



Cellular accumulation and cytotoxicity of macromolecular platinum complexes in cisplatin-resistant tumor cells

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ABSTRACT

The development of resistance is one of the major limitations for the use of platinum (Pt) complexes in cancer chemotherapy. As reduced cellular uptake is a well-known resistance mechanism of cisplatin we explored the potential to overcome resistance in cisplatin-resistant A2780 ovarian carcinoma cells by means of macromolecular prodrugs exploiting endocytosis as alternative uptake mechanism.

Two Pt–albumin (PLO4–HSA, PLO7–HSA) complexes and one Pt–polyethylene glycol complex (PEG_{10k}–(Mal–Pt–DACH)₂) were investigated. Intracellular platinum accumulation was quantified by FAAS. Cytotoxic activity was measured using the MTT assay. Endocytosis mechanisms were investigated by co-incubation experiments with bafilomycin A₁ and methyl-β-cyclodextrin, inhibitors of the clathrin-mediated and caveolae-mediated endocytosis, respectively.

Whereas the intracellular accumulation of the low molecular precursors PLO4 and PLO7 was reduced in the resistant cell variant, no difference between sensitive and resistant cells was observed for the three macromolecular complexes. In the presence of bafilomycin A₁ intracellular accumulation of all investigated macromolecular complexes was decreased whereas methyl-β-cyclodextrin only affected the Pt–PEG complex. The Pt–PEG complex exhibited a higher cytotoxic activity than the albumin conjugates but also showed cross-resistance with cisplatin. In conclusion, cellular accumulation of macromolecular platinum complexes is not altered in cisplatin-resistant A2780 cells as these complexes enter the cells mainly via endocytotic pathways. Macromolecular platinum complexes specially designed to circumvent reduced cellular accumulation may be a promising approach to overcome cisplatin resistance.

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1. Introduction

Platinum complexes are successfully used in clinical practice for the treatment of several solid tumors [1,2]. However, therapeutic outcome of platinum-based chemotherapy can be impaired by intrinsic or acquired resistance. Several mechanisms of resistance have been identified so far including reduced drug accumulation which has been frequently observed in platinum-resistant cell lines [3–5].

A reduction in drug accumulation can result either from reduced uptake, enhanced efflux or both. Three copper transporters (CTR1, ATP7A and ATP7B) have been shown to be involved in the uptake and

efflux of platinum complexes. The human copper uptake transporter CTR1 and the copper efflux P-type ATPases ATP7A and ATP7B belong to the superfamily of transmembrane domain spanning transport proteins responsible for copper homeostasis in mammalian cells. Down-regulation of CTR1 and up-regulation of ATP7A and ATP7B has been associated with acquired platinum resistance [6–11].

Recently, we have characterized the cisplatin-sensitive and -resistant ovarian A2780 carcinoma cell lines regarding cellular accumulation and efflux of cisplatin as well as expression of the proteins mediating drug uptake and efflux. Cellular accumulation and DNA platination were considerably reduced in the resistant variant compared to the respective sensitive cell line, whereas no difference in efflux was observed. Resistant cells expressed lower levels of CTR1 (1.5–1.8-fold) than their sensitive counterparts. The results highlight the relevance of the influx of platinum complexes for sensitivity as there is a clear relationship between lower CTR1 expression, cellular accumulation, and cytotoxicity of cisplatin in this cell line [12].

Macromolecular prodrugs may have two major advantages in anticancer therapy. First, most solid tumors possess unique characteristics that are not observed in normal tissues such as hypervascularity, defective vascular architecture and impaired lymphatic drainage. Consequently, macromolecular compounds primarily accumulate in

Abbreviations: BSA, bovine serum albumin; BOP, (benzotriazole-1-yl)tris(dimethylamino)phosphonium hexafluorophosphate; CME, clathrin-mediated endocytosis; DMSO, dimethylsulfoxide; DACH, trans-1,2-diaminocyclohexane; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; FITC-BSA, fluorescein-isothiocyanate labeled bovine serum albumin; HOBt, hydroxybenzotriazole; HSA, human serum albumin; LDL, low-density lipoprotein; β-MCD, methyl-β-cyclodextrin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PEG, polyethylene glycol; RME, receptor-mediated endocytosis; *t*-BuOH, *tert*-butanol; TFA, trifluoroacetic acid.

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tumor tissue and can hence serve as drug carriers to the tumor. This effect is well known as “enhanced permeability and retention (EPR)” [13–16]. Second, tumor cells often exhibit a high endocytotic rate due to an increased requirement of proteins as nutrients [17–19]. Considering these two effects, macromolecular complexes are expected to be taken up to a higher extent by tumor cells. We hypothesized that endocytosis may serve as an alternative uptake pathway in resistant cells with deficient uptake mechanisms as macromolecules neither enter the cell by simple passive diffusion nor are internalized by copper transport proteins.

The aim of this work was to explore the potential of macromolecular platinum complexes to overcome platinum resistance by entering the cells via endocytic pathways and thereby to provide a better understanding of their mechanism of action. Two albumin-bound platinum complexes (PL04–HSA and PL07–HSA) and a polyethylene glycol-based platinum complex (Fig. 1) were investigated as examples of macromolecular platinum complexes.

2. Materials and methods

2.1. Chemicals

Chemicals and solvents were purchased from Sigma-Aldrich, Fluka and Merck and were used without further purification. Bafilomycin A₁ was dissolved in dimethylsulfoxide at the final concentration of 32 mM and stored at –20 °C.

2.2. Synthesis of macromolecular platinum complexes

The synthesis of the albumin binding platinum complexes PL04 and PL07 was described previously [20]. PL04 and PL07 (100 μM) were incubated each with human serum albumin (600 μM) at 37 °C for 30 min (incubation buffer: 4 mM sodium phosphate, pH 7.4) to obtain the respective Pt–albumin complexes PL04–HSA and PL07–HSA [20].

The PEG–Pt complex PEG_{10k}-(Mal-Pt-DACH)₂ was synthesized in five steps:

1. PEG_{10k} dicarboxylic acid *tert*-butylester

A solution of 50.0 g (5.00 mmol) PEG-diol (10 kDa) in 700 mL of toluene was azeotroped with the removal of 50 mL of distillate. The

reaction mixture was then cooled to 25 °C, followed by the addition of 16.5 mL (16.5 mmol) of a 1.0 M solution of potassium *tert*-butoxide in *t*-BuOH. The mixture was stirred for 1 h at room temperature, followed by the addition of 2.96 mL (20.0 mmol) of *tert*-butyl bromoacetate. The resulting mixture was heated to reflux, followed by removal of the heat and stirring for 18 h at room temperature. Subsequently, the turbid mixture was filtered through celite, and the solvent was removed in vacuo. The residue was recrystallized from DCM/diethyl ether affording 48.3 g (95%) of PEG_{10k} dicarboxylic acid *tert*-butylester.

2. PEG_{10k} dicarboxylic acid

To a solution of 48.3 g (4.74 mmol) PEG_{10k} dicarboxylic acid *tert*-butylester in 350 mL of DCM was added 175 mL of TFA containing 0.3 mL of water. The mixture was stirred at room temperature for 3 h and subsequently poured into 3 L of vigorously stirred diethyl ether. The solids were collected by filtration and the crude product was recrystallized twice from 2-propanol and diethyl ether affording 28.53 g (60%) of pure PEG_{10k} dicarboxylic acid. The degree of COOH-functionalization was 1.87 as determined by titration.

3. PEG_{10k}-[Mal(*tert*-Bu)₂]₂

To a solution of 2.00 g (0.20 mmol) PEG_{10k} dicarboxylic acid in 10 mL of anhydrous DMF was added 164 mg (0.60 mmol) di-*tert*-butyl 2-(3-aminopropyl)malonate, 81 mg (0.60 mmol) HOBt, 102 μL (2.00 mmol) DIEA, and 265 mg (0.60 mmol) BOP. The mixture was stirred for 16 h at room temperature. Subsequently, the product was precipitated by pouring the reaction mixture into 400 mL of vigorously stirred diethyl ether. The solids were collected by filtration affording 2.12 g (100%) of PEG_{10k}-[Mal(*tert*-Bu)₂]₂ that was used in the next step without further purification.

4. PEG_{10k}-(Mal)₂

To a solution of 2.00 g (0.19 mmol) PEG_{10k}-[Mal(*tert*-Bu)₂]₂ in 5 mL of DCM was added 5 mL of TFA. The mixture was stirred at room temperature for 1 h and subsequently poured into 100 mL of vigorously stirred diethyl ether. The solids were collected by filtration and the crude product was recrystallized twice from 2-propanol affording 0.90 g (43%) of pure PEG_{10k}-(Mal)₂. ¹H NMR (400 MHz, CDCl₃): δ 1.61 (bs, 4H, NHCH₂CH₂), 1.91 (bs, 4H, CH₂CH(COOR)₂), 3.56 (bs, ~900H, PEG backbone), 7.11 (bs, 2H, NH). ¹³C NMR (100 MHz,

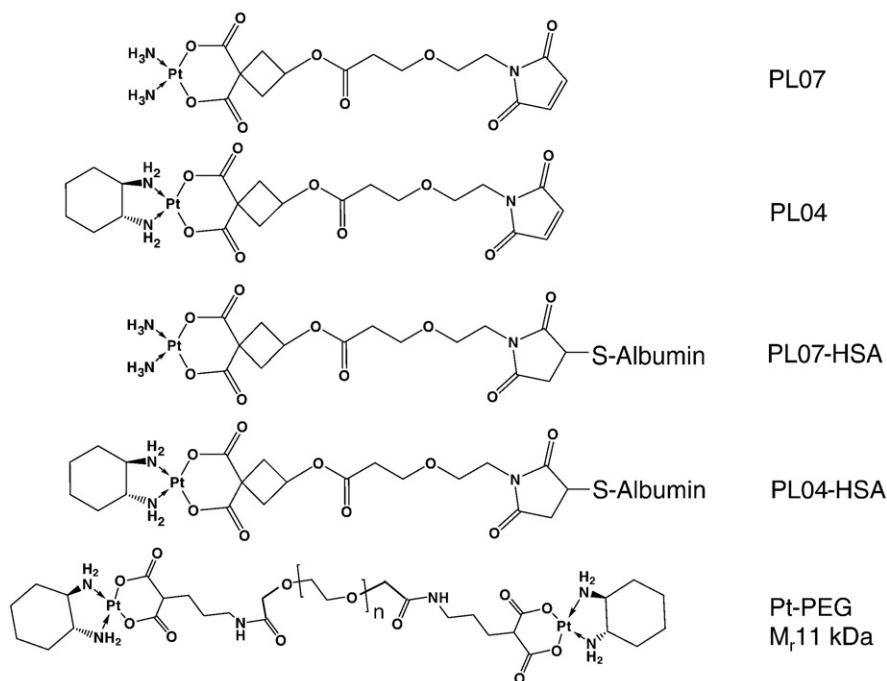


Fig. 1. Structure of PL07 (diammineplatinum(II)-[3-(6-maleimido-4-oxacaproyl)cyclobutane-1,1-dicarboxylate], PL04 (trans-(R,R,S,S)-cyclohexane-1,2-diamino-platinum(II)-[3-(6-maleimido-4-oxacaproyl)cyclobutane-1,1-dicarboxylate], their HSA conjugates and the PEG-conjugated platinum complex.

CDCl₃): δ 26.2, 27.1 (CH₂CH₂CH(COOH)₂), 38.3 (NHCH₂), 50.7 (CH(COOH)₂), 70.3 (b, PEG backbone), 170.1, 170.7 (CONH, (COOH)₂). The ¹H NMR and ¹³C NMR spectra are given as Supplementary material S1. The degree of COOH-functionalization was 3.46 as determined by titration.

5. PEG_{10k}-(Mal-Pt-DACH)₂

48 mg [Pt-DACH](NO₃)₂ (0.11 mmol) was dissolved at 50 °C in 5 mL water. The solution was cooled down to room temperature, and 488 mg PEG_{10k}-(Mal)₂ (44.4 μ mol) dissolved in 5 mL of water was added. The pH was adjusted to pH 5.5 using 0.1 M KOH, and the reaction mixture was stirred in the dark for 1 h. Subsequently, the mixture was extracted with 3 \times 30 mL of DCM. The combined organic layers were dried over MgSO₄ and concentrated in vacuo to a final volume of 20 mL. Subsequently, the product was precipitated by pouring the residue into 200 mL of vigorously stirred diethyl ether to yield 402 mg (82%) of PEG_{10k}-(Mal-Pt-DACH)₂. ¹H NMR (400 MHz, CD₃OD): δ 1.03–1.28 (m, 8H, cyclohexyl-H), 1.43–1.61 (m, 8H, cyclohexyl-H, NHCH₂CH₂), 1.86–1.99 (m, 4H, cyclohexyl-H), 2.09–2.29 (m, 8H, cyclohexyl-H, CH₂CH(COOR)₂), 3.54 (bs, ~900H, PEG backbone), 3.91 (s, 4H, CH₂CONH). ¹³C NMR (100 MHz, CD₃OD): δ 25.6 (cyclohexyl), 28.6, 28.8 (CH₂CH₂CH(COOR)₂), 33.3 (cyclohexyl), 39.6 (NHCH₂), 50.9 (CH(COOR)₂), 64.08/64.14 (cyclohexyl-NH₂), 71.6 (b, PEG backbone), 172.7 (CONH), 180.2 (CH(COOR)₂). The ¹H NMR and ¹³C NMR spectra are given as Supplementary material S1. Elemental analysis for C₄₈₂H₉₅₆N₆O₂₃₈Pt₂: calculated C, 52.46; H, 8.73; N, 0.76; found C, 51.91; H, 8.82; N, 0.76. The content of platinum was determined by AAS to be 3.35% (m/m).

Di-*tert*-butyl 2-(3-aminopropyl)malonate and [Pt-DACH](NO₃)₂ were prepared according to the literature [21,22].

2.3. Cell lines

The human ovarian carcinoma cell line A2780 and its cisplatin-resistant variant A2780cis were studied. The A2780cis cells were obtained by incubation with stepwise increasing cisplatin concentrations [23]. Backups of all cells were frozen with 10% DMSO. A2780/A2780cis cells were grown in RPMI-1640™ medium (Sigma-Aldrich Chemie, Steinheim, Germany) after adding 10% fetal calf serum, 0.6 mL-glutamine, 100 I.E./mL penicillin and 0.1 mg/mL streptomycin. The cells were cultivated as monolayers in a humidified atmosphere at 37 °C and 5% CO₂. Every 10 passages, a new backup of cells was thawed to ascertain that resistance mechanisms were unchanged during long time cultivation.

2.4. MTT assay

The MTT assay was used to assess cytotoxicity [24]. Briefly, cells were plated in 96-well microtiter plates (approximately 10,000 cell/well) and preincubated with growth medium overnight. Cells were exposed to different concentrations of the platinum complexes and incubated for 72 h. Following incubation, cells were treated with MTT solution [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 pEC₅₀ values (pEC₅₀ = -log EC₅₀, EC₅₀ is the drug concentration associated with 50% of the maximum possible response) were estimated using non-linear regression (sigmoidal dose response, variable slope; GraphPad Prism™ analysis software package, GraphPad Software, San Diego, USA). The resistance factor was calculated by dividing the EC₅₀ in the resistant variant by the EC₅₀ in the respective sensitive cell line.

2.5. Measurement of intracellular FITC-BSA concentration

In order to characterize FITC-BSA uptake, 2 \cdot 10⁶ cells were incubated with 20 μ g/mL FITC-BSA up to 120 min in 6-well plates. After pre-determined time points the medium was discarded quickly and the cells were washed twice with 1 mL ice-cold PBS buffer. Then cells were trypsinized, resuspended in fresh drug-free medium and centrifuged for 1 min at 4 °C and 1000 g. The supernatant was discarded and the pellet was washed twice with 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. Immediately after thawing the cells were lysed with 600 μ L 0.1% triton X-100 dissolved in PBS buffer for 30 min. Then the fluorescence was measured at the excitation wavelength of 485 nm and the emission wavelength of 538 nm (Fluoroscan Ascent™ plate reader, Thermo Electron, Dreieich, Germany). The method was validated according to the FDA guideline "Guidance for Industry: Bioanalytical Method Validation" from May 2001. Validation data are provided as Supplementary material S2.

For quality control, 1 mL of medium containing 0.03, 0.06 or 0.3 μ g/mL FITC-BSA, respectively, was filled in empty wells. These samples underwent the same procedure (e.g. centrifugation, freezing) as the cells, except for washing steps. The results were accepted if the deviations of the concentrations of the quality control samples from the respective nominal concentrations were lower than 15%.

2.6. Measurement of intracellular platinum concentration

In order to characterize platinum uptake, the same incubation conditions as described for FITC-BSA were used. Instead of FITC-BSA, cells were incubated with 100 μ M of the respective platinum complex. Immediately after thawing the cells were lysed with 150 μ L concentrated nitric acid at 60 °C for 20 min.

Then intracellular platinum concentrations were measured by flameless atomic absorption spectrometry (FAAS) using a modification of the procedure described by Kloft et al. [25]. In brief, an atomic absorption spectrometer (SpectrAA™ Zeeman 220; Varian, Darmstadt, Germany) equipped with a graphite tube atomisator (GTA 100), a programmable sample dispenser (PSD 100) and a platinum hollow cathode lamp (UltrAA™ lamp) were used. The temperature program comprised a pretreatment temperature of 1300 °C and an atomization temperature of 2700 °C. Platinum concentrations were related to the cell number (measured by Casy™ 1 cell counter, Schärfe System, Reutlingen, Germany).

2.7. Inhibition of endocytosis

A2780 and A2780cis cells were preincubated with PBS in the absence or presence of potential inhibitors of endocytosis. Cells were preincubated with bafilomycin A₁ (100 nM) for 60 min in PBS buffer containing 0.1% dimethylsulfoxide (DMSO) and with methyl- β -cyclodextrine (2.5 mM) for 10 min in PBS buffer. The same vehicles were used for the control experiments. Bafilomycin A₁ was used only in the preincubation buffer and was not added to the uptake buffer according to the protocol of Yumoto et al. [26].

2.8. Hydrolytic activation and DNA platination

DNA platination of PL04-HSA, PL07-HSA and Pt-PEG was studied in an equilibrium microdialysis system consisting of two chambers separated by a semipermeable membrane (5 kDa cut-off). This setting was chosen to study the hydrolytic formation of reactive low molecular platinum species and subsequent DNA binding in one experiment. A solution of each macromolecular platinum complex (150 μ M) in incubation buffer (12 mM sodium phosphate, 100 mM sodium chloride, pH 7.4) was added to the first chamber. The second

chamber was filled with 200 µg/mL calf thymus DNA solution in incubation buffer. The system was kept at 37 °C for 120 h. At pre-determined time points the DNA was isolated by solid-phase extraction (QIAamp™, Qiagen) and quantified by UV-spectrometry (260 nm). The amount of platinum was measured by flameless atomic absorption and related to the DNA nucleotides.

The hydrolytic stability of the Pt-PEG complex without binding to DNA was studied in the same system. A solution of the Pt-PEG complex (150 µM) in incubation buffer was added to the first chamber. The second chamber was filled with incubation buffer only. The system was kept at 37 °C for 48 h. After pre-determined time points the platinum concentration in the second chamber was measured and related to the platinum concentration in the first chamber.

2.9. Statistical analysis

Statistical analysis was performed by the nonparametric Mann-Whitney *U*-test. A *p* value < 0.05 was considered statistically significant.

3. Results

3.1. New synthesized platinum complexes

The structures of the new platinum complexes PL04 and PL07, their albumin conjugates PL04-HSA and PL07-HSA and the new platinum PEG-conjugate PEG_{10k}-(Mal-Pt-DACH)₂ are shown in Fig. 1. The new Pt-PEG complex was synthesized in five steps with a good yield. The compound was characterized by ¹H NMR, ¹³C NMR and elemental analysis. The ¹H NMR spectrum of the Pt-PEG complex shows a broad signal of the PEG backbone at 3.5 ppm as well as signals at 1.03–1.28 ppm, 1.43–1.61 ppm, 1.86–1.99 ppm and 2.09–2.29 ppm corresponding to the DACH ligand. These data confirm the presence of both PEG and Pt-DACH moieties in the Pt-PEG complex.

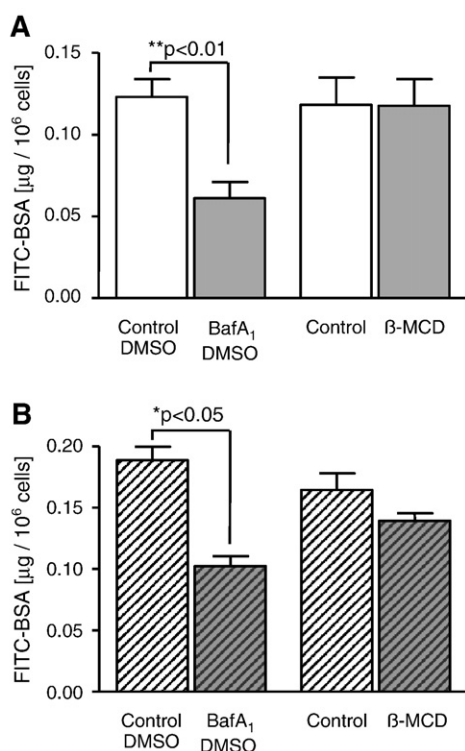


Fig. 2. Influence of bafilomycin A₁ and methyl-β-cyclodextrin on the intracellular concentrations of FITC-BSA after 60 min incubation with 20 µg/mL FITC-BSA in A2780 (A) and A2780cis (B) cells (n=6–10; mean±SE).

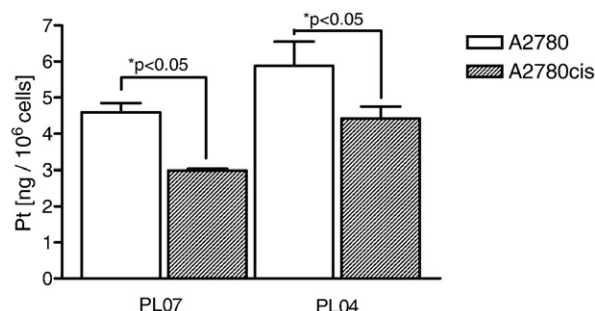


Fig. 3. Platinum concentrations in A2780 and A2780cis cells after 120 min incubation with 100 µM PL04 and PL07 (n=3; mean±SE).

3.2. Intracellular FITC-BSA concentrations

Intracellular FITC-BSA concentrations were measured up to 120 min in order to reveal differences in the endocytosis of albumin between the sensitive (A2780) and resistant (A2780cis) cell line. No difference was observed (data not shown).

To further elucidate albumin uptake by A2780 cells, the effects of two endocytosis inhibitors were examined. Fig. 2A shows the influence of methyl-β-cyclodextrin (β-MCD) and bafilomycin A₁ on the intracellular concentration of FITC-BSA in sensitive A2780 cells. Treatment with bafilomycin A₁ significantly lowered the accumulation of FITC-BSA whereas methyl-β-cyclodextrin had no influence. Comparable results were obtained for the resistant cell variant A2780cis (Fig. 2B).

3.3. Intracellular platinum concentrations

In the next step we investigated the intracellular platinum concentrations after exposure with the unconjugated platinum complexes PL04 and PL07 in order to compare them with their macromolecular counterparts. PL04 concentrations were slightly higher compared to PL07. The resistant variant exhibited significantly lower intracellular platinum concentrations after 120 min of incubation with PL04 and PL07 (Fig. 3). Subsequently, intracellular platinum concentration after incubation with macromolecular platinum complexes was measured. Intracellular concentrations were reduced compared to the low molecular platinum complexes. However, no difference between sensitive and resistant cell lines was observed (Fig. 4).

In order to elucidate the uptake mechanism of macromolecular platinum complexes, the same experiments as described above for FITC-BSA were performed with PL04-HSA, PL07-HSA and PEG_{10k}-(Mal-Pt-DACH)₂. Treatment with bafilomycin A₁ influenced the accumulation of all macromolecular platinum complexes. Fig. 5A and B shows a decrease of intracellular platinum concentrations in A2780 and A2780cis cells after 120 min incubation with bafilomycin A₁. The differences were significant except for PL07-HSA in A2780 cells.

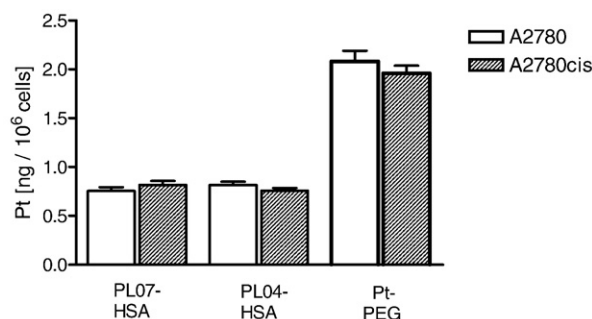


Fig. 4. Platinum concentrations in A2780 and A2780cis cells after 120 min incubation with 100 µM PL04-HSA, PL07-HSA and Pt-PEG (n=6–10; mean±SE).

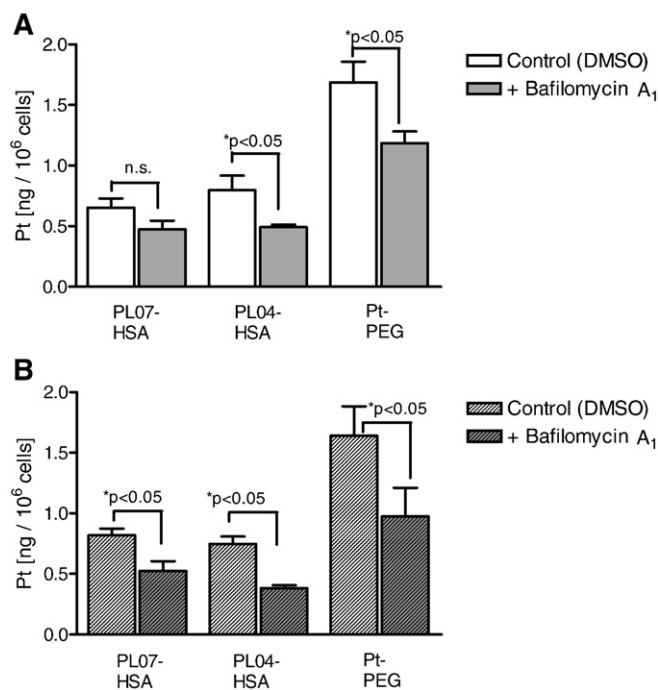


Fig. 5. Influence of bafilomycin A₁ on platinum concentrations in A2780 (A) and A2780cis (B) cells after 120 min incubation with 100 μM PL07-HSA, PL04-HSA and Pt-PEG ($n=3-6$; mean ± SE).

Whereas methyl-β-cyclodextrin treatment did not change the intracellular platinum concentration after incubation with PL07-HSA or PL04-HSA, it significantly lowered the platinum concentrations in A2780 and A2780cis cells after exposure to Pt-PEG (Fig. 6A, B). The reduction of intracellular platinum concentrations was more pronounced in the sensitive cells.

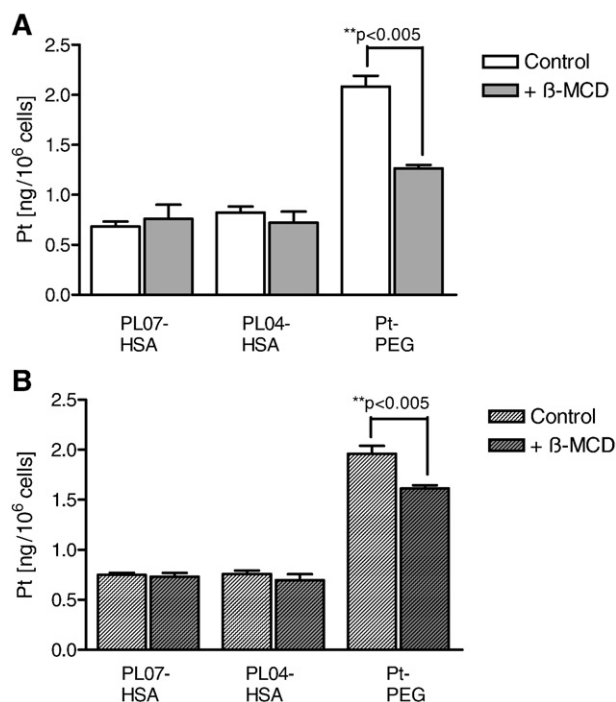


Fig. 6. Influence of methyl-β-cyclodextrin on platinum concentrations in A2780 (A) and A2780cis (B) cells after 120 min incubation with 100 μM PL07-HSA, PL04-HSA and Pt-PEG ($n=3-6$; mean ± SE).

Table 1

Results of the MTT assay ($n=3-6$; mean ± SE)

| | PL04 | PL07 | PL04-HSA | PL07-HSA | Pt-PEG |
|---------------------------------------|-------------|-------------|-------------|-------------|-------------|
| A2780 | 5.19 ± 0.11 | 4.42 ± 0.07 | 3.58 ± 0.10 | 3.54 ± 0.16 | 5.21 ± 0.11 |
| pEC ₅₀ (EC ₅₀) | (6.5 μM) | (38.0 μM) | (263 μM) | (288 μM) | (6.2 μM) |
| A2780cis | 4.68 ± 0.08 | 3.84 ± 0.06 | 3.47 ± 0.12 | 3.53 ± 0.11 | 4.65 ± 0.11 |
| pEC ₅₀ (EC ₅₀) | (20.9 μM) | (144 μM) | (338 μM) | (295 μM) | (22.4 μM) |
| Resistance factor | 3.2 | 3.8 | 1.3 | 1.0 | 3.6 |

In order to assure that these results were not partly affected by released or unconjugated molecular platinum complexes a control experiment with PL04 and PL07 was performed. Neither methyl-β-cyclodextrin nor bafilomycin A₁ affected the accumulation of these compounds in both cell lines (data not shown).

3.4. Cytotoxic activity

The MTT assay was performed to assess the cytotoxic activity of the platinum complexes and to compare the resistance factors for macromolecular platinum complexes (Table 1) and their low molecular counterparts. Although PL04 shows higher cytotoxicity compared to PL07, the resistance factors were quite similar and calculated to be 3.2 for PL04 and 3.8 for PL07. Albumin binding clearly lowered cytotoxic activity in both cell lines. However, both HSA conjugates exhibited comparable activity in the sensitive and resistant cells. In contrast to the HSA conjugates, the Pt-PEG complex showed higher cytotoxic activity but it was susceptible to cisplatin resistance with a resistance factor of 3.6 (Table 1).

3.5. Hydrolytic activation and DNA platination

Hydrolytic activation and reactivity towards the cellular target, the DNA, were studied in an equilibrium microdialysis system consisting of two chambers separated by a semipermeable membrane (5 kDa cut-off). Due to the molar mass of the prodrugs only low molecular platinum complexes formed in the first chamber by hydrolysis of the ester moiety could reach the DNA. Therefore, the measured DNA platination reflected both hydrolytic activation as well as reactivity of the hydrolysis products towards the DNA. The Pt-PEG complex caused a high degree of DNA platination indicating rapid and substantial activation by hydrolysis whereas the HSA conjugates exhibited only a low degree of platination reflecting high hydrolytic stability (Fig. 7).

Since the hydrolytic instability of the Pt-PEG complex could lead to misinterpretations of the intracellular accumulation and cytotoxicity measurements we investigated the hydrolysis rate of this conjugate in more detail. The released low molecular fraction was studied in the same system without the presence of DNA in the second chamber. The Pt-PEG complex exhibited a platinum release following zero-order kinetics with a hydrolysis rate of $2.735 \cdot 10^{-8}$ mol/L/h. After 2 h

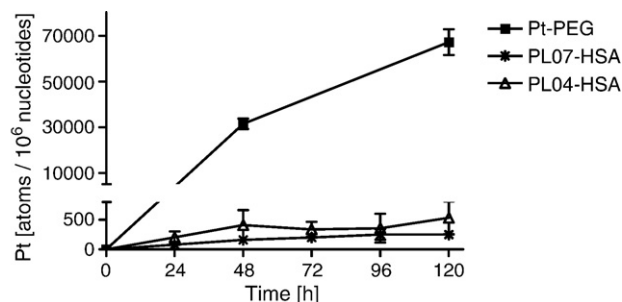


Fig. 7. Platinum-DNA adduct formation after hydrolytic activation of PL04-HSA, PL07-HSA and PEG_{10k}-(Mal-Pt-DACH)₂ ($n=3$; mean ± SD).

(maximum time used for uptake experiments) less than 3% of the Pt-PEG complex was hydrolyzed indicating only marginal impact on the measured intracellular concentrations. After 48 h more than 65% of the Pt-PEG complex was hydrolyzed. Thus, the cytotoxic activity of this compound measured after 72 h incubation using the MTT assay was considerably affected by the hydrolysis products.

4. Discussion

Cellular resistance to cisplatin is multifactorial. However, reduced accumulation of platinum complexes seems to be a common mechanism of resistance. This project was conducted to study the possibility to overcome resistance in a well-characterized cell line with a known uptake defect utilizing endocytosis as an alternative uptake pathway. To our knowledge, this is the first report describing experiments with macromolecular platinum compounds in well-characterized cisplatin-resistant tumor cells.

Our results show that the uptake of the unconjugated low molecular platinum complexes PL04 and PL07 is decreased in the resistant A2780cis cell variant indicating that these substances exhibit cross-resistance to cisplatin. In the A2780cis cell line reduced platinum accumulation was associated with a lower expression of CTR1 [12]. It has been reported that the copper transporter CTR1 does not distinguish between different platinum complexes. Therefore, the decreased intracellular platinum concentration after incubation with PL04 and PL07 is likely to result from the reduced CTR1 expression. Two mechanisms have been described for the transport of platinum complexes by this protein: endocytosis of the platinum-transporter complex and diffusion of platinum complexes across a stabilized channel. As an initial step, binding of platinum complexes to methionine-rich domains of the CTR1 transport protein is required for both mechanisms [27,28].

In order to verify our hypothesis that macromolecular platinum complexes can overcome this uptake defect, endocytosis was examined in A2780 and A2780cis cells using FITC-BSA as a model compound. There are only a few studies concerning endocytosis in platinum-resistant cells. The reported results are contradictory. Reduced uptake of horseradish peroxidase (HRPO) and Texas Red dextran was observed in cisplatin-resistant human epidermoid carcinoma KB-CP-r cells indicating defects in fluid-phase endocytosis. However, the kinetics of EGF uptake, a marker of receptor-mediated endocytosis (RME), was similar in both cells [29,30]. Other authors did not observe differences in fluid-phase pinocytosis determined with lucifer yellow between cisplatin-sensitive ovarian carcinoma 2008 cells and their resistant variant C13 [31]. Furthermore, an enhanced accumulation of fluorescence-labeled LDL as a marker for RME in C13 cells was reported [32]. Considering all these results it is likely that alterations of endocytotic pathways are cell-type specific. Nevertheless, reduced RME was not reported so far. In our study, accumulation of FITC-BSA was not reduced in the resistant tumor cells indicating unchanged endocytotic pathways. Encouraged by these findings we examined the accumulation of the three macromolecular platinum complexes PL04-HSA, PL07-HSA and Pt-PEG. No difference in intracellular platinum concentrations was found between sensitive and resistant cells. However, the accumulation of macromolecular platinum complexes was reduced compared with the low molecular counterparts PL04 and PL07. Although this finding may limit the potential of our concept to overcome accumulation defects, it should be considered that two other effects may counteract *in vivo*. Both, accumulation of macromolecular platinum complexes in tumor tissue due to the EPR effect and an enhanced endocytosis of tumor cells compared with normal cells may serve as compensatory mechanisms. We have previously shown that PL04 and PL07 bound to circulating albumin show enhanced antitumor efficacy compared to carboplatin *in vivo* [20]. Moreover, after incubation with Pt-PEG the concentration of platinum was more than 2-fold higher, compared

with PL04-HSA and PL07-HSA. However, it should be considered that two Pt moieties are bound to the PEG backbone. Therefore, macromolecular backbones loaded with multiple platinum complexes are presumably capable of further enhancing the amount of platinum taken up by endocytosis.

As a next step we studied the effect of endocytosis inhibitors in order to confirm that endocytosis is a crucial uptake pathway for macromolecular platinum complexes and to examine the mechanisms involved. Endocytosis encompasses four basic mechanisms: macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis. CME was previously referred to as 'receptor mediated' endocytosis (RME) [33]. Since caveolae-mediated and clathrin-mediated endocytosis have been reported as albumin uptake pathways, we chose bafilomycin A₁ and methyl- β -cyclodextrin as endocytosis inhibitors in our experiments. Bafilomycin A₁ is a specific inhibitor of a vacuolar H⁺-ATPase localized in the endosomal membrane and responsible for lowering pH inside the endosome. This is an essential process for the dissociation of ligands and receptors after clathrin-mediated endocytosis [34]. Methyl- β -cyclodextrin inhibits caveolae-mediated endocytosis by interacting with cholesterol in the plasma membrane [35–37]. Caveolae are cholesterol- and sphingolipid-rich microdomains.

The accumulation of FITC-BSA was inhibited by bafilomycin A₁ whereas methyl- β -cyclodextrin had no influence. This finding was in accordance with results reported by Sasaki et al. and Yumoto et al. [26,38] indicating that FITC-BSA is taken up by clathrin-mediated endocytosis. Considering the results of Yumoto et al. who found no difference in mechanisms of endocytosis between native and chemically modified albumin, it is not surprising that we also found reduced accumulation of the albumin conjugates PL04-HSA and PL07-HSA after treatment with bafilomycin A₁. These results show that the uptake of the HSA conjugates is mainly influenced by the albumin backbone. Interestingly, the accumulation of the Pt-PEG complex was also decreased by methyl- β -cyclodextrin, indicating that other endocytotic mechanisms are additionally involved, e.g. caveolae-mediated endocytosis. The fact that the reduction of intracellular platinum concentrations by methyl- β -cyclodextrin was more pronounced in sensitive cells suggests an altered content of cholesterol in the membrane of the resistant cell variant, which was also described for other cell lines [39,40].

Control experiments showed that bafilomycin A₁ and methyl- β -cyclodextrin have no influence on the uptake of unconjugated low molecular platinum complexes PL04 and PL07. Therefore, our results indicate that only the macromolecular platinum complexes enter tumor cells primarily by endocytosis. However, different mechanisms depending on the structure of the macromolecule seem to be involved. To identify the main responsible endocytotic mechanism, further investigations with other inhibitors are required.

The fact that accumulation of macromolecular platinum complexes remains unchanged does not necessarily mean that these compounds elicit comparable cytotoxic activity in cisplatin-resistant cells. Therefore, the MTT assay was used to assess the resistance factor values for the three macromolecular platinum complexes and compare these values with those of the unconjugated counterparts. The results show that the Pt-HSA conjugates indeed overcome cisplatin resistance. Their cytotoxic activity is comparable indicating that the macromolecular characteristics of the compounds determine cytotoxicity. However, the cytotoxic activity was decreased as compared to their unconjugated counterparts PL04 and PL07. This is only partly the result of their lower intracellular accumulation. In addition, the Pt-HSA conjugates are relatively stable with regard to hydrolysis and probably need to be activated by lysosomal degradation. In contrast, hydrolytic instability may explain the higher cytotoxic activity as well as the cross-resistance of the Pt-PEG complex. Whereas intracellular accumulation was measured after 120 min, the cells were incubated for 72 h before the MTT assay was applied. It has to be considered that after 48 h

more than 65% of the Pt-PEG complex was hydrolyzed. Therefore, the measured cytotoxic activity of the Pt-PEG complex is probably caused by the released low molecular platinum species which are probably susceptible to resistance.

5. Conclusions

In conclusion, our results show that macromolecular platinum complexes primarily enter tumor cells via endocytotic pathways which are not impaired in resistant cells. The relatively low intracellular accumulation of macromolecular platinum complexes may be compensated by the EPR effect and enhanced endocytosis as well as by the design of macromolecular backbones loaded with multiple platinum species. Such tailor-made macromolecular platinum complexes specially designed to circumvent reduced intracellular accumulation may be a promising approach to overcome cisplatin resistance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2008.07.017.

References

- [1] M.S. Highley, A.H. Calvert, Clinical experience with cisplatin and carboplatin, in: L. Kelland, N. Farrell (Eds.), *Platinum Based Drugs in Cancer Therapy*, Humana Press, Totowa, New Jersey, 2000, pp. 171–194.
- [2] P.J. O'Dwyer, J.P. Stevenson, S.W. Johnson, Clinical experience: DACH-based platinum drugs, in: L. Kelland, N. Farrell (Eds.), *Platinum Based Drugs in Cancer Therapy*, Humana Press, Totowa, New Jersey, 2000, pp. 231–245.
- [3] Z.H. Siddik, Cisplatin: mode of cytotoxic action and molecular basis of resistance, *Oncogene* 22 (47) (2003) 7265–7279.
- [4] S. Akiyama, Z.S. Chen, T. Sumizawa, T. Furukawa, Resistance to cisplatin, *Anticancer Drug Des.* 14 (2) (1999) 143–151.
- [5] P. Jordan, M. Carmo-Fonseca, Molecular mechanisms involved in cisplatin cytotoxicity, *Cell. Mol. Life Sci.* 57 (8–9) (2000) 1229–1235.
- [6] A.K. Holzer, G. Samimi, K. Katano, W. Naerdemann, X. Lin, R. Safaei, S.B. Howell, The copper influx transporter human copper transport protein 1 regulates the uptake of cisplatin in human ovarian carcinoma cells, *Mol. Pharmacol.* 66 (4) (2004) 817–823.
- [7] A.K. Holzer, G.H. Manorek, S.B. Howell, Contribution of the major copper influx transporter CTR1 to the cellular accumulation of cisplatin, carboplatin, and oxaliplatin, *Mol. Pharmacol.* 70 (4) (2006) 1390–1394.
- [8] K. Katano, A. Kondo, R. Safaei, A. Holzer, G. Samimi, M. Mishima, Y.M. Kuo, M. Rochdi, S.B. Howell, Acquisition of resistance to cisplatin is accompanied by changes in the cellular pharmacology of copper, *Cancer Res.* 62 (22) (2002) 6559–6565.
- [9] M. Komatsu, T. Sumizawa, M. Mutoh, Z.S. Chen, K. Terada, T. Furukawa, X.L. Yang, H. Gao, N. Miura, T. Sugiyama, S. Akiyama, Copper-transporting P-type adenosine triphosphatase (ATP7B) is associated with cisplatin resistance, *Cancer Res.* 60 (5) (2000) 1312–1316.
- [10] R. Safaei, S.B. Howell, Copper transporters regulate the cellular pharmacology and sensitivity to Pt drugs, *Crit. Rev. Oncol. Hematol.* 53 (1) (2005) 13–23.
- [11] R. Safaei, Role of copper transporters in the uptake and efflux of platinum containing drugs, *Cancer Lett.* 234 (1) (2006) 34–39.
- [12] J. Zisowsky, S. Koegel, S. Leyers, K. Devarakonda, M.U. Kassack, M. Osmak, U. Jaehde, Relevance of drug uptake and efflux for cisplatin sensitivity of tumor cells, *Biochem. Pharmacol.* 73 (2) (2007) 298–307.
- [13] J. Fang, T. Sawa, H. Maeda, Factors and mechanism of “PR” effect and the enhanced antitumor effects of macromolecular drugs including SMANCS, *Adv. Exp. Med. Biol.* 519 (2003) 29–49.
- [14] K. Greish, J. Fang, T. Inutsuka, A. Nagamitsu, H. Maeda, Macromolecular therapeutics: advantages and prospects with special emphasis on solid tumour targeting, *Clin. Pharmacokinet.* 42 (13) (2003) 1089–1105.
- [15] H. Maeda, J. Fang, T. Inutsuka, Y. Kitamoto, Vascular permeability enhancement in solid tumor: various factors, mechanisms involved and its implications, *Int. Immunopharmacol.* 3 (3) (2003) 319–328.
- [16] Y. Noguchi, J. Wu, R. Duncan, J. Strohal, K. Ulbrich, T. Akaike, H. Maeda, Early phase tumor accumulation of macromolecules: a great difference in clearance rate between tumor and normal tissues, *Jpn. J. Cancer Res.* 89 (3) (1998) 307–314.
- [17] H. Sinn, H.H. Schrenk, E.A. Friedrich, U. Schilling, W. Maier-Borst, Design of compounds having an enhanced tumour uptake, using serum albumin as a carrier, Part I, *Int. J. Rad. Appl. Instrum. B* 17 (8) (1990) 819–827.
- [18] G. Stehle, H. Sinn, A. Wunder, H.H. Schrenk, J.C. Stewart, G. Hartung, W. Maier-Borst, D.L. Heene, Plasma protein (albumin) catabolism by the tumor itself – implications for tumor metabolism and the genesis of cachexia, *Crit. Rev. Oncol. Hematol.* 26 (2) (1997) 77–100.
- [19] T. Tanaka, S. Shiramoto, M. Miyashita, Y. Fujishima, Y. Kaneo, Tumor targeting based on the effect of enhanced permeability and retention (EPR) and the mechanism of receptor-mediated endocytosis (RME), *Int. J. Pharm.* 277 (1–2) (2004) 39–61.
- [20] A. Warnecke, I. Fichtner, D. Garmann, U. Jaehde, F. Kratz, Synthesis and biological activity of water-soluble maleimide derivatives of the anticancer drug carboplatin designed as albumin-binding prodrugs, *Bioconjug. Chem.* 5 (6) (2004) 1349–1359.
- [21] O. Aronov, A.T. Horowitz, A. Gabizon, D. Gibson, Folate-targeted PEG as a potential carrier for carboplatin analogs. Synthesis and in vitro studies, *Bioconjug. Chem.* 14 (3) (2003) 563–574.
- [22] A. Pasini, C. Caldirola, S. Spinelli, M. Valsecchi, Comments on different synthetic methods for the preparation of diamine and bis(amine) organodichloro(oxo)platinum(II) complexes, *Synth. React. Inorg. Met.-Org. Chem.* 23 (6) (1993) 1021–1060.
- [23] B.C. Behrens, T.C. Hamilton, H. Masuda, K.R. Grotzinger, J. Whang-Peng, K.G. Louie, T. Knutsen, W.M. McKoy, R.C. Young, R.F. Ozols, Characterization of a cis-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues, *Cancer Res.* 47 (2) (1987) 414–418.
- [24] H. Mueller, M.U. Kassack, M. Wiese, Comparison of the usefulness of the MTT, ATP, and calcein assays to predict the potency of cytotoxic agents in various human cancer cell lines, *J. Biomol. Screen.* 9 (6) (2004) 506–515.
- [25] C. Kloft, H. Appelius, W. Siegert, W. Schunack, U. Jaehde, Determination of platinum complexes in clinical samples by a rapid flameless atomic absorption spectrometry assay, *Ther. Drug Monit.* 21 (6) (1999) 631–637.
- [26] R. Yumoto, H. Nishikawa, M. Okamoto, H. Katayama, J. Nagai, M. Takano, Clathrin-mediated endocytosis of FITC-albumin in alveolar type II epithelial cell line RLE-6TN, *Am. J. Physiol. Lung Cell Mol. Physiol.* 290 (5) (2006) L946–L955.
- [27] Y. Guo, K. Smith, M.J. Petris, Cisplatin stabilizes a multimeric complex of the human Ctr1 copper transporter: requirement for the extracellular methionine-rich clusters, *J. Biol. Chem.* 279 (45) (2004) 46393–46399.
- [28] A.K. Holzer, K. Katano, L.W. Klomp, S.B. Howell, Cisplatin rapidly down-regulates its own influx transporter hCTR1 in cultured human ovarian carcinoma cells, *Clin. Cancer Res.* 10 (19) (2004) 6744–6749.
- [29] S.S. Chauhan, X.J. Liang, A.W. Su, A. Pai-Panandiker, D.W. Shen, J.A. Hanover, M.M. Gottesman, Reduced endocytosis and altered lysosome function in cisplatin-resistant cell lines, *Br. J. Cancer* 88 (8) (2003) 1327–1334.
- [30] X.J. Liang, S. Mukherjee, D.W. Shen, F.R. Maxfield, M.M. Gottesman, Endocytic recycling compartments altered in cisplatin-resistant cancer cells, *Cancer Res.* 66 (4) (2006) 2346–2353.
- [31] G. Marverti, P.A. Andrews, Stimulation of cis-diamminedichloroplatinum(II) accumulation by modulation of passive permeability with genistein: an altered response in accumulation-defective resistant cells, *Clin. Cancer Res.* 2 (6) (1996) 991–999.
- [32] K. Ayesu, P.A. Andrews, Role of receptor mediated endocytosis in cisplatin accumulation, *Proc. Am. Assoc. Cancer Res.* 37 (1996) 403.
- [33] S.D. Conner, S.L. Schmid, Regulated portals of entry into the cell, *Nature* 422 (6927) (2003) 37–44.
- [34] N. Nelson, W.R. Harvey, Vacuolar and plasma membrane proton-adenosinetriphosphatases, *Physiol Rev.* 79 (2) (1999) 361–385.
- [35] D.H. Hryciw, J. Ekberg, C. Ferguson, A. Lee, D. Wang, R.G. Parton, C.A. Pollock, C.C. Yun, P. Poronnik, Regulation of albumin endocytosis by PSD95/Dlg/ZO-1 (PDZ) scaffolds. Interaction of Na⁺-H⁺ exchange regulatory factor-2 with CIC-5, *J. Biol. Chem.* 281 (23) (2006) 16068–16077.
- [36] E.B. Neufeld, A.M. Cooney, J. Pitha, E.A. Dawidowicz, N.K. Dwyer, P.G. Pentchev, E.J. Blanchette-Mackie, Intracellular trafficking of cholesterol monitored with a cyclodextrin, *J. Biol. Chem.* 271 (35) (1996) 21604–21613.
- [37] P.A. Orlandi, P.H. Fishman, Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains, *J. Cell Biol.* 141 (4) (1998) 905–915.
- [38] Y. Sasaki, J. Nagai, Y. Kitahara, N. Takai, T. Murakami, M. Takano, Expression of chloride channel, ClC-5, and its role in receptor-mediated endocytosis of albumin in OK cells, *Biochem. Biophys. Res. Commun.* 282 (1) (2001) 212–218.
- [39] P.A. Andrews, Cisplatin accumulation, in: L. Kelland, N. Farrell (Eds.), *Platinum based Drugs in Cancer Therapy*, Humana Press, Totowa, New Jersey, 2000, pp. 89–113.
- [40] S.Y. Sharp, P. Mistry, M.R. Valenti, A.P. Bryant, L.R. Kelland, Selective potentiation of platinum drug cytotoxicity in cisplatin-sensitive and -resistant human ovarian carcinoma cell lines by amphoterin B, *Cancer Chemother. Pharmacol.* 35 (2) (1994) 137–143.