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Relevance of drug uptake and efflux for cisplatin sensitivity of tumor cells

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ABSTRACT

Platinum sensitivity and platinum resistance may involve altered activity of transport proteins. In order to assess the role of drug uptake and efflux in this phenomenon, we compared the expression of three copper transporters, intracellular platinum accumulation, DNA platination and cytotoxicity of cisplatin in two cisplatin-sensitive and -resistant tumor cell line pairs (ovarian A2780/A2780cis and cervical HeLa/HeLaCK cells). Gene expression of importer CTR1, and ATP7A and ATP7B efflux transporters (with and without cisplatin treatment) was investigated using quantitative real-time PCR and platinum concentrations were determined by flameless atomic absorption spectrometry.

After incubation with cisplatin, DNA platination was significantly lower in the resistant variants compared to the respective sensitive cell lines, whereas no obvious difference in DNA repair was found. Accordingly, the resistant variants exhibited lower intracellular platinum concentrations than their respective parental cells (2.5- and 2.9-fold lower in A2780cis and HeLaCK cells, respectively). No differences in efflux were observed. Resistant cells expressed lower levels of *CTR1* (1.5–1.8-fold) than their sensitive counterparts. Expression differences of *ATP7A* and *ATP7B* between resistant and sensitive cells were cell type-specific.

The results highlight the relevance of CTR1 for cisplatin sensitivity as there is a clear relationship between lower *CTR1* expression, intracellular concentration, DNA platination and cytotoxicity of cisplatin in both resistant cell lines. Our data provide the basis for a quantitative understanding of alterations in uptake and efflux processes leading to cisplatin resistance and might hence facilitate the development of *ex vivo* assays that can predict cisplatin sensitivity in tumor specimens of patients.

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Abbreviations: ACTB, β-actin; ATP7A, ATPase, copper transporting, alpha polypeptide; ATP7B, ATPase, copper transporting, beta polypeptide; B2M, beta-2-microglobulin; hCTR1, human copper transporter 1; DEPC, diethyl pyrocarbonate; GSH, glutathione; GU, glucuronidase beta; HUPO, 60S (human) acidic ribosomal protein P1; HPRT1, hypoxanthine-phosphoribosyltransferase 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PhosA2, phospholipase A2; RPL13, ribosomal protein L13; TFR, transferrin receptor (p90; CD71); UBE2D2, ubiquitin-conjugating enzyme E2D2

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1. Introduction

Cisplatin [*cis*-diamminedichloroplatinum(II)] is a widely used platinum complex with high cytotoxic activity against several tumors [1]. The cytotoxic effect of cisplatin is based upon the formation of platinum-DNA adducts. As one consequence of DNA platination, the cell cycle is arrested in order to allow the cell to repair the damage. If repair fails apoptosis is induced by activation of various pathways [2,3].

The therapeutic outcome of cisplatin-based chemotherapy can be impaired by intrinsic or acquired resistance. Cisplatin resistance is the consequence of multifactorial events. Several molecular mechanisms of resistance may occur in the same cell population. Intensive efforts have been made to clarify these mechanisms. They include decreased accumulation and increased detoxification of cisplatin, more efficient removal of platinum-DNA adducts, enhanced capacity to replicate past adducts, as well as inhibition of apoptosis [4–7].

An increasingly recognized factor affecting DNA platination is the change of intracellular platinum concentrations. Reduced drug accumulation is frequently observed in cisplatin-resistant cell lines but the mechanism has remained uncertain. It could result either from reduced uptake or enhanced efflux or both. Recently, three copper transporters (CTR1, ATP7A and ATP7B) have been found to be involved in the uptake and efflux of platinum complexes [8–11]. The results of Komatsu et al. [12] provided the first connection between copper transporters and cellular pharmacology of platinum complexes. Their data indicated that overexpression of the copper efflux transporter *ATP7B* may lead to cisplatin resistance. Lin et al. [8] confirmed this relation and showed that *CTR1*-deficient yeast cells developed cisplatin resistance. The results of Ishida et al. [9] showed that CTR1 can transport copper as well as cisplatin in yeast and mammals. The human copper uptake transporter CTR1 and the copper efflux P-type ATPases ATP7A and ATP7B belong to the superfamily of transmembrane domain spanning transporter proteins responsible for the copper homeostasis of mammalian cells. Down-regulation of *CTR1* and up-regulation of *ATP7A* and *ATP7B* has been associated with acquired platinum resistance [12–14].

The aim of this work was to reveal the relevance of copper transporters CTR1, ATP7A, and ATP7B on intracellular cisplatin accumulation, DNA platination and cisplatin sensitivity, since their relationships have been only partly resolved so far (Fig. 1). We used two well-characterized tumor cell line pairs: cisplatin-sensitive and -resistant ovarian A2780 and cervical HeLa carcinoma cells. The results may help develop strategies to predict and affect platinum sensitivity and hence increase the therapeutic outcome of platinum-based chemotherapy.

2. Materials and methods

2.1. Cell lines

The human ovarian carcinoma cell lines A2780 and A2780cis and cervical carcinoma cell lines HeLa and HeLaCK were used. The cisplatin-resistant variants were obtained due to incubation with stepwise increasing cisplatin concentrations [15,16]. A2780/A2780cis cells were grown in RPMI-1640™ medium (Sigma–Aldrich Chemie, Steinheim, Germany) after adding 10% fetal calf serum, 0.6 mM L-glutamine, 100 I.E./mL penicillin and 0.1 mg/mL streptomycin. HeLa/HeLaCK cells were grown in D-MEM with GlutaMAX™ (Invitrogen Gibco, Karlsruhe, Germany) after adding 10% fetal calf serum, 100 I.E./mL penicillin and 0.1 mg/mL streptomycin. The cells were cultivated as monolayer in a humidified atmosphere at 37 °C and 5% CO_2. The resistant cell lines were incubated with 1–3 μM cisplatin every 3–5 passages to maintain resistance.

2.2. MTT assay

MTT assays were performed as recently described [17]. Briefly, cells were plated in 96-well microtiter plates (approximately 10.000 cell/well) and pre-incubated with growth medium overnight. Cisplatin (Sigma–Aldrich, Steinheim, Germany) was dissolved in 0.9% saline and cells were exposed to different cisplatin concentrations and incubated for 72 h. Following incubation cells were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich, Steinheim, Germany) solution [0.5 mg/mL dissolved in phosphate-buffered saline] for approximately 60 min. The formazan crystals formed were dissolved using isopropanol containing 0.04 M hydrochloric acid. Absorbance of the converted dye was measured at 595 nm with background subtraction at 690 nm using a Polarstar-Galaxy™ microtiter plate reader (BMG-Lab-Technologies, Offenburg, Germany). The results were analyzed and the EC_{50} values (molar concentration which produces 50% of the maximum possible response) were estimated using the software Prism™ (GraphPad Software, San Diego, USA). The resistance factor was calculated by dividing EC_{50} in the resistant variant by the EC_{50} in the respective sensitive cell line.

2.3. Measurement of DNA platination

5×10^6 cells were incubated with cisplatin (10–125 μM) up to 4 h and washed three times with 5 mL ice-cold PBS buffer. Afterwards, cells were trypsinized, resuspended in fresh drug-free medium and centrifuged for 4 min at 4 °C and 260 × g. The supernatant was discarded and the pellet was washed twice in 1 mL ice-cold PBS buffer. After centrifugation for 1 min at 6000 × *g* the supernatant was discarded again and the cell pellet was frozen at −20 °C until further analysis.

In order to study DNA repair the cells were incubated with different cisplatin concentrations (100–300 μM) for 2 h. The cisplatin concentration was chosen based on previous results in order to obtain a comparable extent of DNA platination. Subsequently, the cells were incubated up to 4 h with drug-

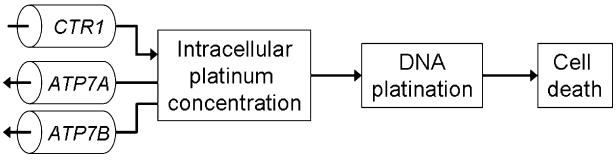

Fig. 1 – Relationship between gene expression of selected transporters known to be involved in cisplatin uptake and efflux, intracellular platinum concentration, DNA platination and cell death.

free medium. The further procedure was the same as described before.

DNA platination was measured by using a previously published method [18] adapted to tumor cells: DNA concentration was measured by UV photometry after isolation with solid-phase extraction (QIAmp©, Qiagen, Hilden, Germany) and platinum concentration was measured by flameless atomic absorption spectrometry (FAAS). Based on these two concentrations the platinum–nucleotide ratio [Pt atoms:10^4 nucleotides] was calculated as measure for the extent of DNA platination.

2.4. Measurement of intracellular platinum concentration

In order to characterize platinum uptake 5×10^6 cells were incubated with 100 μM cisplatin up to 120 min. After certain time points the medium was discarded quickly and the cells were washed three times with 5 mL ice-cold PBS buffer. Then cells were trypsinized, resuspended in fresh drug-free medium and centrifuged for 4 min at 4 °C and 260 × g. The supernatant was discarded and the pellet was washed twice in 1 mL ice-cold PBS buffer. After centrifugation for 1 min at 6000 × *g* the supernatant was discarded again and the cell pellet was frozen at −20 °C until analysis. Immediately after thawing the cells were lysed with 200 μL concentrated nitric acid for 20 min on the water bath at 60 °C. Then intracellular platinum concentrations were measured by flameless atomic absorption spectrometry (FAAS). The results were related to the total cell volume (measured by Casy™1 cell counter, Schärfe System, Reutlingen, Germany).

In order to characterize the efflux of platinum 5×10^6 cells were incubated with 100 μM cisplatin for 120 min. Subsequently cells were washed three times with 5 mL ice-cold PBS buffer and incubated up to 120 min with drug-free medium. The further procedure was as described before.

2.5. Gene expression analysis

Depending on the cell growth, 10^5 to 10^6 cells (A2780, A2780cis, HeLa, HeLaCK) were plated in six-well plates (Greiner, Frickenhausen, Germany) and incubated with 2 mL of growth medium at 37 °C and 5% CO_2 over night. On the next day, the growth medium was replaced by 2 mL of either fresh medium (control) or medium containing cisplatin (6–20 μM), and cells were incubated at 37 °C and 5% CO_2 for 2 h. Afterwards, cells were washed twice with 1 mL of medium. Then 2 mL of drug-free medium were added, and the cells were again incubated at 37 °C and 5% CO_2. After 24 h, cells were washed with ice-cold phosphate buffered saline (7.7 mM Na_2HPO_4, 2.3 mM NaH_2PO_4, 154 mM NaCl, pH 7.2), and RNA was isolated using TRI reagent (0.5 mL/well, Sigma, Taufkirchen, Germany) according to the manufacturer's instructions. Each lysate was transfered to a reaction tube, centrifuged at 20,000 × *g* at 4 °C for 20 min, and the aqueous phase was transfered to a fresh tube. 25 μL of absolute isopropanol were added, mixed and centrifuged. The supernatant was transfered to a fresh tube. RNA was precipitated by adding 225 μL of absolute isopropanol and subsequent centrifugation (22,000 × *g*, 4 °C, 20 min). The pellet was washed with 75% ethanol, air-dried, and dissolved in 20 μL of DEPC-treated water (diethyl pyrocarbonate, Sigma). The OD ratio (260/280 nm) of all RNA samples was between 1.95 and 2.18. RNA integrity was confirmed by agarose gel electrophoresis (2% in HEPES buffer containing 13 mM EDTA, 50 mM sodium acetate, 183 mM HEPES, pH 7.4, and 0.6% formaldehyde). Two micrograms of RNA were mixed with 6× loading dye (Fermentas, St. Leon-Rot, Germany.) and heated to 65 °C for 5 min. Then 2 μL of SYBR-GREEN II (Sigma, Taufkirchen, Germany) were added and the samples loaded onto the gel. Reverse transcription was performed with 1 μg RNA by using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA) and 35 pM oligo$(dT)_{23}$ anchored primer (Sigma). Sequences of the used primers are listed in Table 1. Reverse transcription was performed at 37 °C for 2 h. Reverse transcription mixtures were diluted with 1× TRIS–EDTA buffer pH 7.4, and quantitative real-time PCR (qRT-PCR) was performed using the QUANTITECT SYBR-GREEN PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Housekeeping genes were identified using GENORM [19]. Analysis of differential gene expression was

Table 1 – Sequences of the primers used in qRT-PCR

Gene	Accession number	Left 5′ → 3′	Right 5′ → 3′
β-Actin (*ACTB*)	AY582799	TCCTTCCTGGGCATGGAGT	GCACTGTGTTGGCGTACAG
Beta-2-microglobulin (*B2M*)	BC064910	CACCCCCACTGAAAAAGATG	CAAACCTCCATGATGCTG
Glucuronidase beta (*GU*)	NM_000181	TTCACCAGGATCCACCTCTG	AGCACTCTCGTCGGTGACTG
Hypoxanthine-phosphoribosyltransferase 1 (*HPRT1*)	NM_000194	CTGGCGTCGTGATTAGTG	CACACAGAGGGCTACAATG
60S (human) acidic ribosomal protein P1 (*HUPO*)	BC070194	AGCTCTGGAGAAACTGCTG	CAGCAGCTGGCACCTTATTG
Phospholipase A2 (*PhosA2*)	M86400	GACAGCTTTTGATGAAGCCATTG	TCCACAATGTCAAGTTGTCTCTCAG
Ribosomal protein L13 (*RPL13*)	BC071929	GCTCATGAGGCTACGGAAAC	TATTGGGCTCAGACCAGGAG
Transferrin receptor (p90, CD71) (*TFR*)	BC001188	GACTTTGGATCGGTTGGTG	CAGTAACCGGATGCTTCAC
Ubiquitin-conjugating enzyme E2D2 (*UBE2D2*)	NM_003339	ACCACCTAAGGTTGCATTTAC	TAGATCCGAGCAATCTCAGG
Human copper transporter 1 (*hCTR1*)	BC061924	AGCTGGAGAAATGGCTGGAG	AGGTGAGGAAAGCTCAGCATC
ATPase, copper transporting, alpha polypeptide (*ATP7A*)	NM_000052	ATGATGAGCTGTGTGGCTTG	TGCCAACCTGAGAAGCAATAG
ATPase, copper transporting, beta polypeptide (*ATP7B*)	NM_001005918	TACCCATTGCAGCAGGTGTC	ACTTGAGCTGCAGGGATGAG

carried out using the ΔΔCt-method [20]. For statistical comparison, Student's *t*-test was performed.

3. Results

3.1. MTT assay

The MTT assay was performed to assess the cytotoxic activity of cisplatin in the cell lines used. The EC_{50} values obtained were 1.72×10^{-6} M and 7.62×10^{-6} M in the sensitive A2780 line and the resistant variant A2780cis, respectively. In HeLa cells, EC_{50} values were 3.57×10^{-6} and 12.8×10^{-6} M in the sensitive cells and in the resistant variant HeLaCK, respectively. Resistance factors were calculated as 4.4 for A2780cis and 3.6 for HeLaCK cells.

3.2. DNA platination

After incubation with increasing cisplatin concentrations there was a proportional increase of DNA platination both in sensitive and resistant cell lines (Fig. 2). However, in the resistant cell lines the amount of DNA platination was significantly lower than in the corresponding sensitive cell line. The resistant cell lines formed on average 5.4-fold (A2780cis) and 1.9-fold (HeLaCK) less platinum-DNA adducts than their respective parental cells.

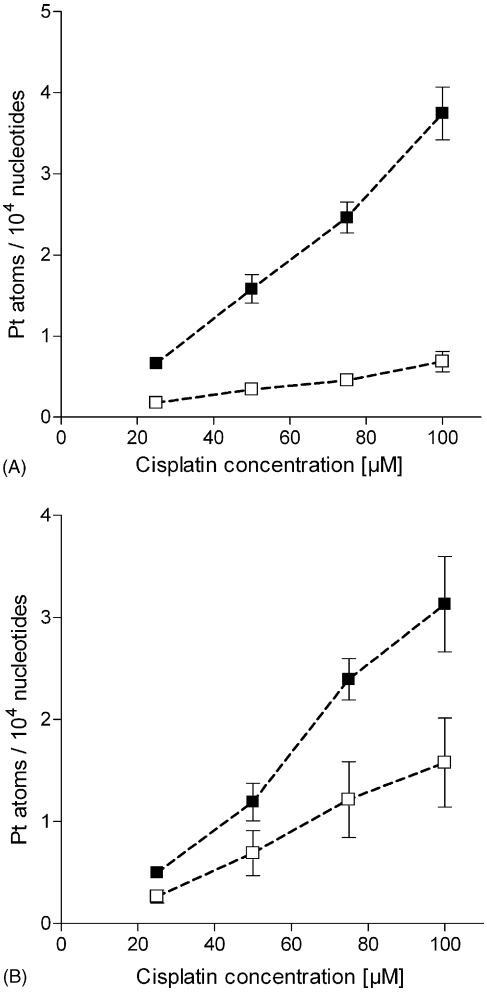

Fig. 2 – DNA platination A in A2780 (■) and A2780 cis (□) and B in HeLa (■) and HeLa CK (□) after incubation with cisplatin for 4 h ($n = 3$, mean ± S.D.).

To investigate whether the differences in DNA platination are due to an increased DNA repair the alteration of DNA platination after incubation with cisplatin and subsequent incubation with drug-free medium was investigated. The extent of DNA platination at the end of the cisplatin incubation was set to 100% and the subsequent decline was monitored (Fig. 3). There was no obvious difference in DNA repair between sensitive and resistant cell lines.

3.3. Intracellular platinum concentrations

After incubation with 100 μM cisplatin the intracellular platinum concentrations increased continuously. However, the resistant variants showed lower intracellular platinum concentrations than their respective parental cells (Fig. 4). On average, the concentrations were 2.5- and 2.9-fold lower in A2780cis and HeLaCK cells, respectively.

In order to study drug efflux, intracellular platinum concentrations were also measured after incubation with cisplatin and subsequent incubation with drug-free medium. The intracellular platinum concentration at the end of cisplatin incubation was set to 100% and the subsequent decline was monitored. Whereas A2780 and A2780cis cells exhibited a biphasic decline, intracellular concentrations did not decline in HeLa and HeLaCK cells (Fig. 5). However, no difference between sensitive and resistant cell lines was observed.

3.4. Gene expression analysis of CTR1, ATP7A, and ATP7B

Among a set of nine possible housekeeping genes (Table 1), the three most stably expressed genes were identified by GENORM [19]: *RPL13*, *GU*, *HPRT1* for A2780/A2780cis; *β-Actin*, *GU*, *HUPO* for HeLa/HeLaCK. Expression of the copper transporters was normalized to the expression levels of the respective housekeeping genes, and presented in Fig. 6 in a rescaled way that untreated sensitive cells (i.e., A2780- and HeLa-) obtained a normalized expression value of 1 (Fig. 6A–F). Cisplatin-induced and resistance-mediated changes between cell pairs (fold change values) were then calculated and are presented in Table 2. Cisplatin concentrations of >20 μM were avoided for expression experiments to prevent a possible interference of cisplatin with RNA and subsequent reverse transcription and PCR.

Sensitive cells (A2780 and HeLa) expressed significantly higher levels of *CTR1* (1.5–1.8-fold, Table 2) than their corresponding resistant counterparts (A2780cis, HeLaCK, Fig. 6A and B). Upon cisplatin treatment, sensitive cells still expressed more *CTR1* than resistant cells but the difference was significant only in HeLa cells. Cisplatin treatment did not change the expression level of *CTR1* neither in A2780 nor A2780cis nor HeLa nor HeLaCK (Fig. 6A and B).

Further, treated and untreated resistant A2780cis cells expressed 2.13- and 2.66-fold more copper exporter *ATP7A* than sensitive A2780 cells (Fig. 6C, Table 2). Whereas cisplatin treatment did not change the expression of *ATP7A* in A2780 or

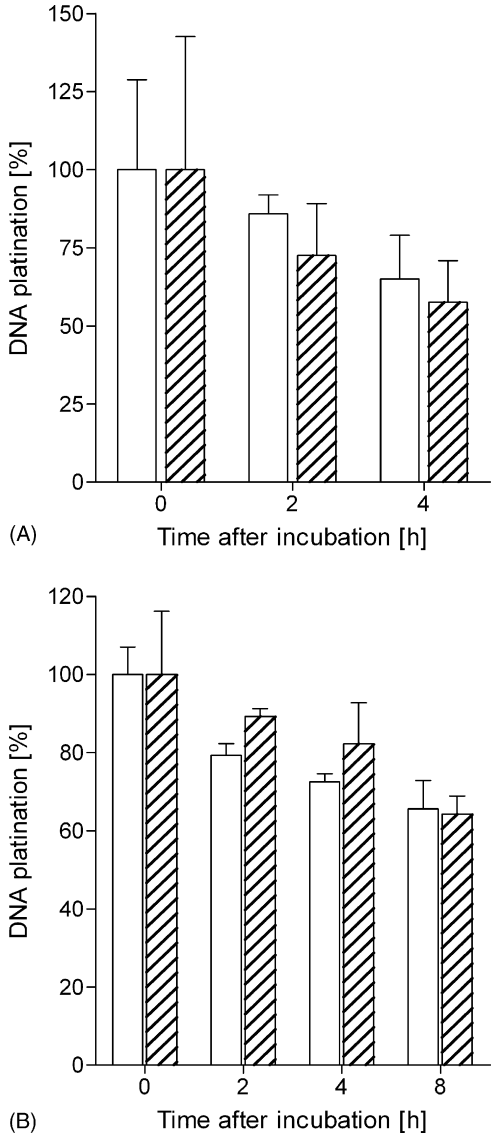

Fig. 3 – DNA platination A in A2780 (open) and A2780 cis (striped) and B in HeLa (open) and HeLa CK (striped) after incubation with cisplatin and subsequent incubation with drug-free medium ($n = 3$, mean ± S.D.).

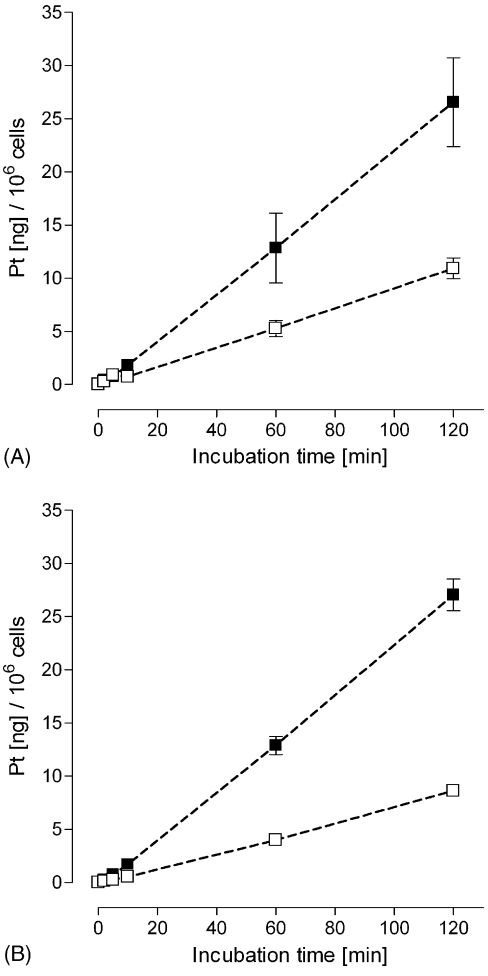

Fig. 4 – Intracellular platinum concentration A in A2780 (■) and A2780 cis (□) and B in HeLa (■) and HeLa CK (□) during incubation with 100 μM cisplatin ($n = 3$, mean ± S.D.).

A2780cis, it did reduce the expression of *ATP7B* in sensitive A2780 (~2-fold, but not significant) and resistant A2780cis (~4-fold, significant) compared to untreated cells (Fig. 6E, Table 2). *ATP7B* expression was, however, fairly similar in sensitive versus resistant A2780 cells (treated or untreated). In HeLa cells, *ATP7A* expression remained fairly unchanged except an ~2-fold lower expression in resistant versus sensitive cells (both untreated, Fig. 6D). Treatment with cisplatin showed no significant effect in sensitive cells but caused a significant increase in *ATP7A* expression in resistant HeLaCK (Fig. 6D). Resistant HeLaCK cells showed a 2.4-fold (untreated) and a 3.9-fold (treated) lower expression of *ATP7B* than their sensitive counterpart HeLa (Fig. 6F, Table 2). Treatment with cisplatin had either no effect (HeLaCK) or caused an increase (1.6-fold in sensitive HeLa) in *ATP7B* expression (Fig. 6F, Table 2).

4. Discussion

This project was conducted to assess the role of drug uptake and efflux in platinum sensitivity using an integrated and quantitative approach. In two cell line pairs from different origin (ovarian and cervical cancer) differences in gene expression of selected transport proteins, intracellular cisplatin accumulation, DNA platination and cytotoxic activity were determined and compared.

Intracellular concentration–time profiles are the result of uptake and efflux processes that take place simultaneously. In both resistant cell lines lower intracellular platinum concentrations were found compared to the sensitive parental cells. On the other hand, efflux studies did not reveal any obvious differences between sensitive and resistant cells. Therefore, we conclude that alterations in platinum uptake primarily account for the differences in intracellular platinum concentrations between cisplatin-sensitive and resistant cell lines. A decreased uptake and lower intracellular platinum concentrations in cisplatin-resistant cells were reported by other investigators as well [21–23]. Whereas most authors found a

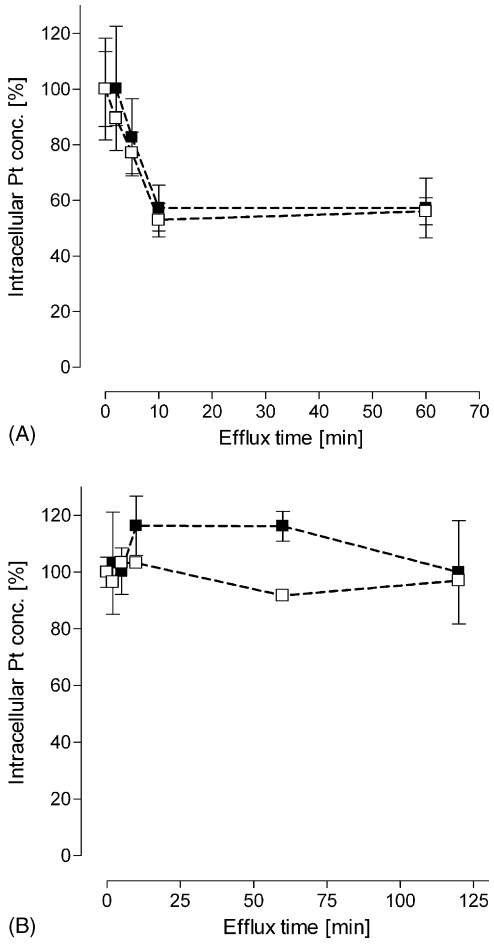

Fig. 5 – Intracellular platinum concentration A in A2780 (■) and A2780 cis (□) and B in HeLa (■) and HeLa CK (□) after incubation with cisplatin and subsequent incubation with drug-free medium ($n = 3$, mean ± S.D.).

decreased uptake without changes in efflux [24,25] also the opposite situation (no difference in uptake but increased efflux) was reported [26].

A reduction in uptake has consequently great influence on the degree of DNA platination. Therefore it is not surprising that we also found reduced formation of platinum-DNA adducts. in the resistant cells. As the cytotoxic activity of platinum complexes is based upon DNA platination, the increase of EC_{50} values in resistant cells is the consequence of this alteration. Other authors (except one [27]) reported reduced DNA platination in cisplatin-resistant cells as well [21,22,26,28,29].

In order to interpret the quantitative relationships between uptake, DNA platination and cell death it is important to know whether DNA repair is different between sensitive and resistant cells. In our study we did not determine different DNA repair rates. The results of other studies concerning DNA repair are contradictory: Some authors reported a faster DNA adduct repair rate in resistant cell lines [22,26,30], others did not observe differences between sensitive and resistant cells [31]. Further investigations concerning DNA adduct repair are necessary to explain these conflicting data.

As there were no apparent alterations in efflux and DNA repair between sensitive and resistant cells in our experiments, it might be justified to directly compare the quantitative differences in uptake, DNA platination and EC_{50} values to assess the contribution of uptake to platinum sensitivity in the two cell line pairs. In the A2780/A2780cis cells the reduction of DNA platination was in good agreement with the resistance factor, i.e., the increase in EC_{50} values (reduced DNA platination: 4.4-fold, resistance factor: 5.4) but the reduction in uptake was only 2.5-fold. In the HeLa/HeLaCK cells, the resistance factor was 3.6, but the DNA platination was reduced only 1.9-fold and the reduction in uptake was 2.9-fold. These results suggest that reduced uptake is an important resistance mechanism in both cell line pairs, but that further mechanisms of resistance are likely to exist as well. As an example, higher glutathione levels were found in A2780cis cells compared to the respective parental cell line (data not shown).

In order to understand the mechanisms leading to altered intracellular platinum concentrations it is crucial to reveal the role of transport proteins. Therefore we analysed the expression of three genes encoding copper transport proteins that have recently been shown to be involved in uptake and efflux of platinum complexes [10]. As there is strong evidence that CTR1 can transport cisplatin into the cell a decreased expression of the *CTR1* gene may cause decreased intracellular platinum accumulation. In our both resistant cell lines the expression of *CTR1* was reduced by almost a factor of 2 compared to the respective sensitive cell line. The reduced expression was observed in both untreated and treated cells. This finding is consistent with the differences observed in cisplatin uptake and is also in accordance with results reported by Song et al. [32]. They showed that transfection of the *hCTR1* gene into human cells increased uptake of cisplatin and sensitized the cells to this drug. Holzer et al. [13] reported that the enhanced expression of *hCTR1* in transfected A2780 cells was associated with increased cisplatin accumulation without increase in DNA platination and only marginal increase in cisplatin sensitivity. In contrast, Beretta et al. [33] showed that enhanced expression of *hCTR1* by transfection was not accompanied by changes in cisplatin uptake and sensitivity. The main difference between those and our results is that we did not use transfected cells which may display a generally altered gene expression profile but cisplatin-sensitive and -resistant cell pairs. Since we obtained similar results in both cell line pairs we may conclude that the expression level of *CTR1* plays a major role in platinum sensitivity in A2780 and HeLa cells by influencing intracellular platinum concentrations and DNA platination substantially.

However, with respect to efflux our results suggest the importance of the cell type. No difference in efflux rate was observed between sensitive and resistant cells (Fig. 5). However, the concentration–time profiles were different in the two cell pairs. The experiments in A2780/A2780cis cells showed a strong decrease in intracellular platinum concentration in the first minutes after removal of cisplatin with a subsequent plateau phase. This observation is consistent with the results of Chao, Loh et al. and Parker et al. [25,26,30] and might reflect a fast initial back diffusion of platinum species with low molecular weight and a long-term intracellular retention of

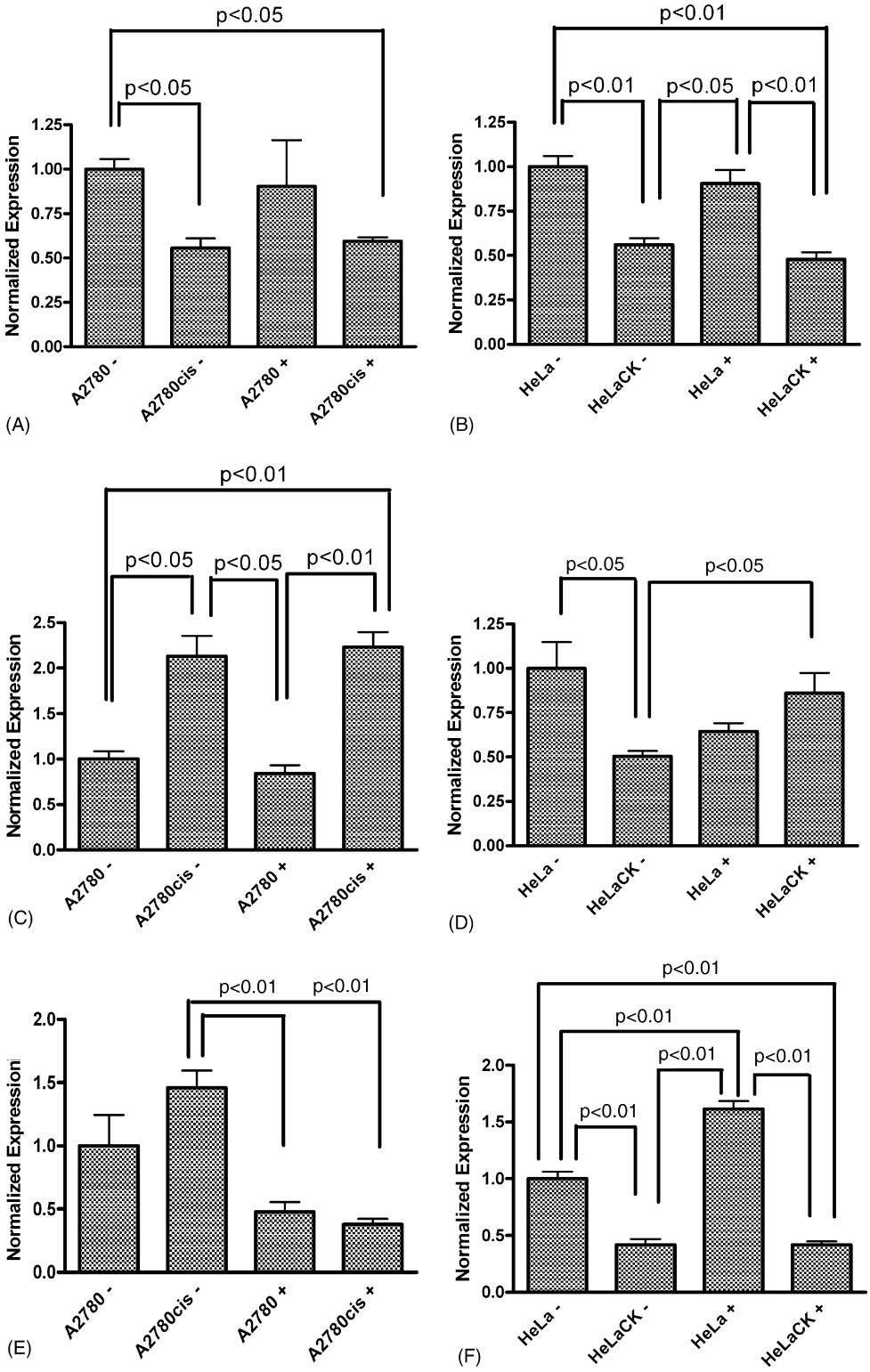

Fig. 6 – Normalized expression of *CTR1* (A and B), *ATP7A* (C and D), and *ATP7B* (E and F) in cisplatin (6–20 μM) treated (+) and untreated (−) A2780 (A, C, E) and HeLa (B, D, F) cell lines ($n = 3$, mean ± S.D.).

macromolecular platinum species. In HeLa/HeLaCK cells, no substantial drug efflux could be observed at all.

Our results show that the genes encoding ATP7A and ATP7B respond differently on cisplatin treatment, and the expression profile differs depending on the cell type (ovarian A2780 or cervical HeLa cells). Although both transporters have been shown to transport platinum drugs [34], their expression and role in cisplatin sensitivity might be different. Samimi et al. [35] reported an increased expression of *ATP7A* in resistant ovarian carcinoma cells, but no reduced cisplatin

Table 2 – Cisplatin-induced (treated 6–20 μM vs. untreated) and resistance-mediated (resistant vs. sensitive) changes in the expression of *CTR1*, *ATP7A*, and *ATP7B* in A2780 and HeLa cells

	CTR1	ATP7A	ATP7B
A2780 treated/untreated	0.90 ± 0.46	0.84 ± 0.22	0.48 ± 0.44
A2780cis treated/untreated	1.07 ± 0.10	1.05 ± 0.48	0.26 ± 0.25
Untreated A2780cis/A2780	0.56 ± 0.14	2.13 ± 0.42	1.46 ± 0.48
Treated A2780cis/A2780	0.66 ± 0.45	2.66 ± 0.33	0.79 ± 0.16
HeLa treated/untreated	0.91 ± 0.10	0.64 ± 0.26	1.61 ± 0.11
HeLaCK treated/untreated	0.85 ± 0.09	1.70 ± 0.20	1.00 ± 0.10
Untreated HeLaCK/HeLa	0.56 ± 0.12	0.51 ± 0.26	0.42 ± 0.14
Treated HeLaCK/HeLa	0.53 ± 0.07	1.34 ± 0.20	0.26 ± 0.05

accumulation. Our results in A2780 cells are in accordance with their observation: the expression of *ATP7A* was significantly higher in resistant A2780cis cells compared to sensitive A2780 cells, with a higher cisplatin accumulation in sensitive cells which may however be explained by the higher *CTR1* expression in sensitive cells since the efflux rate remained unchanged (Fig. 5). In HeLaCK cells, no overexpression of *ATP7A* was observed. In a recent review Safaei speculated that the function of ATP7A is rather vesicular sequestration of platinum drugs than efflux [36]. This would mean that platinum remained in the cell, however without access to the DNA.

In some previous studies higher expression of *ATP7B* was found to be related to cisplatin resistance [12,37,38]. In contrast, our results do not suggest this relationship for A2780 and HeLa cells under the used experimental conditions. In both investigated cell pairs the *ATP7B* expression under cisplatin treatment was comparable or even lower in the resistant cell lines. The reason for this discrepancy is unclear. One explanation may be that the different experimental conditions (such as presence or absence of cisplatin and the cell line used) influenced the expression pattern of genes encoding the efflux transporters. The differences in the results obtained by other groups using different clonal cell lines highlight the advantage and the importance to investigate gene expression, intracellular cisplatin concentrations, DNA platination and cytotoxic activity in the same cells under comparable conditions.

Our results provide the basis for a quantitative understanding of alterations in uptake and efflux processes leading to cisplatin resistance integrating important steps from gene expression changes to cell death (Fig. 1). Further studies should focus on the kinetics of gene expression after different incubation times and concentrations. Furthermore the exact localization and trafficking of cisplatin within the cell should be investigated to understand better the intracellular processing of cisplatin [39].

Changes in the expression of genes encoding efflux transporters have also been shown to be related to clinical observations [40–42]. In order to obtain a better insight into the situation in patients, it would be desirable to investigate gene expression, uptake and efflux in tumor cells isolated from individual patients (*ex vivo*) and to correlate the results with clinical outcome of platinum-based chemotherapy. Provided that these investigations lead to parameters with high predictive power an individual sensitivity profile could be generated for each patient. Such a sensitivity profile could be an important additional marker for detection of clinically relevant cisplatin resistance and to individualize cancer chemotherapy accordingly.

In conclusion, resistance to cisplatin is based on multiple molecular mechanisms, and is dependent on the cell type. In this study we examined the role of drug uptake and efflux in cisplatin sensitivity and resistance of ovarian and cervical cancer cells. The results highlight the relevance of copper import transporter *CTR1* for platinum sensitivity in the investigated cell lines as there is a good correlation between lower *CTR1* expression, lower intracellular concentration, lower DNA platination and lower platinum sensitivity in both types of resistant cell lines. In contrast, alterations in the gene expression level of efflux transporters strongly depend on the cell line. Therefore, the manipulation of drug uptake might be a more promising approach to overcome platinum resistance than to influence drug efflux. The results may contribute to the development of new strategies to overcome platinum resistance and individualize platinum-based chemotherapy.

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