line was specifically cross-resistant to carboplatin (RF: 12.0), tetraplatin (RF: 8.1) and oxaliplatin (RF: 6.1) which was associated with a reduced cellular platinum accumulation (50%, 54% and 58% of A2780, respectively). No cross-resistance was found for a variety of other anticancer agents.

Further experiments to determine the cause of the platinum resistance of the A2780-Pt cell line revealed that: (1) impaired cellular platinum accumulation could not be attributed to aberrant expression of MRP2 (ABCC2), CTR1 (SLC31A1), ATP7A or ATP7B, (2) resistance was not associated with platinum inactivation by metallothionein and glutathione, (3) the platinum efflux rate was similar to that of A2780, (4) the defect in cellular accumulation and the resistance could be overcome by treatment with cisplatin nanocapsules, consistent with impaired influx, and (5) the defect in accumulation is specific for platinum compounds in the cis-configuration, since A2780-Pt cells did not show reduced accumulation of transplatin. This specificity suggests that not passive diffusion but an inward transporter is impaired in A2780-Pt.

In conclusion, we generated an A2780 subline that showed a uniquely stable platinum resistance phenotype, which could theoretically be caused by an impaired inward transporter specific for cis-configurated platinum compounds.

INTRODUCTION

Platinum-based drugs are among the most active anticancer agents. The use of cisplatin and its less toxic analog carboplatin has influenced the chemotherapeutic management of many solid tumors including testicular, ovarian, head and neck, and lung cancer.[1] As a result of the clinical use of platinum compounds, metastatic germ cell tumors are predominantly curable,[1] and the prognosis for patients with ovarian cancer has improved significantly.[2]

Since the development of cisplatin and carboplatin, a large number of platinum analogs have been synthesized to increase the spectrum of activity, to reduce the toxicity and/or to overcome cellular resistance. Two of these analogues oxaliplatin and tetraplatin, both 1,2-diamminocyclohexane (DACH) derivatives, were selected for preclinical development because they showed substantially different resistance profiles when compared to cisplatin and carboplatin.[3,4] This suggests that the DACH derivatives have a different mechanism of cytotoxicity probably because they form a bulkier DNA adduct due to the large DACH moiety. The clinical development of tetraplatin was stopped due to severe neurotoxicity observed in clinical studies. However, the effectiveness of oxaliplatin in the treatment of colorectal cancer which is primary resistant to cisplatin and carboplatin, has been demonstrated by in vitro and in vivo studies, as well as clinical studies.[4]

Unfortunately, the effectiveness of platinum drugs in the treatment of cancer is hindered by intrinsic and acquired resistance to each of the clinically used platinum compounds (cisplatin, carboplatin and oxaliplatin). A clear example to highlight this limitation is ovarian cancer. Although the initial response to platinum-based chemotherapy is high, about 20% of the patients never achieve a complete response and the majority of patients will relapse and eventually die of drug-resistant disease.[5]

Four major mechanisms of resistance to cisplatin have been reported, i.e., (1) inactivation of cisplatin by sulfur-containing molecules like glutathione and metallothionein, (2) increased repair of cisplatin-DNA adducts, (3) increased cisplatin adduct tolerance and failure of apoptotic pathways, and (4) reduced platinum accumulation by a decreased drug uptake or an increased drug efflux. Although the significance or nature of these mechanisms has not been clearly established, it is generally believed that reduced drug accumulation is a clinically important mechanism of resistance to platinum compounds.[6] In addition, the majority of the resistant sublines generated in vitro by cisplatin selection, accumulate less platinum than the drug sensitive cell line they were derived from.[7] As a possible explanation for a decreased accumulation, some putative cisplatin transporters have been described in the literature i.e., MRP2 (ABCC2) and the copper transporters CTR1 (SLC31A1), ATP7A and ATP7B.

The ATP dependent efflux pump MRP2 has been reported to mediate active efflux of cisplatin conjugated to glutathione.[8] In addition, increased intracellular glutathione levels were associated with cisplatin resistance.[9] Further support for a role of MRP2 in cisplatin resistance came from carcinoma cell line studies demonstrating that an increased MRP2 expression, intrinsic or enforced by transfection, was associated with resistance to cell death induced by cisplatin, decreased cellular drug accumulation and decreased DNA adduct formation.[8,10,11]

Recently, it has been reported that three transporters involved in maintaining the cellular copper homeostasis are also involved in the transport of cisplatin, carboplatin and oxaliplatin (reviewed by Safaei et al.).[12] One of these transporters is CTR1 that transports copper across the plasma membrane in an energy-independent manner. Two studies showed that deletion of CTR1 in yeast and mice leads to a decreased accumulation of and resistance to copper and cisplatin.[13,14] In addition, hCTR1 transfected in the ovarian carcinoma cell line A2780 resulted in an increased cisplatin accumulation associated with an increased sensitivity to the drug.[12] These results provide evidence that CTR1 is involved in the cellular uptake of cisplatin.

The two other copper transporters are the P-type ATPase transporters ATP7A or ATP7B[15] which are located in the final compartment of the Golgi-apparatus and transport excess copper out of the cell. Several cisplatin resistant human ovarian carcinoma cell lines (derived by in vitro selection from the sensitive parental cell line), have been shown to be cross-resistant to copper and showed a decreased accumulation of copper as well as cisplatin which was associated with a decreased DNA adduct formation by cisplatin.[15] Two of these cell lines showed increased expression of ATP7A, while the other cell line showed increased expression of ATP7B.[15] Moreover, transfection of ATP7B in human carcinoma cell lines caused resistance to cisplatin, carboplatin, oxaliplatin and copper accompanied by a decreased accumulation of these agents.[12,16,17] ATP7A deficient human fibroblast cell lines obtained from patients with Menkes disease, showed an increased accumulation of copper and cisplatin associated with hypersensitivity to both agents.[12] Furthermore, transfection of the ovarian carcinoma cell line 2008 with ATP7A resulting in only a small increase of ATP7A expression, was sufficient to cause resistance to cisplatin induced cell death.[18] These results indicate that both ATP7A and ATP7B could be involved in the efflux of cisplatin.

Despite the numerous reported in vitro cisplatin resistance mechanisms, the cause of clinical resistance to this drug has not been fully elucidated yet. In an attempt to identify new cellular mechanisms of cisplatin resistance, we generated resistant sublines with a short one-step cisplatin selection procedure. A cisplatin resistant derivative of the platinum sensitive ovarian cancer cell line A2780 was obtained and the characterization of this cell line showed a unique stable resistance phenotype with an impaired influx of cis-configurated platinum compounds that could not be attributed to one of the above described genes and that could be circumvented by the use of cisplatin nanocapsules (nanoprecipitates of cisplatin coated with a lipid bilayer).[19]

MATERIAL AND METHODS

Cell lines and culture condition. The ovarian carcinoma cell line A2780 and its cisplatin resistant variant A2780-Pt were grown as monolayers and maintained at 37°C in a humidified incubator with 8.5% CO_2 in HEPES buffered RPMI 1640 supplemented with 10% FCS (GIBCO BRL, Paisley, UK), 100 U/ml penicillin (Sigma-Aldrich) and 100 μg/ml streptomycin (Sigma).

Generation of a cisplatin-resistant A2780 cell line. A total of 1 x 10^8 A2780 cells were subjected to drug selection with 3.3 μM cisplatin (1 μg/ml) for ten consecutive days. In total, 11 colonies survived treatment and were picked, cloned and expanded. In the present study we characterized one of these resistant sublines (referred to as A2780-Pt) that harbors a significantly reduced ability to accumulate cisplatin and consequently shows a clear cisplatin-resistant phenotype.

Drug sensitivity assay. The MTT colorimetric assay, which measures the number of viable cells capable of reducing the tetrazolium compound to a blue formazan product, was used to determine the chemosensitivity of the cell lines as described previously.[20] With this assay, we determined the chemosensitivity to cisplatin, carboplatin, bleomycin and doxorubicin (Pharmachemie, Haarlem, The Netherlands), tetraplatin, cadmium chloride, mitomycin C, chlorambucil and taxol (Sigma-Aldrich, Steinheim, Germany), melphalan and busulfan (GlaxoSmithKline, Zeist, The Netherlands), oxaliplatin (Sanofi-Synthelabo, Maasluis, The Netherlands), carmustin (BCNU) (Bristol-Myers, Woerden, The Netherlands), thiotepa (Lederle, Gosport Hampshire, UK), 5-fluorouracil (TEVA Pharma, Mijdrecht, The Netherlands), copper sulfate (Merck, Darmstadt, Germany) and Ecteinascidin 743 (ET-743) (PharmaMar, Madrid, Spain). The chemosensitivity to cisplatin nanocapsules was determined with the sulforhodamine B (SRB) assay.[21] The absorbance value measured in the absence of drug was set at 100% cell survival in both the MTT and the SRB assay. Subsequently, drug concentrations were plotted against cell survival using the Inhibitory Effect Sigmoid Emax model, as implemented in WinNonLin version 4.0 (Pharsight Corp., Mountain View, CA), as follows:

$$E = Emax \times (1 - (Concentration \wedge Gamma / (Concentration \wedge Gamma + IC_{50} \wedge Gamma)$$

The chemosensitivity is expressed as the IC_{50} value, i.e., the drug concentration at which a 50% cell survival is observed. The resistance factor is calculated by dividing the IC_{50} value for A2780-Pt by the IC50 value for A2780.

Clonogenic survival assays were basically performed as described previously.[22] Briefly, cells were plated at specified concentrations in six-well plates and cultured for 24 hours. The exponentially growing cultures were exposed to cisplatin (range: 0–50 μM) for two hours.

The cisplatin-containing culture medium was replaced by drug-free medium and surviving colonies were scored after eight to ten days. Each drug concentration was tested in duplicate and at least three independent survival experiments were performed for each cell line. Plating efficiencies for A2780 and its cisplatin-resistant variant A2780-Pt were similar (~70%). To plot surviving fractions as a function of the cisplatin dose level on a logarithmic scale, each point at the survival curve was normalized against the plating efficiency of untreated control cells.

Intracellular platinum accumulation. Triplicate 25 cm^2 tissue culture flasks with exponentially growing cells were exposed to cisplatin (100 μM), oxaliplatin (100 μM), tetraplatin (100 μM), carboplatin (600 μM) and transplatin (100 μM) for two, four and six hours. Subsequently the cells were washed to remove free drug, harvested by trypsinization and washed with ice cold PBS. One third of the cell pellet was used to measure the protein concentration using a Bio-Rad protein assay kit. Total platinum content was determined in duplicate in the remaining two thirds of the cell pellet by flameless atomic absorption spectrometry (AAS) using a Perkin-Elmer (Foster City, CA) 4110 ZL spectrophotometer. Intracellular platinum levels were expressed as μg of platinum per mg of protein (μg Pt/mg protein). In addition to the intracellular platinum accumulation, the total DNA platination was also determined by measuring the amount of platinum bound to DNA following cisplatin exposure (2-hour incubation; 33 μM). Preparation of genomic DNA and subsequent determination of platinum-DNA adducts using AAS were performed as described previously.[20] The DNA platination levels were expressed as pg of Pt per μg of DNA (pg Pt/μg DNA).

Quantitative real-time RT-PCR. The mRNA expression levels of BAX, BCL2, BCL-XL, MDR1 (ABCB1), MRP1 (ABCC1), MRP2 (ABCC2), BCRP (ABCG2), MVP (LRP), ATP7A, ATP7B and SLC31A1 (CTR1) were measured by quantitative real-time RT-PCR analysis based on TaqMan chemistry using an ABI PRISM 7000 sequence detector system (Applied Biosystems, Foster City, CA). Primer pairs and fluorescent hybridization probes for MDR1, MRP1, MRP2, BCRP, LRP, PBGD and GAPDH have been described previously.[23] The primer pairs and probe for CTR1 are: forward 5'-CAGTGTTTTTACTAGCAATG-3', reverse 5'-GAAAGCTCCAGCATCTCT-3', probe 5'-FAM-CGTAAGTCACAAGTCAGCATTC-TAMRA-3', for BAX: forward 5'-GAGCTGCAGAGGATGATT-3', reverse 5'-GAGGCCGTCCCAAC-3', probe 5'-FAM-CTGATCAGTTCCGGCACCTT-TAMRA-3', for BCL2: forward 5'-TCGGTGGGGTCATGT-3', reverse 5'-GGGCCAAACTGAGCA-3', probe 5'-FAM-TCAACCGGGAGATGTCGC-TAMRA-3' and for BCL-X_L: forward 5'-CCCAGGGACAGCATATC-3', reverse 5'-GCTGCATTGTTCCCATAG-3', probe 5'-FAM-TGAATGAACTCTTCCGGGATG-TAMRA-3'. The 20x assay-on-demand primers and FAM-TAMRA labeled probe-mix (Applied Biosystems) were used for ATP7A (Hs00163707_m1) and ATP7B (Hs00163739_m1). Expression of the studied genes was normalized to an internal control gene, i.e., GAPDH. To compare the relative expression levels of target genes between A2780 and A2780-Pt, the comparative C_T method was used as previously described.[23] The C_T value is defined as the fractional cycle number at which the emitted sample fluorescence passes a fixed threshold above the baseline. The ΔC_T value is defined as the difference in C_T value for the target and reference gene GAPDH. Accordingly, ΔC_T = (mean target gene C_T) - (mean of C_T values for GAPDH). The relative gene expression in A2780-Pt (normalized to GAPDH) is compared with the relative expression of the target gene in A2780 (also normalized to GAPDH) which was used here as a calibrator. The relative amount of target in A2780-Pt is calculated by the formula $2^{-\Delta\Delta CT\ value}$. The $\Delta\Delta C_T$ value in this formula is defined as the ΔC_T value of the target gene for A2780-Pt minus the ΔC_T value of the target gene for A2780 ($\Delta\Delta C_T = \Delta C_{T(A2780\text{-}Pt)} - \Delta C_{T(A2780)}$). Thus the expression of the target gene in A2780 (the calibrator) is set at 1 and the amount of target in A2780-Pt is given relative to A2780."

Glutathione content. The ApoGSH Glutathione Colorimetric Detection Kit (BioVision) was used to determine the glutathione (GSH) content in A2780 and A2780-Pt cells that were incubated with or without 33 μM cisplatin for 2.5 hours. The cell pellets were washed with ice-cold PBS and lysed with the glutathione buffer supplied by the manufacturer. The sample solutions and the supplied glutathione standard solutions were incubated with the reaction mix for five minutes at room temperature. The reaction mix contains the GSH substrate DTNB that reacts with GSH to generate GSSG and 2-nitro-5-thiobenzoic acid. Since 2-nitro-5-thiobenzoic acid is a yellow colored product, GSH concentration can be determined by measuring absorbance at 405 nm using a microplate reader. The amount of glutathione in the sample solution was determined using the standard glutathione calibration curve and was normalized to the number of cells used for the analysis (ng GSH/1 x 10^6 cells).

Preparation of cisplatin nanocapsules. Cisplatin (SigmaAldrich) was dissolved in MilliQ water and incubated in the dark overnight at 37°C. Lipid dispersions (1.2 mM) were prepared by adding 5 mM cisplatin to a dry film of DOPS and DOPC (1:1) phospholipids (Avanti Polar Lipids, Birmingham, Alabama). The lipid dispersions were then incubated at 37°C for 30 minutes, followed by ten freeze-thaw cycles using ethanol/dry ice (-70°C) and a water bath (55°C). Nonencapsulated cisplatin was removed by centrifugation (two times, 4 minutes, 20°C, 500 g) and the pellets were resuspended in MilliQ. Subsequently, the nanocapsules were incubated for two hours at 37°C, collected by centrifugation, resuspended in MilliQ and used in the experiments. The platinum content was quantified by AAS on a SpectrAA-400 Zeeman spectrometer (Varian, Palo Alto, CA), using a modifier solution of 0.5% Triton X-100 in MilliQ and K_2PtCl_6 as a standard.[24] Phospholipid content was determined by phosphate (Pi) analysis after destruction with perchloric acid.[25]

RESULTS

Generation of a cisplatin-resistant cell line. A2780 cell cultures (1 x 10^8 cells) were treated with 3.3 μM cisplatin (1 μg/ml) for ten consecutive days. This relatively short (one-step) cisplatin selection resulted in the survival of 11 independent cell clones. Clones were further characterized for level of resistance and growth parameters. In the present study we selected one of these clones, the A2780-Pt subline that exhibits a clear and stable cisplatin-resistant phenotype. A2780-Pt cells show no differences in intrinsic growth properties under normal culture conditions (i.e., duration of the cell cycle and distribution over the cell cycle phases) as compared to the parental A2780 cell line. Moreover, the cisplatin resistance phenotype is maintained in cell culture without drug selection and stayed stable during six months of drug-free culturing (data not shown), suggesting a stable genetic alteration in the genome of this cell line.

To determine the level of cisplatin resistance of the A2780-Pt cell line we performed MTT and clonogenic survival assays. The latter assay measures the capacity of the cells to form colonies in the presence of serial diluted cisplatin, i.e., cell survival and outgrowth. Both

assays showed that the resistant A2780-Pt cells are significantly less sensitive to cisplatin than the parental A2780 cell line (Fig. 1A and B). The IC_{50} values derived from a representative MTT assay for A2780 (IC50: 131 nM) and A2780-Pt (IC50: 1126 nM), showed that A2780-Pt variant cells are 8.6-fold more resistant than parental A2780 cells (Table 1). In addition, the IC_{50} values derived from the clonogenic assay show a 3.8-fold difference between A2780 (IC_{50}: 1.2 μM) and A2780-Pt (IC_{50}: 4.5 μM). Moreover, the clonogenic survival curves show that the difference in survival and cell outgrowth between parental A2780 and A2780-Pt cells is more than one log (e.g., at 10,7 μM the survival difference is 27-fold: A2780: 0.39% and A2780-Pt: 10.64%, respectively). These results demonstrate that A2780-Pt is less sensitive to cisplatin than the parental A2780 cell line.

A2780-Pt is cross-resistant to other platinum-containing compounds. Next, we tested whether the cisplatin-resistant A2780-Pt cells are cross-resistant to other chemical compounds including other platinum-containing drugs (Table 1). MTT assays showed that A2780-Pt cells were cross-resistant to carboplatin (RF 12.0), tetraplatin (RF 8.1) and oxaliplatin (RF 6.1). In contrast, A2780-Pt was not cross-resistant to other compounds such as cadmium chloride, which is bound by metallothionein. Interestingly, the A2780-Pt cells are also not cross-resistant to copper sulfate, which is transported by the copper/cisplatin transporters CTR1, ATP7A and ATP7B (Table 1). In addition, no cross-resistance was seen for a variety of anticancer drugs that belong to the alkylating agents, vinca alkaloids, anthracyclines, microtubule antagonists and antimetabolites (Table 1). Dose-response curves representative for the cross-resistant compounds, i.e., tetraplatin (Fig. 1C) and not cross-resistant compounds, i.e., ET-743 (Fig. 1D) are depicted in Figure 1.

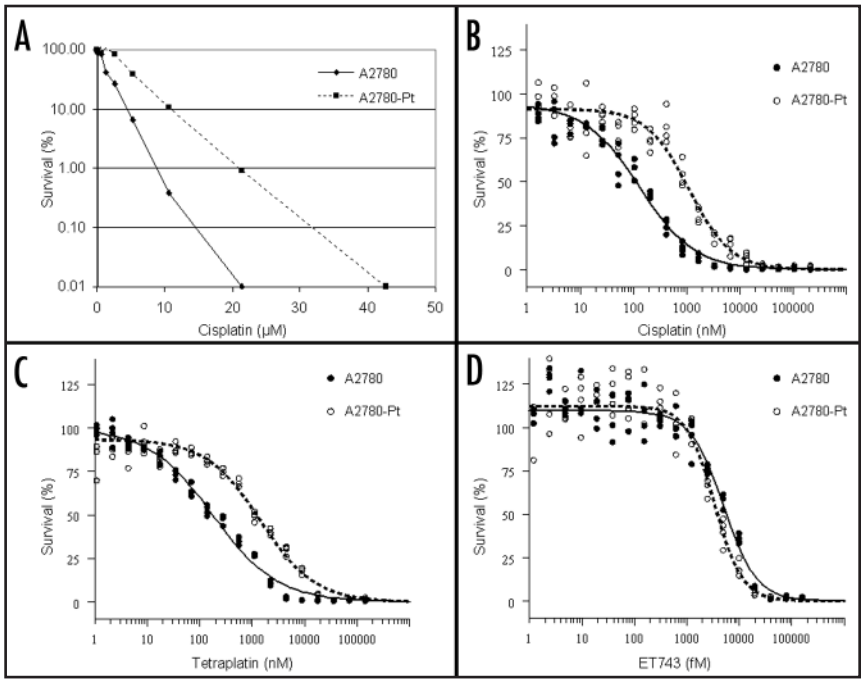

Figure 1. Dose-response curves of A2780 and A2780-Pt determined by a clonogenic assay for cisplatin (A) or by an MTT assay for cisplatin (B). In addition, representative dose-response curves (determined by an MTT assay) for cross-resistant, i.e., tetraplatin (C) and not cross-resistant drugs, i.e., ET743 (D) are depicted.

Known drug-resistance mechanisms are not responsible for the cisplatin-resistant phenotype of A2780-Pt. To determine the possible involvement of known drug resistance mechanisms in A2780-Pt cells, we performed quantitative real-time RT-PCR for the following genes: MDR1 (ABCB1), MRP1 (ABCC1), MRP2 (ABCC2), BCRP (ABCG2), ATP7A, ATP7B, SLC31A1 (CTR1), MVP (LRP), BCL2, BAX and BCL-X_L. All genes were readily detectable in both cell lines, except for MDR1, and not differentially expressed between A2780 and A2780-Pt (Table 2). These data indicate that known platinum transporters like MRP2, ATP7A, ATP7B and CTR1 are probably not involved in the resistance phenotype of A2780-Pt cells as well as the other drug pumps MDR1, MRP1 and BCRP. In addition, there was no difference in the expression of MVP (LRP), a gene associated with the resistance of ovarian cancer cells to cisplatin,[26] and BCL2, BAX and BCL-X_L, genes involved in the apoptotic response to cisplatin treatment.[27]

Since it has been reported that cellular glutathione (GSH) might be a critical determinant in cisplatin resistance, we compared the GSH content of A2780 and A2780-Pt cells. In untreated cells the GSH content was not significantly different between the cisplatin resistant A2780-Pt and parental A2780 cell lines (2.0 ng/μl and 2.3 ng/μl respectively, p = 0.145). In addition, we found no difference in GSH concentration after a 2.5 hour incubation with 33 μM cisplatin for A2780-Pt and A2780 (respectively, 5.4 and 5.0 ng/μl, p = 0.294), although a clear ~2.5-fold induction in cellular GSH could be detected in both cell lines after drug treatment.

Intracellular accumulation of platinum is decreased in A2780-Pt cells. We measured the intracellular platinum content to determine whether an impaired intracellular drug accumulation is responsible for the observed cisplatin resistance phenotype of A2780-Pt. A2780 and A2780-Pt cells were incubated for two, four or six hours with 100 μM cisplatin after which the intracellular accumulation of platinum was measured with AAS. In both cell lines the accumulation increased in time but was significantly reduced in A2780-Pt cells for each time point ($p < 0.05$) as compared to the parental cells (Fig. 2A: 6 hour time point). The decreased cellular platinum accumulation in A2780-Pt (36% of A2780) was paralleled by a decrease of total platinum bound to genomic DNA (31% of A2780) (Fig. 2B). This similar difference in accumulation and platinum-DNA adduct formation was repeatedly found in independent and parallel performed experiments. The mean ± the standard deviation of platinum bound to genomic DNA was 12 ± 3 pg Pt/μg DNA and 39 ± 10 pg Pt/μg DNA for A2780-Pt and A2780 cells, respectively (p = 0.035). In addition to the impaired accumulation of cisplatin, incubation with oxaliplatin (100 μM), tetraplatin (100 μM) or carboplatin (600 μM) for two, four and six hours, demonstrated that the intracellular accumulation of these compounds was also markedly decreased in A2780-Pt, as compared to the parental A2780 (Fig. 2A, 6 hour time point).

All platinum compounds, which accumulated less in the A2780-Pt cells compared to the parental cell line and tested positive for cross-resistance, are in the cis-configuration (Table 1). To investigate whether the reduction in accumulation was specific for platinum compounds with a cis-configuration, we incubated the A2780-Pt and the A2780 cells with 100 μM of the noncytotoxic trans-variant of cisplatin i.e., transplatin. We subsequently measured the intracellular

Table 1 **The sensitivity of A2780 and A2780-Pt determined with an MTT-assay**

	A2780		A2780-Pt		Resistance
	IC_{50} (nM)	SE	IC_{50} (nM)	SE	Factor
Resistant					
Carboplatin	2169	394	25949	4156	12.0
Cisplatin	131	12	1126	112	8.6
Tetraplatin	179	11	1445	123	8.1
Oxaliplatin	88	12	535	80	6.1
Not resistant					
Cadmium chloride[a]	513	45	784	74	1.5
Coppersulfate[a]	67270	4687	44249	4415	0.7
Busulfan[b]	26074	1964	30989	3644	1.2
Carmustine (BCNU)[b]	46394	3720	69506	5765	1.5
Chlorambucil[b]	1519	176	1335	137	0.9
Melphalan[b]	4126	290	9604	670	2.3
Mitomycin C[b]	29	3	40	5	1.4
Thiotepa[b]	2295	257	3047	388	1.3
5-Fluorouracil[c]	3085	286	3329	257	1.1
Doxorubicin[d]	6.4	0.4	8.1	0.6	1.3
Taxol[e]	1.93	0.09	1.74	0.07	0.9
ET-743	5.0*E-3	0.4*E-3	3.4*E-3	0.3*E-3	0.7
Bleomycin	7.4	1.1	12.1	1.8	1.6

The 50% cytotoxicity values (IC_{50}) ± standard error (SE) and the resistance factor of A2780-Pt versus A2780 are listed for each drug. [a]Heavy metals; [b]Alkylating agents; [c]Antimetabolite; [d]Topoisomerase II inhibitor; [e]Microtubule inhibitor.

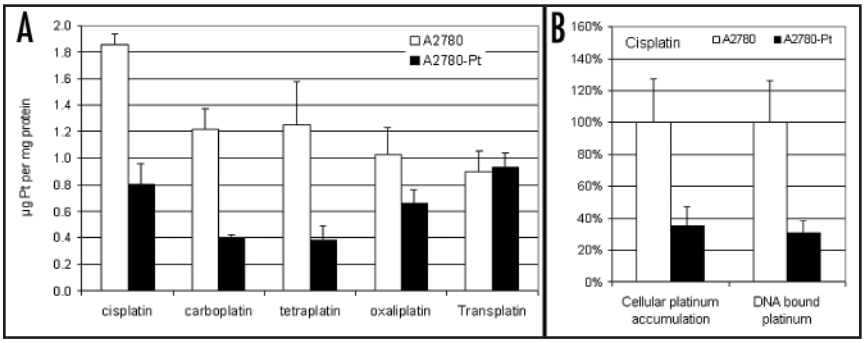

Figure 2. Platinum accumulation in the cell and bound to DNA. (A) Cellular platinum accumulation after a six hour incubation with 100 µM cisplatin, 600 µM carboplatin, 100 µM tetraplatin, 100 µM oxaliplatin and 100 µM transplatin (n = 3). The error bars represent the standard deviations. (B) The percentage of cellular platinum and platinum bound to the DNA after a two hour incubation with 33 µM cisplatin compared to A2780 (A2780 values are set at 100%, n=6 for the cellular accumulation and n = 3 for the DNA bound platinum). The error bars represent the standard deviations.

platinum accumulation after two, four and six hours of incubation. Although transplatin is a non-cytotoxic platinum compound, it readily accumulates in A2780 cells, indicating that it enters the cells as efficiently as cisplatin. However, in contrast to the other platinum drugs tested, transplatin accumulation was not significantly different in A2780-Pt as compared to A2780 (Fig. 2A). This suggests that the impaired intracellular platinum accumulation in A2780-Pt cells is specific for platinum drugs with a cis-configuration.

A defect in cellular influx accounts for the cisplatin resistance of A2780-Pt cells. To determine whether an increased efflux mechanism might be responsible for the observed reduced accumulation of platinum compounds, the platinum efflux was followed over time. For this experiment, cells were exposed to 33 µM (A2780) and 100 µM (A2780-Pt) cisplatin for one hour. This resulted in similar intracellular platinum levels in both cell lines (Fig. 3, t = 0). After replacement of the medium, the time-dependent decrease in intracellular platinum was comparable for both cell lines (Fig. 3), indicating similar efflux rates.

Since we did not see an increased efflux of platinum, it is very likely that an impaired influx is responsible for the observed reduced accumulation of platinum in the A2780-Pt cells. Previously a new method was developed to effectively encapsulate cisplatin in a lipid formulation. Cisplatin nanocapsules are bean-shaped cisplatin nanoprecipitates surrounded by a single phospholipid bilayer, which have an unprecedented drug-to-lipid molar ratio of approximately ten to one.[19] The cisplatin nanocapsules most likely enter the cell by endocytosis,[19] and may thereby circumvent the natural cellular uptake mechanisms for free cisplatin. To investigate whether treatment with cisplatin nanocapsules can by-pass the cisplatin-resistance in A2780-Pt cells, A2780 and A2780-Pt cells were incubated with the nanocapsules and the chemosensitivity as well as the intracellular platinum accumulation were determined. The dose response curves derived from the SRB assay, demonstrate that there is no difference in chemosensitivity between the resistant A2780-Pt and parental A2780 cells when incubated with cisplatin nanocapsules (Fig. 4A). Moreover, the intracellular platinum accumulation measured by AAS after a two-hour exposure to cisplatin nanocapsules at two concentrations (33 µM and 100 µM cisplatin) was identical for both cell lines (Fig. 4B). These result show that the circumvention of the natural cisplatin influx mechanisms by administrating cisplatin nanocapsules instead of free cisplatin results in a comparable platinum accumulation and chemosensitivity for both cell lines.

DISCUSSION

To identify new cellular mechanisms of cisplatin resistance we used a short one-step cisplatin-selection procedure and obtained a mutant subline (A2780-Pt) of the ovarian cancer A2780 cell line that shows resistance to cisplatin accompanied by a strong reduction in cellular platinum accumulation as compared to the parent cells. Characterization of the A2780-Pt mutant cell clone revealed a specific cross-resistance pattern to carboplatin, tetraplatin, and oxaliplatin that was also associated with impaired cellular drug accumulation. In contrast, no cross-resistance was found for a variety of other clinically used anticancer drugs that belong to the alkylating agents, vinca alkaloids, anthracyclines, microtubule antagonists, and antimetabolites.

In the past many cisplatin resistant cell lines have been generated by long-term exposure of mammalian cells to stepwise increased concentrations of platinum.[28-33] This procedure mostly resulted in mutant cell lines with pleiotropic resistance phenotypes caused by a variety of drug-induced cellular aberrations. In contrast, we obtained a drug resistant cell clone after a relatively short selection period with only one concentration of cisplatin. The resistant A2780-Pt cells are not cultured in the presence of cisplatin or any

Table 2 **Comparison of relative mRNA expression levels in A2780 parental cells and in A2780-Pt cells measured by quantitative RT-PCR**

	Relative Expression		Relative Amount of Target Compared to A2780
	A2780	A2780-Pt	
	(ΔCt)	(ΔCt)	($2^{-\Delta\Delta Ct}$)
BCL-2	12.4	12.5	0.93
BAX	5.3	5.2	1.07
BCL-X_L	10.1	9.3	1.74
LRP	6.6	6.4	1.15
MRP1	5.0	4.8	1.15
MRP2	16.5	17.1	0.66
BCRP	14.1	13.3	1.74
MDR1	0.0	0.0	1.00
ATP7A	8.0	8.3	0.81
ATP7B	4.2	5.0	0.57
CTR1	5.0	5.7	0.62

other platinum-containing compound. The cells maintain their resistance even after six months of drug-free propagation in cell culture, indicating that the phenotype is stable. These characteristics suggest that A2780-Pt is an appropriate model for studying clinical resistance to platinum.

A2780-Pt cells exhibit a unique resistance phenotype that apparently was not caused by the known molecular mechanisms of platinum resistance. Inactivation of platinum by thiol-containing compounds (glutathione and metallothionein) was ruled out by the results of the MTT assay with cadmium chloride and the intracellular glutathione (GSH) determinations. Cadmium chloride is bound by metallothionein and overexpression of metallothionein results in tolerance to cadmium toxicity[34] and the sensitivity to cadmium chloride and the GSH content were similar for both cell lines.

We did not detect a difference in the mRNA expression levels of several proteins involved in the early steps of apoptosis activation following the DNA damage recognition (BAX, BCL-2 and BCL-X_L). We did detect a difference in the platinum accumulation. In the resistant cell line a reduced cellular accumulation of platinum compounds resulted in a similar reduction of platinum bound to the DNA. The observed difference in cellular platinum accumulation can only be caused by an increased efflux and/or a decreased influx. Based on gene expression data, the involvement of the outward copper/cisplatin transporters ATP7A and ATP7B in the resistance

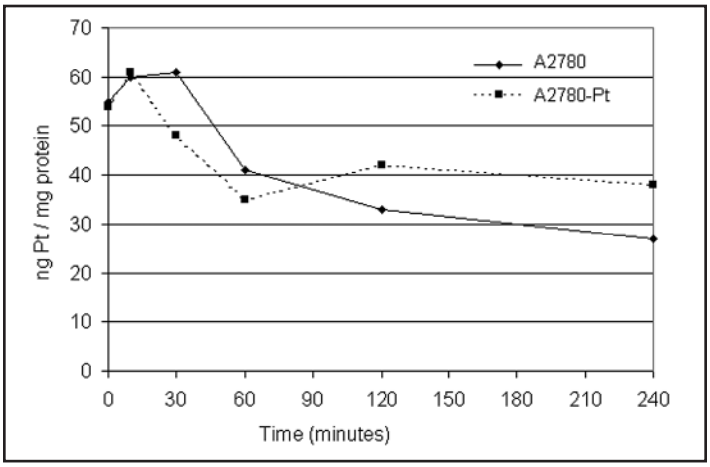

Figure 3. The efflux of cisplatin for A2780 and A2780-Pt. Cellular platinum content was followed in time after a one hour incubation with 33 µM and 100 µM cisplatin for A2780 and A2780-Pt, respectively.

phenotype of A2780-Pt cells is very unlikely. We also found no differences in expression of MRP2 that might function as an outward cisplatin transporter. The similar efflux of platinum in the resistant cells when placed in drug-free medium is in accordance with these observations.

Since there is no clear difference in platinum efflux, the reduced accumulation must be caused by an impaired influx of the platinum drug. It is generally believed that cisplatin enters the cell by passive diffusion as well as by facilitated transport.[7] A recent notion is that various anticancer drugs can enter mammalian cells by facilitated transport using solute carriers (SLCs).[35] The SLC family has about 300 members and one of the members is the copper inward transporter CTR1 (SLC31A1) that has recently been implicated to be involved in cellular uptake of platinum.[12] However our gene expression data demonstrated that the levels of CTR1 are comparable in the sensitive and resistant cell line. It is possible that the overall or membrane localized protein level of CTR1 is reduced in the A2780-Pt cells, thus causing the cisplatin resistant phenotype. However, no cross-resistance for copper sulfate was found. This strongly suggests that downregulation of copper-inward transporters is not responsible for the platinum resistance of A2780-Pt cells.

Previously, it was shown that cisplatin nanocapsules most likely enter the cell via endocytosis,[19] and may thereby circumvent the natural influx mechanisms of cisplatin. The platinum-accumulation and the IC_{50} values were similar for A2780 and A2780-Pt cells treated with nanocapsules. These data also suggests that nanocapsules circumvent the impaired cisplatin influx mechanism that causes the reduced

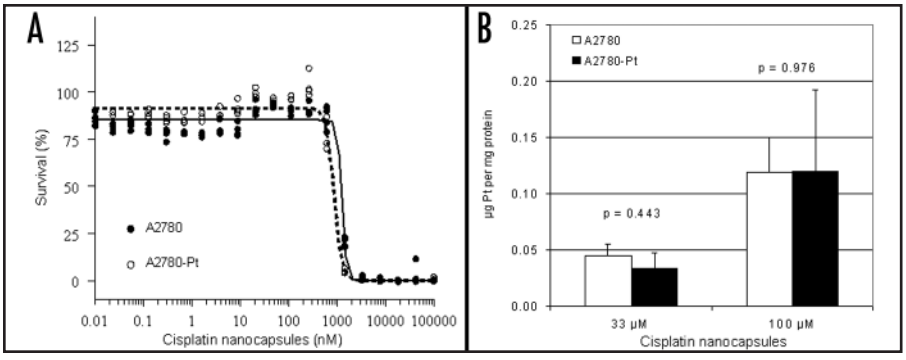

Figure 4. The dose-response curves and platinum accumulation for A2780 and A2780-Pt treated with cisplatin nanocapsules. (A) The dose-response curves were determined with an sulforhodamine B assay. (B) Cellular platinum accumulation was determined for A2780 and A2780-Pt after a two hour incubation with cisplatin nanocapsules (33 µM and 100 µM, n = 3). The significance of the difference in platinum accumulation between A2780 and A2780-Pt is given in the figure. The error bars represent the standard deviations.

platinum accumulation and resistance in A2780-Pt. This study is the first to show that the cisplatin nanocapsules can circumvent platinum resistance caused by an impaired platinum influx in vitro, suggesting that cisplatin nanocapsules could prove useful in the treatment of platinum-resistant tumors in vivo.

Our data support the hypothesis that an impaired cisplatin influx mechanism causes the reduced platinum accumulation and resistance in A2780-Pt cells. Characterization of the specificity of this platinum-specific influx mechanism showed that after treatment with transplatin, the noncytotoxic trans-variant of cisplatin, the platinum accumulation in A2780-Pt did not significantly differ from that in A2780. This suggests that the platinum-specific influx mechanism is specific for the cis- and not the trans-configuration of platinum drugs. The striking specificity of this influx mechanism only for platinum compounds in the cis-configuration suggests that not passive diffusion but a platinum specific inward transporter is involved in the observed impaired platinum influx and resistance in A2780-Pt.

In conclusion, we selected an A2780 subline that showed a uniquely stable platinum resistance phenotype, which could theoretically be caused by an impaired inward transporter specific for cis-configurated platinum compounds. The existence of another SLC capable of transporting platinum compounds like the copper/cisplatin inward transporter CTR1 (SLC31A1), is an attractive idea that deserves further investigation. Moreover, this study is the first to show that cisplatin resistance due to an impaired influx can be circumvented by the use of cisplatin nanocapsules.

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