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Overcoming cisplatin resistance of ovarian cancer cells by targeted liposomes in vitro

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ABSTRACT

The clinical application of cisplatin to treat solid tumours is often limited by the development of tumour cell resistance against this cytostatic agent. Although liposomal carriers of cisplatin are currently in clinical development, approaches to functionally overcome cisplatin resistance by liposomes have hardly been reported. We prepared PEGylated cisplatin-containing liposomes with diameters of about 110 nm and targetability to transferrin receptors (TfR) to correlate cisplatin cell uptake with cytotoxicity in sensitive and cisplatin resistant ovarian cancer cells A2780 compared to the free drug. Whereas the cell entry of free cisplatin was reduced by factor 4 after 24 h in resistant cells, liposomal uptake was similar in both cell lines and not affected by resistance. Cytotoxicity was clearly related to intracellular platinum levels, which were even higher for liposomal vs. free cisplatin in the resistant cells after 24, 48, and 72 h and slightly lower in the sensitive cells. However, TfR targeting was of less impact on activity in comparison to non-targeted liposomes. Detection of cellular ATP levels within 24 h allowed postulations on the intracellular fate of the liposomes. Altogether, this study strongly supports approaches to overcome cisplatin resistance by a liposomal application of the drug.

1. Introduction

Cisplatin (cis-diamminedichloroplatinum II/cDDP) is one of the most widely used cytotoxic agents in the treatment of malignancies of lung, head and neck, and ovarian cancers. Activated via hydrolysis after cell entry, a positively charged and highly reactive platinum complex is formed causing DNA adducts, which subsequently lead to cell death (Siddik, 2003). The success of platinum-based chemotherapy is limited by the appearance of severe side effects and the pre-existence or development of cancer cell resistance against this cytostatic agent.

Concerning the side effects, the high binding capacity of cisplatin to proteins and enzymes is believed to be the main reason for these damages, especially ototoxicity and nephrotoxicity (Andrews and Howell, 1990). Since these effects intrinsically accompany the cytotoxicity in the tumour tissue, attempts to avoid the side effects by chemical modifications of the agent interfere with cytostatic activities.

One strategy to effort effective intracellular doses of cisplatin in the tumour tissues while reducing systemic toxicities is the use of liposome-based drug formulations. PEGylated small unilamellar liposomes tend to passively accumulate in the tumour tissue according to the EPR effect (Gabizon et al., 2006). Two different liposome formulations of cisplatin, SPI 077 and lipoplatin, have reached clinical trials. While the former failed to exhibit significant antitumour activities in patients in clinical phase I and II trials (Harrington et al., 2001; White et al., 2006), promising data on lipoplatin activity and reduction of side effects in clinical phase III trials were reported (Stathopoulos et al., 2005; Jehn et al., 2008).

The intracellular drug release from the liposomes appears as critical parameter. Therefore, further efforts were put on creating trigger mechanisms to induce a controlled intracellular drug release, such as thermosensitivity (Woo et al., 2008) or pH-sensitivity of cisplatin liposomes (Carvalho Júnior et al., 2007) or destruction by ultrasound (Schroeder et al., 2009).

Nevertheless, the existence or development of tumour cell resistance against cisplatin is the dominant limitation in the clinical application of cisplatin. Several mechanisms have been postulated

Abbreviations: Alb, albumin; BSA, bovine serum albumin; cDDP, cisplatin/cis-diamminedichloroplatinum II; Chol, cholesterol; EPR, enhanced permeability and retention effect; FAAS, flameless atomic absorption spectrometry; Holo, holotransferrin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid; mPEG-PE, polyethyleneglycol-phosphatidylethanolamine; R_f, resistance factor; Rhod-PE, rhodamin-phosphatidylethanolamine; SPC, soy phosphatidylcholine; TfR, transferrin receptor.

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to contribute to the resistance phenomenon (Siddik, 2006). Recent data refer to the interconnection of the insulin-like receptor signalling pathway and cisplatin resistance in various ovarian cancer cell lines (Eckstein et al., 2009).

However, a reduced intracellular accumulation of cisplatin seems to be a common reason and consequence of resistance (Shen et al., 2000). The mechanisms of cisplatin entry into the cells and the intracellular trafficking are complex. Besides a passive transport of cisplatin through the cell membranes, a protein-mediated uptake and processing involving copper transporters seems to dominantly contribute to cisplatin cellular activity (Ishida et al., 2002; Holzer et al., 2004). Therefore, changes in the endocytic recycling compartment (Liang et al., 2006) or the localisation of the copper transporters ATP 7A/B (Kalayda et al., 2008) was demonstrated to be accompanied with the cisplatin resistance.

Depending on the kind of target cells and several other factors, liposomal drug carriers can be taken up by cells on active pathways, mostly endocytosis. A recent study using cisplatin nanocapsules or liposomes investigated the impact of the uptake pathway on the cytotoxicity in different tumour cell lines (Hamelers et al., 2009). A caveolin-dependent endocytosis of carriers was found to be most effective.

Although liposomal cell entry appears as an attractive strategy to overcome the limitations of cisplatin resistance, only two studies yet reported about the use of cisplatin liposomes interacting with cisplatin resistant tumour cells. Carvalho Júnior et al. (2007) applied non-targeted cisplatin liposomes to a sensitive and resistant lung carcinoma cell line and found comparable cytotoxicity of liposomes in both cells in contrast to the free drug. Similar findings were reported using bile acid cisplatin complexes in a liposomal form (Briz et al., 2000).

However, several questions remain to be answered, such as the impact of targeting an internalising receptor by cisplatin liposomes in sensitive vs. resistant cells on overcoming the resistance, or following the cytotoxicity of those liposomes in a time range, which is relevant for a liposomal therapeutic approach.

Therefore, we applied transferrin targeted vs. non-targeted PEGylated cisplatin liposomes to a cisplatin sensitive and resistant line of ovarian cancer cells A2780 and followed the uptake kinetics within the first 24 h and correlated the data with cytotoxicity, obtained from both, MTT and ATP assays. The data confirm that liposomes have the potential to overcome the chemoresistance of tumour cells, which might have important consequences for supporting the arguments of a liposomal drug cancer therapy.

2. Materials and methods

2.1. Materials

Lipids were purchased from the following sources: soy phosphatidylcholine (SPC) was a gift from Lipoid AG (Ludwigshafen, Germany); rhodamin-phosphatidylethanolamine (Rhod-PE) and polyethyleneglycol-phosphatidylethanolamine (mPEG-PE) were obtained from Avanti Polar Lipids (Alabastar, AL, USA). Cyanur-PEG-PE, the lipid anchor for protein coupling was synthesized in our group as described (Bendas et al., 1999).

Cyanuric chloride, cisplatin, bovine serum albumin (BSA), holotransferrin (Holo), cholesterol (Chol) and sephadex G-50 were purchased from Sigma–Aldrich Chemie (Steinheim, Germany). The FITC-conjugated mouse anti-human transferrin mAb (L11339-CD71) and the ATP Determination Kit were obtained from Invitrogen (Karlsruhe, Germany). The BCA-kit was purchased from Thermo Fisher Scientific (Schwerte, Germany). All other chemicals were obtained from Applichem (Darmstadt, Germany) unless otherwise stated.

2.2. Liposome preparation

Liposomes were prepared using the film hydration method. The lipid composition was SPC/Chol/mPEG-PE in 65/30/5 mole ratio.

For protein coupled liposomes, 2 mol% of mPEG-PE were replaced by Cyanur-PEG-PE. Liposomes for flow cytometry contained 0.5 mol% of Rhod-PE. All lipids were dissolved in chloroform. A thin lipid film of the lipids was formed by removing the solvents in a rotary evaporator at 40 °C followed by drying under vacuum for at least 60 min. The lipid film was hydrated either with aqueous 0.9% NaCl solution or NaCl solution containing cisplatin (8 mg/mL, at 65 °C) to reach a final lipid concentration of 60 mM. Unilamellar liposomes were prepared by extrusion (Lipex extruder; Vancouver) three times through a 200 nm and seven times through a 100 nm polycarbonate membrane. Cisplatin-containing liposomes were homogenised by sonicating (Bandelin Sono Plus, Berlin) seven times, each with 30 seconds cycles and with a relative power of 60%.

To couple albumin (Alb) or holotransferrin (Holo) to the surface of liposomes, the Cyanur-PEG-PE-anchor was used to connect the terminal end of the PEG chain to certain amino acids of the protein (Bendas et al., 1999). Briefly, the calculated amount of protein (protein:lipid 1:1000 mol ratio) was added to liposomes in borate buffer of pH 8.8 and the mixture was incubated at room temperature for about 16 h in a shaker under exclusion of light. Unbound protein or non-entrapped cisplatin were removed by gel permeation chromatography using sephadex G-50. Platinum content of the liposomes was measured by flameless atomic absorption spectrometry (FAAS) (SpectrAA^{TM} Zeeman 220; Varian; Darmstadt, Germany) as described recently (Kloft et al., 1999; Garmann et al., 2008). Cisplatin-containing liposomes were used for the experiments within a time range of 48 h after preparation.

The particle size of the liposomes was characterised by dynamic light scattering (Malvern Autosizer IIc, Malvern, UK). The phospholipid concentration was determined with a standard phosphate assay (Ames and Dubin, 1960), and the concentration of coupled proteins was obtained by a modified Lowry assay (Peterson, 1977).

2.3. Cell culture

The human ovarian carcinoma cell lines A2780 and A2780cis were obtained from ECACC, UK, No. 93112519 [A2780] and No. 93112517 [A2780 cis]. The cell lines were cultured in RPMI 1640 medium (PAN^{TM}-biotech, Passau, Germany) supplemented with 10% fetal calf serum (Sigma–Aldrich Chemie), 5% (50 μg/mL streptomycin and 50 U/mL penicillin G, Sigma–Aldrich Chemie) and 1.5% (365 μg/mL L-glutamine, Sigma–Aldrich Chemie) at 37 °C in a 5% CO_2 incubator.

2.4. Fluorescence activated cell sorting (FACS)

Liposomal binding to the A2780 and A2780cis cells was determined by flow cytometry. Briefly, cells were rinsed once with PBS and detached with trypsin and resuspended in KHP. At a final concentration of 1×10^5 cells/100 μL, cells were dispensed in a 96-well-plate with V-bottom.

Three different, Rhod-PE labeled liposome preparations were investigated: plain mPEG-PE liposomes, albumin-coupled (Alb) and holotransferrin-coupled (Holo)-liposomes. The liposomes (100 nmol lipid/1×10^5 cells) were incubated with the cells for 90 min at 4 °C in the dark. Unbound liposomes were removed by washing two times with PBS and cells were resuspended in 100 μL PBS. Subsequently, the liposomal binding was analysed using a flow cytometer (FACS Calibur, BD Bioscience, San Jose, CA, USA).

To analyse the expression levels of TfR on A2780 cells, cells were rinsed one time with PBS and detached with trypsin; rinsed

twice with washing-buffer (PBS containing 0.5% BSA, iced). Cells were counted and resuspended in ice washing-buffer (density: 1×10^5 cells in 20 μL per well). In the next step, 5 μL FITC-conjugated mouse anti-human transferrin antibody (dilution 1:5 in PBS) were added. After incubation for 40 min on ice in the dark, samples were centrifugated and washed two times with 100 μL buffer, each washing step followed by centifugation. Afterwards, cells were resuspended in 100 μL buffer and analysed.

2.5. Quantification of intracellular platinum accumulation

For characterisation of cellular cisplatin uptake, 1×10^6 cells were seeded out in 6-well plates overnight. Afterwards, the medium was changed and cisplatin or liposomal cisplatin was added in a final concentration of 20 μM per well. After each incubation time point (1–12 h, and 24 h) medium was siphoned off quickly and the cells were washed once with 1 mL ice cold PBS (4 °C). After removing PBS, cells were trypsinised for 2 min and resuspended in fresh cold medium (4 °C) and put into a 2 mL Eppendorf tube and centrifuged at $1500 \times g$ for 1 min. The supernatant was discarded and the cell pellet was resuspended in 1 mL ice cold PBS. In the next step, 20 μL were taken from this mixture, put into a 0.5 mL Eppendorf tube and frozen at −20 °C until further analysis of total protein of the cells with the BCA-kit. The 2 mL Eppendorf tube was centrifuged again at $24{,}100 \times g$ for 1 min and the supernatant was removed. Afterwards the pellet was washed with 1 mL ice cold PBS followed by an additional centrifugation at $24{,}100 \times g$. Subsequently the supernatant was removed again and the pellet was frozen at −20 °C until further analysis.

Toward thawing the cell pellet was lysed with 50 μL 65% HNO_3 (suprapure) for 60 min at 80 °C in a water bath. These samples were diluted with aqua millipore and analysed by FAAS as described before (Kloft et al., 1999; Garmann et al., 2008). The platinum concentrations were related to the total protein concentration of the cells, which were determined by BCA-kit.

2.6. Cytotoxicity assays

The MTT assay was used to assign cytotoxicity, and an ATP assay was used to kinetically follow the cell viability (Müller et al., 2004).

For the MTT assay cells were seeded out in colourless flat bottom 96-well-plates at densities of 1×10^4 cells per well, 2×10^4 cells per well, or 4×10^4 cells per well in 90 μL medium and preincubated overnight. Cells were exposed with different concentrations of cisplatin or liposomal cisplatin for 24, 48 and 72 h. Subsequently, the assays were developed by adding 20 μL MTT reagent (5 mg MTT/1 mL PBS) for approximately 1 h until development of formazan crystals. The supernatant in the wells was removed and the cells were dissolved in 100 μL DMSO. Absorbance of the dye was measured at 570 nm with background subtraction at 690 nm, using a plate reader (Thermo multiskan EX, Thermo, Schwerte, Germany). The obtained sigmoidal dose–response curves were calculated by nonlinear regression using the four-parameter logistic equation with variable Hill slope (GraphPad Prism 5.0 software, San Diego, CA, USA). The resistance factor was obtained by dividing the IC_{50} of a certain probe in the resistant A2780 cells by the appropriate IC_{50} in the sensitive cells.

For the ATP assay, cells were seeded out in white, white flat bottom 96-well-plates (density: 2×10^4 cells per well) in 90 μL medium and preincubated overnight. Cells were spiked with 10 μL cisplatin or liposomal cisplatin for 1–24 h in a final concentration of 20 μM per well. Afterwards, cisplatin-containing medium was removed and ATP levels were measured as described in the ATP kit instruction (plate reader Lumistar™; BMG-Lab Technologies, Offenburg, Germany).

To correlate the cellular ATP levels with total protein concentrations, a parallel cellular setup was identically handled and used for protein detection in colourless flat bottom 96-well-plates. Cisplatin-containing medium was removed and cells were washed once with 100 μL PBS per well. Subsequently PBS was removed again and the plate was frozen at −20 °C until further analysis of total protein using the BCA-kit. Toward thawing the plate, protein assay was performed as described. The plate was developed in a cabinet dryer at 60 °C for 1 h. Absorbance of the converted dye was measured at 570 nm using a plate reader.

2.7. Immunoblotting

10 μg of total protein per lane were subjected to gel-electrophoresis on 12% SDS-PAGE. For immunoblotting, standard procedures were used as described elsewhere (Eckstein et al., 2008). To ensure uniformity of protein loading, blots were stripped with NaOH (2 mM) and reprobed with an antibody specific for β-actin. As primary antibody, mouse anti-human transferrin antibody CD71 was used. Western blots were developed with the enhanced chemoluminescence system (Amersham Biosciences, Buckinghamshire, UK).

2.8. Statistics

Data represent means ± standard deviations (SD), or standard error of the mean (SEM) in case of the MTT assays, of at least three independent experiments. Comparisons were performed by a non-parametric two-tailed Mann–Whitney test.

3. Results

3.1. Characterisation of target cells and liposome cell interactions

The ovarian carcinoma cell line A2780 (sens) compared to the cisplatin resistant line (A2780cis) are established models to investigate several aspects of cisplatin resistance (Garmann et al., 2008; Kalayda et al., 2008). A2780 cells have recently been described to lack a caveolin-dependent pathway of cell uptake (Hamelers et al., 2009), which favours a clathrin-mediated endocytosis of liposomal uptake. Since the transferrin receptor (TfR) is an indicator for this route of uptake, both cell lines were characterised with respect to TfR expression by Western blot. It was evident (Fig. 1A) that both cell lines express the TfR. Although the A2780cis displayed a slightly lower degree of TfR expression, the liposomal coupling of holotransferrin to target the TfR appeared as promising approach to reach both cell lines in a comparable intensity.

PEGylated SUV were prepared and holotransferrin (Holo) was coupled terminal to PEG chains via the cyanuric chloride linker (Bendas et al., 1999). For comparison, uncoupled liposome (mPEG) or BSA coupled liposomes (Alb) were used. Comparison of the liposomes with respect to particle size and protein coupling efficiency displayed similar characteristics of empty and cisplatin-containing liposomes (Table 1). The cisplatin entrapment of the different liposomes was comparable and not affected by the protein coupling procedure. Entrapment stability was detected and followed for a time range of several days (data not shown). To exclude deviations by liposomal cisplatin release, liposomes were used within 48 h after preparation for the experiments.

To evaluate the impact of coupled transferrin on cell receptor binding, the targetability of the three empty liposome preparations to A2780sens and A2780cis cells was determined after a 90 min liposome incubation at 4 °C. As illustrated in Fig. 1B, the Holo-liposomes displayed the highest binding ability in both cell lines.

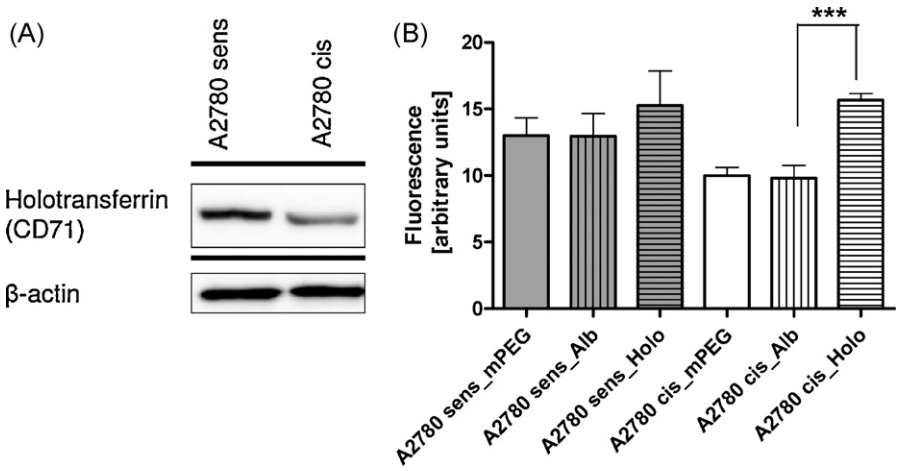

Fig. 1. Expression of TfR by A2780sens and A2780cis cells and targeting the TfR by empty liposomes compared to non-targeted liposomes; (A) immunoblot analysis of TfR expression, using β-actin as housekeeping protein. (B) The binding of the Holo-liposomes displayed a significant difference to the control liposomes in case of A2780cis ($p < 0.0001$), while a relative high degree of unspecific liposome binding was evident. Data represent mean of at least three independent experiments ($n = 3$ in triplicates ± SD).

Table 1
Characterisation of the liposomes used for the targeting approaches. Data are means ± SD ($n > 5$).

	Size [nm] ± SD	Coupling rate [μg protein/μmol phospholipid] ± SD	Entrapment [mmol Pt ± SD/mmol lipid ± SD]
Holo	129.0 ± 4.3	58.5 ± 11.9	–
Alb	129.0 ± 4.3	76.5 ± 8.6	–
mPEG	128.0 ± 3.2	–	–
cDDP Holo	116.0 ± 15.1	80.1 ± 12.6	0.24 ± 0.05/8.33 ± 0.34
cDDP Alb	107.9 ± 5.9	53.6 ± 4.7	0.29 ± 0.04/9.31 ± 0.50
cDDP mPEG	99.6 ± 8.4	–	0.27 ± 0.13/8.89 ± 2.75

Although the binding differences between Holo and control liposomes were significant in the resistant cells, the relatively high binding of control liposome referred to unspecific binding mechanisms. Binding differences between the liposomes in the sensitive cell line were less distinguished. However, the slight differences in expression levels were not reflected in the binding data.

To exclude a down regulation of TfR due to long term saturation by Holo-liposomes, which could compensate their binding benefit, both cell lines were incubated with Holo-liposomes for 72 h. Flow cytometry data (Fig. 2) confirm on one hand the slight differences in TfR expression in untreated cells (Fig. 1A) and on the other hand an upregulation of TfR with Holo incubation, which strongly support the targeting approach.

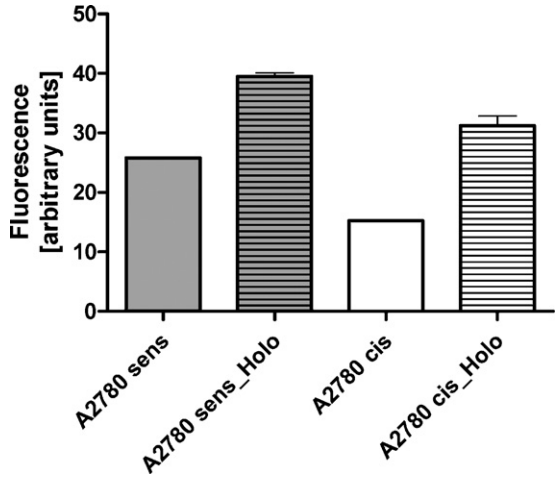

Fig. 2. Influence of holotransferrin incubation (cells incubated with 2 μM holotransferrin-coupled liposomes over 72 h) on the TfR expression on A2780 cells; analysed by flow cytometry of about 10,000 cells, data are means ± SD, $n = 3$.

3.2. Cellular uptake of free or liposomal cisplatin

Cisplatin resistance of cells is most likely associated with reduced intracellular levels of the drug, caused by decreased uptake and/or increased efflux. To verify this for the A2780 cell pair, the uptake kinetics of free cisplatin (20 μM) by A2780sens and A2780cis cells was compared within 24 h. A stricking difference was evident (Fig. 3A) indicating a much stronger uptake of cisplatin by the sensitive cells leading to a fourfold accumulation after 24 h. Considering the uptake of identical concentrations of liposomal cisplatin into the sensitive cells (Fig. 3B), no significant differences within the three liposome preparation became evident. Although all liposomes induced a comparable cisplatin cell uptake up to 8 h in comparison to the free drug, and slightly increased levels at the earlier time points, the increase between 10 and 24 h suggested a higher uptake of free vs. liposomal cisplatin.

In contrast, the liposomes exceeded the cisplatin uptake of the free drug in the resistant cells at all time points strikingly (Fig. 3C). Although a targeting effect of the Holo-liposomes was again not evident, all liposomal preparations displayed nearly identical levels of cell uptake in both, the sensitive and resistant cell lines.

3.3. Cytotoxicity of free and liposomal cisplatin

To evaluate the cytotoxicity of free and liposomal cisplatin, MTT assays were applied. MTT assays were usually evaluated after a cell incubation period of 72 h. To emphasise potential consequences from the different cisplatin uptake kinetics (Fig. 3) for the cytotoxicity at earlier time points, we evaluated the MTT data already after 24, 48 h, and finally 72 h.

Referring to the free drug (Fig. 4) at all three time points the striking differences in the activity of cisplatin in the sensitive vs. the resistant cell line were nearly identical. This supports the informational value of MTT assay already after the shorter incuba-

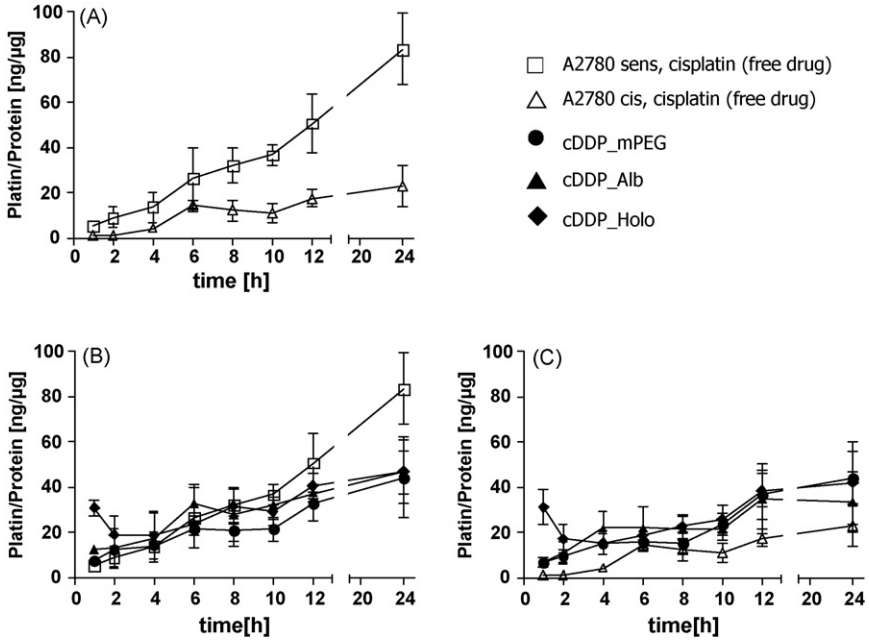

Fig. 3. Kinetics of intracellular platinum accumulation in A2780 cells. (A) Uptake of free cisplatin in A2780sens in comparison to A2780cis cells. (B) Cellular uptake of free vs. liposomal cisplatin in A2780sens and (C) in A2780cis cells. Cisplatin concentration was 20 μM in each experiment. Data points are means ± SD of three experiments performed in triplicates.

tion periods. Interestingly, the differences in activity (IC_{50} values) clearly reflect the uptake differences of cisplatin shown in Fig. 3A.

For evaluating the cytotoxicity of liposomal cisplatin, potential lipid effects to the cells were evaluated first by incubating the cells with empty liposomes. The data in Fig. 5A indicate that up to a Holo liposome concentration of 10^{-3} M no toxic effect is evident after 24 h, which is also true for the other liposome preparation and an evaluation after 48 h (data not shown). After 72 h (Fig. 5B) a decrease in cell viability was detectable when liposome concentration exceeded 10^{-4} M. However, taking a liposomal cisplatin concentration of 20 μM, corresponding to a liposome concentration of about 600 μM, an intrinsic cytotoxicity of the liposomes themself can be excluded at the two earlier time points in the following experiments, and is even at the sensitive concentration after 72 h.

On that basis, the cytotoxicity of all three types of cisplatin liposomes was detected in A2780 cis and A2780sens cells for the three time points. As exemplarily demonstrated in Fig. 5C for the 72 h range, the liposomes displayed a significantly higher cytotoxicity compared to the free drug in the resistant cells, while the activity of liposomal cisplatin is slightly lower than the free drug in sensitive cells.

A complete comparison of all cytotoxicity data derived from the MTT assays of the free and the three liposomal cisplatin preparations is given in Table 2. In general, the data are in agreement with further experiments using different cell numbers (10,000 and 40,000; data not shown, except for 24 h).

At all three time points, the free drug displayed a strong difference in activity in the sensitive and the resistant cell line. The resistance factors of about 4 clearly reflects the findings of the different uptake kinetics, as shown in Fig. 3A. In contrast, the liposomal effects were independent on the cell resistance. Liposomes displayed comparable cytotoxicities in both cell lines, which correspond to higher activities in the resistant and slightly lower activity in the sensitive cells compared to the free drug. Within the different liposome preparation, no significant differences became evident, although the TfR-targeted cisplatin-containing Holo-liposomes displayed the highest efficiency. Some of the 24 h cytotoxicity data of liposomes could not exactly be registered and were not implicated in the calculations. This should be related to limits in the practicability of MTT assays after short incubation period.

Although the MTT data give a comprehensive insight into the time dependent cytotoxic activity of cisplatin and support the benefit of liposomes in resistant cells, the sensitive time range of cisplatin cell uptake within the first 24 h cannot adequately be reflected. Therefore, an ATP assay was applied as alternative viability test, which allows to follow the cellular ATP level as indicator

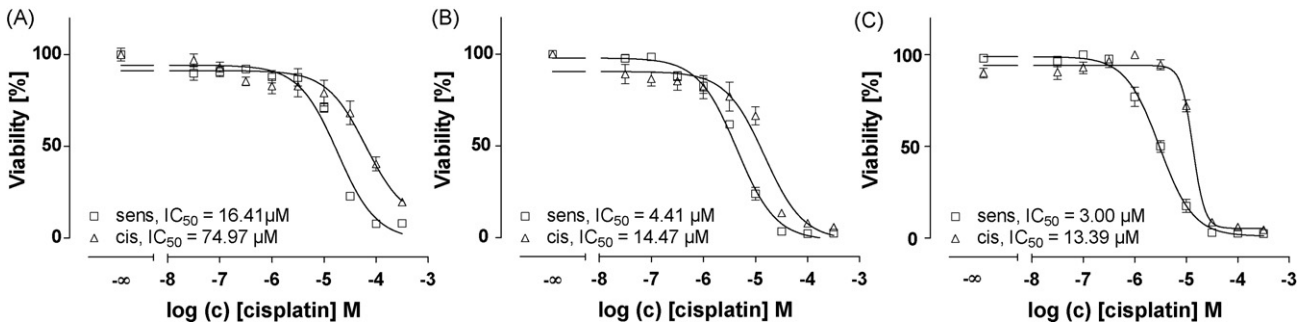

Fig. 4. Comparison of the cytotoxicity of free cisplatin in resistant and sensitive A2780 cells (20,000 cells/well) after different periods of incubation; 24 h (A), 48 h (B), 72 h (C). Data points are means ± SEM of three experiments performed in triplicates.

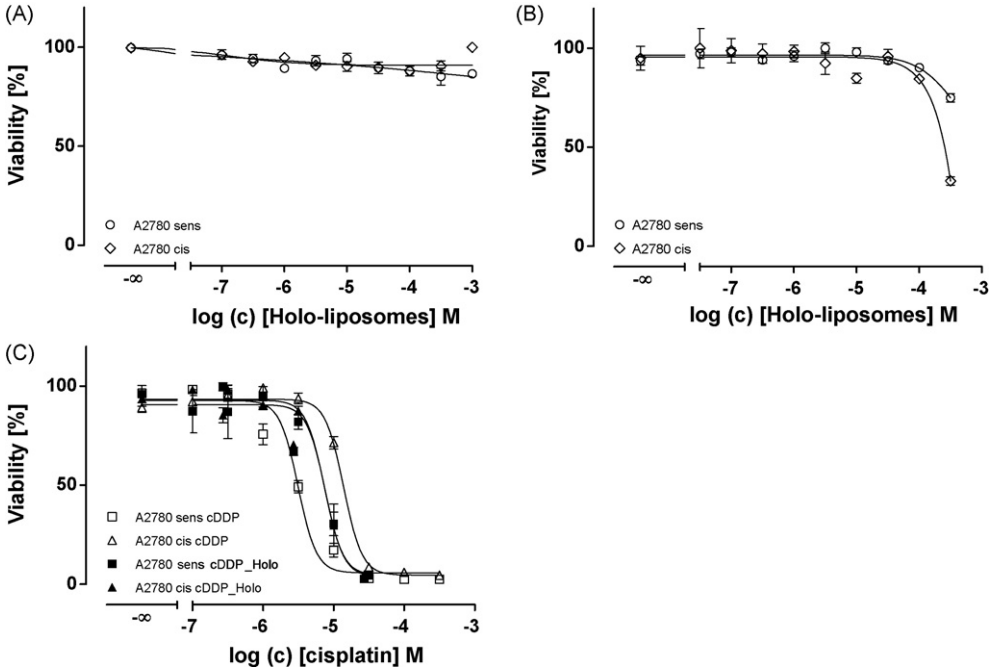

Fig. 5. Cell viability data from MTT assays (20,000 cells/well) to evaluate potential cytotoxic effects of liposomes. (A) After 24 h, no toxic effect of empty Holo-liposomes was evident. (B) After 72 h, a certain decrease in cell viability after incubating the cells with liposome concentrations exceeding the millimolar range was evident. (C) Comparison of the cytotoxicity of free (open symbols) vs. liposomal cisplatin (filled symbols) after 72 h in sensitive and cisplatin resistant A2780 cells. The liposomal formulations displayed a significantly higher toxicity in resistant cells. Data are means ± SEM of three experiments performed in triplicates.

for cell activity within the time range of 24 h. Considering the cellular ATP levels in both untreated cell lines (Fig. 6A), the higher levels in resistant A2780 cells indicate a higher cellular activity.

The percentage of ATP in resistant and sensitive cells treated with 20 μM free cisplatin (Fig. 6B) illustrates that the ATP level in the resistant cells was hardly affected, while ATP increased in the sensitive cells within the first 10 h, likely as a kind of defence reaction against the drug, to finally drop after 12 h. The differences between both cell lines after 24 h clearly reflect the findings in Fig. 4A. Following the ATP levels after liposome application to the sensitive cells (Fig. 6C), the increase in ATP within the initial time period was not evident. Furthermore, the higher ATP data at 24 h for all liposomal preparations compared to the free drug correspond to the differences in cytotoxicity at 24 h. In contrast,

Table 2
Cytotoxicity data of free and liposomal cisplatin in sensitive vs. cisplatin resistant A2780 cells obtained by MTT assays. In some cases of the 24 h data, the curve fitting was ambiguous (A) and therefore was not used for further calculations. Data represent means of three independent experiments.

		A2780 sens IC_{50} [μM]	A2780 cis IC_{50} [μM]	R_F
24 h	Free drug cDDP			
	20,000 cells/well	16.41	74.97	4.57
	40,000 cells/well	18.57	64.38	3.47
	cDDP Holo			
	20,000 cells/well	A: 10.71	A: 11.02	
	40,000 cells/well	A: 222.82	16.11	–
	cDDP Alb			
	20,000 cells/well	A: 10.08	26.23	
	40,000 cells/well	66.2	3.93	0.06
	cDDP mPEG			
	20,000 cells/well	12.06	24.40	2.02
	40,000 cells/well	A: 31,97	A: 16,11	–
48 h	Free drug cDDP			
	20,000 cells/well	4.41	14.47	3.28
	cDDP Holo			
	20,000 cells/well	7.89	8.28	1.05
	cDDP Alb			
	20,000 cells/well	8.00	13.06	1.63
	cDDP mPEG			
	20,000 cells/well	4.50	8.32	1.85
72 h	Free drug cDDP			
	20,000 cells/well	3.00	13.39	4.46
	cDDP Holo			
	20,000 cells/well	6.63	7.04	1.06
	cDDP Alb			
	20,000 cells/well	6.29	8.50	1.35
	cDDP mPEG			
	20,000 cells/well	6.51	8.01	1.23

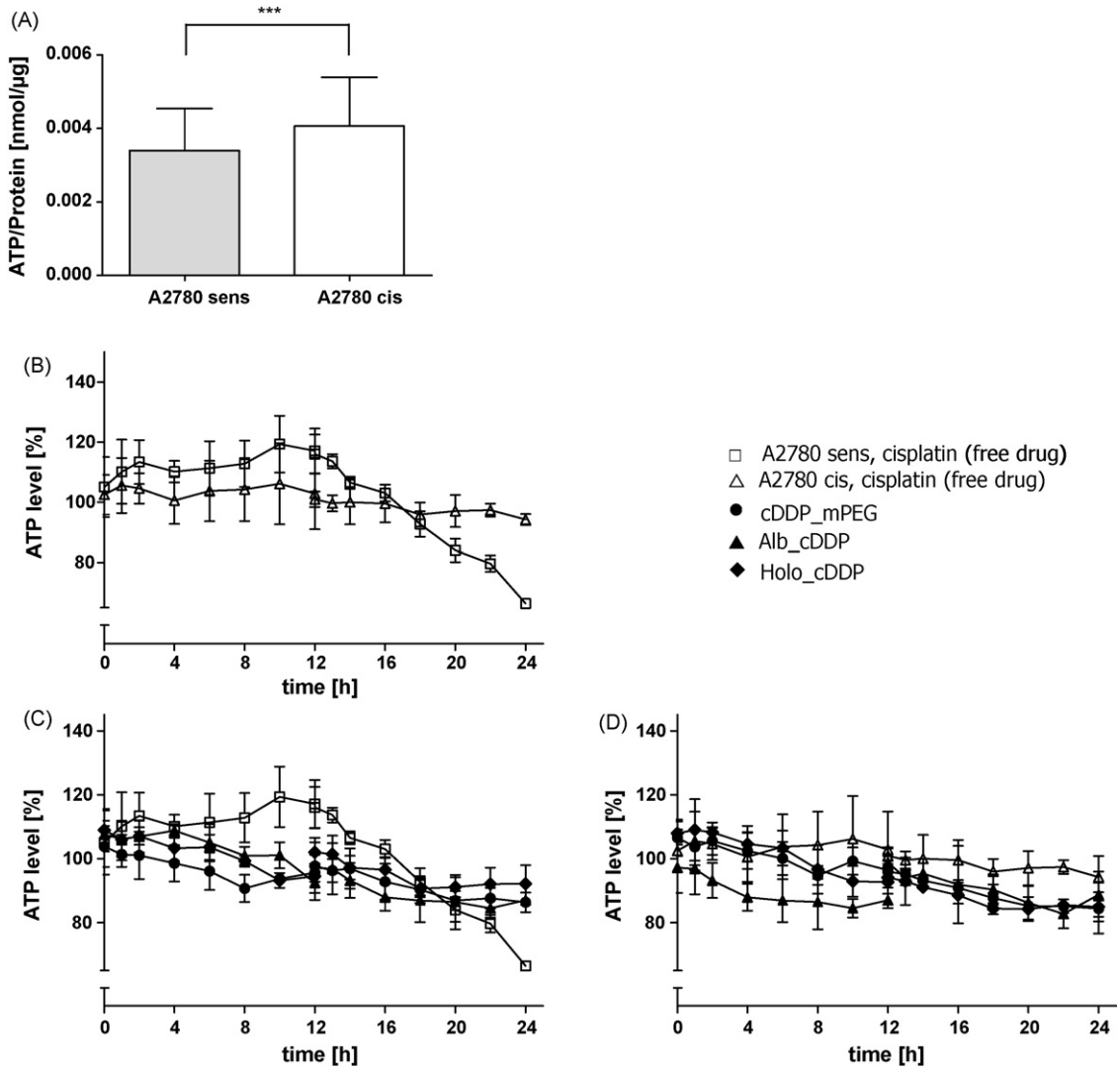

Fig. 6. (A) ATP levels (nmol ATP/µg protein) in untreated A2780sens and A2780cis cells, detected with an ATP assay (20,000 cells/well), $n = 26 \pm$ SD; $p < 0.0001$. (B–D) Tumour cell vitality detected according to the ATP levels after incubating the cells with free and liposomal cisplatin (percentage in relation to untreated cells), (B) comparison of free cisplatin activity in A2780sens and A2780cis cells. (C) Comparison of the cellular ATP levels in A2780sens after incubation with free or liposomal cisplatin; (D) in A2780cis cells. Cisplatin concentration was 20 µM in each experiment. Data points are means ± SD of two experiments performed in triplicates.

liposomes induced lower ATP levels compared to free cisplatin into the resistant cells (Fig. 6D), which reflect the findings of cytotoxicity (Table 2).

4. Discussion

The liposomal targeting of solid tumours is a promising therapeutic strategy to increase the drug index of the targeted cytostatic agents, while reducing unwanted side effects. Due to the EPR effect, long circulating liposomes tend to accumulate in the tumour tissue, which is accompanied by a drug release in the tissue or an uptake of the liposomes by the tumour cells. However, chemoresistance of tumour cells is the major obstacle in clinical treatment of tumours, which can be induced by manifold molecular mechanisms. Most prominently, multidrug resistance induced by the P-glycoprotein (P-gp) efflux pump is relevant for the resistance against most cytostatic agents (Bates et al., 2006). Cisplatin resistance of cells is independent of P-gp. Other mechanisms have been described, which finally lead to reduced intracellular cisplatin concentrations (Siddik, 2006). Thus, cisplatin resistance is a matter of unsufficient intracellular drug levels. Therefore, in the present in vitro study we aimed to overcome cisplatin resistance of tumour cells by an endocytotic uptake of cisplatin-containing liposomes. The ovarian cancer cell line A2780 was regarded as tumour model. Although liposomal targeting strategies lead to a local, and consequently intracellular accumulation of drugs, a surprising low number of studies focussed on the use of liposomes to investigate or functionally overcome resistance phenomena.

Although our findings refer to a model cell line under in vitro conditions, we provide evidence that a liposomal application is a promising strategy to overcome cisplatin resistance of tumours. This assumption is further supported by the fact that not all parameters in our study appeared ideal, such as the use of the TfR as internalising receptor for liposomes. TfR has been used in numerous liposomal tumour targeting studies, (Suzuki et al., 2008; Li et al., 2009, as recent examples). Nevertheless, the marginal increased cellular binding of Holo coupled liposomes vs. uncoupled ones did not reflect in higher intracellular platinum levels. On one hand, this could be explained by the rapid endocytic recycling of TfR and thus, TfR bound liposomes might also be discharged from the cell. On the other hand, Garmann et al. recently reported about strong endocytotic uptake of PEG- or albumin-coupled platinum containing macromolecules by A2780 cells (Garmann et al., 2008),

which could explain the similar uptake of Holo, Alb, and mPEG liposomes.

Since intracellular platinum levels correlated with cytotoxicity, all three types of liposomes displayed a much higher efficiency in the resistant cells compared to the free drug. Considering the resistance factors in Table 2, liposome activity is equilibrated in both cell lines approximating factors around 1. Holo-liposomes tended to lower factors indicating the highest efficiency in the resistant cells, which cannot directly be explained from the uptake data (Fig. 3C).

Referring to the relevant time range of liposomal cell uptake (Fig. 2B and C) and the consequences for cytotoxicity, it is critically to discuss whether the MTT data really reflect all differences between free and liposomal drugs sensitively. Thus, following the ATP kinetics in Fig. 6 seems to be a further parameter to focus on the liposomal effects and to obtain a certain insight into the resistance mechanisms. The sensitive cells increase the ATP activity up to 12 h in contact with the free drug, which refers to a defence reaction. Since the liposomal cisplatin did not induce this increase of ATP, this might be interpreted with a restricted drug release from the liposome or the endocytic compartments within this time range. Therefore, further efforts should be directed to liposomal modifications that support a stronger cisplatin release within the cells.

The higher level of ATP in resistant cells is a clear indicator for increased cell activity and thus part of an intrinsic cellular resistance. Interestingly, we could find a cross-resistance of the A2780cis against doxorubicin (pIC_{50} of 6.10 vs. 6.65 in sensitive cells), although we could exclude the expression of P-gp in the A2780cis cells as the typical reason for doxorubicin resistance (flow cytometry data, not shown). Therefore, both a specific reduction in cisplatin uptake, such as by down regulation of copper transporter Ctr-1, and an increase in metabolic activity as general cellular resistance appear responsible for cisplatin resistance of tumour cells. However, both factors can partly be circumvented by the application of cisplatin in a liposomal form.

5. Conclusions

The liposomal application of cisplatin is a promising approach to overcome the therapeutic limitations by chemoresistance of tumour cells. TfR-targeted, but also non-targeted PEGylated cisplatin liposomes caused increased drug levels in cisplatin resistant A2780 ovarian carcinoma cells and thus provided higher cytotoxicities than the free drug in a therapeutically relevant time range. The similar activity of liposomes after endocytotic uptake by both, the sensitive and resistant cells refer to limitations in the cellular uptake of the free drug as dominant resistance factor. Insights into the intracellular trafficking of the liposomes referred to a limited drug release from the endosomes. Therefore, further studies are needed to accelerate the intracellular availability of the drug and to optimise the targeting approaches.

Altogether, these data give reasons to continue this strategy in further, therapeutically relevant studies.

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