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RESEARCH PAPER

A Study of Liposomal Formulations to Improve the Delivery of Aquated Cisplatin to a Multidrug Resistant Tumor

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ABSTRACT

Purpose This study was aimed at exploring the use of liposomes to deliver aquated cisplatin (ACP), a metabolite of CDDP, with increased potency and toxicity. Three liposomal formulations were compared for delivery of ACP to a multidrug resistant tumor.

Methods Three different liposomes (DMPC, DPPC and DSPC as the main lipid components) were loaded with ACP by the thin-film hydration method. *In vitro* drug release was assessed over 72 h at 37°C in PBS. The pharmacokinetics of free CDDP and the three ACP liposomes was determined using ICP-AES and their efficacy against EMT6-AR1 multidrug resistant murine breast tumor was compared.

Results The DSPC formulation, composed of a C18 acyl chain lipid, exhibited the slowest drug release (~2%) after 72 h at 37°C, compared to the other two formulations with decreased carbon chain lengths (C16 and C14; 7 and 25% release respectively). The pharmacokinetic profile was improved with all liposomal formulations relative to free CDDP, with clearance reduced by 500-fold for DSPC, 200-fold for DPPC and 130-fold for DMPC. The DSPC formulation displayed the highest drug accumulation in the tumor with 2-fold, 3-fold and 100-fold increases compared to DPPC, DMPC and free CDDP respectively. The DSPC formulation significantly inhibited the EMT6-AR1 tumor growth by ~90%, while the other formulations displayed no statistically significant improved activity compared to saline.

Conclusion These results suggest that the DSPC liposomal formulation is a promising formulation for MDR tumor therapy over DMPC and DPPC formulations and free drug.

KEY WORDS aquated cisplatin · cisplatin · long circulating liposome · multidrug resistant tumor

ABBREVIATIONS

ACP	Aquated cisplatin
AUC	Area under the concentration curve
CDDP	Cisplatin
CHOL	Cholesterol
Cl	Clearance
CTR1	Copper transporter 1
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle's medium
DMPC	1, 2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine
DPPC	1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine
DSPE-PEG	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethyleneglycol)-2000]
EPR-effect	Enhanced permeability and retention effect
FBS	Fetal bovine serum

Yucheng Zhao and Jonathan P. May contributed equally to this work.

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ICP-AES	Inductively Coupled Plasma Atomic Emission Spectroscopy
ID/g	Injected dose per gram of tissue
LCL	Long circulating liposomes
MDR	Multi-drug resistance
MLV	Multi-lamellar vesicles
MWCO	Molecular weight cut-off
NPs	Nanoparticles
PBS	Phosphate buffered saline
PDI	Polydispersity index
Pt	Platinum
RES	Reticuloendothelial system
$t_{1/2}$	Half life
V_{ss}	Steady state volume of distribution

INTRODUCTION

Cisplatin (CDDP) is used for the treatment of many cancers, including melanoma, lymphoma, lung, bladder, testicular, cervical, and head and neck cancers (1–3). CDDP enters tumor cells through active transporters such as the copper transporter, CTR1 (4). After being administered into the bloodstream, the chloride ligands of CDDP initially remain intact, as the chloride concentration in plasma is sufficiently high (~100 mM). However, the environment inside the cell exhibits a much decreased chloride concentration (4–20 mM), leading to the substitution of CDDP chloride ligands by water to form the monoaquated and diaquated platinum species (5). Aquated cisplatin (ACP) reacts with the bases of DNA (particularly N7 of guanosine) found in the nucleus, forming intrastrand DNA adducts, activating an apoptosis pathway (6,7) (Fig. 1). ACP species are more potent than CDDP, suggesting that it could be an attractive cytotoxic agent; however, the higher toxicity of ACP is noted for severe side effects including renal toxicity, gastrointestinal toxicity, nephrotoxicity, ototoxicity, and optic neuropathy which may limit its clinical use (6,7). We hypothesized a drug delivery system might reduce the toxicity and enable ACP to be used as a potent chemotherapeutic drug.

Nanoparticles (NPs) have been employed to deliver anticancer drugs, because they can target drugs selectively to tumors via the Enhanced Permeability and Retention effect (EPR-effect), while minimizing accumulation in many normal tissues (8). One liposomal formulation of CDDP, SPI-077 (HSPC/CHOL/DSPE-PEG, 51:44:5 mol%), has been evaluated for treatment of squamous cell cancer of the head and neck, reaching phase I-II clinical trials (9). Even though SPI-077 exhibited prolonged blood circulation and was well tolerated, it had limited therapeutic activity, with an overall response rate of 4.5% (10). Data have shown a lack of efficacy due to the insufficient release of CDDP, leading to drug concentrations below the threshold required to exert a therapeutic effect, causing the trials to be terminated (10,11). Although one of the advantages of liposomal drug delivery is to reduce the side effects of a drug by minimizing its interaction with healthy tissues, a liposome that is too stable can result in reduced bioavailability for the target cells, leading to poor efficacy. This highlights the critical role of the drug release kinetics for the therapeutic activity and toxicity of liposomes. Additionally, a low drug-to-lipid ratio (0.014 weight ratio) is another limitation for SPI-077, due to the limited solubility of CDDP (~1 mg/mL) (12). For low drug-to-lipid ratio formulations, an increased amount of lipid excipients will be injected to achieve the therapeutic dose, which might lead to increased toxicity from these excipients, such as hypersensitivity reactions.

There are examples in the literature of using different lipid compositions to change the drug release characteristics of liposomes for other drugs (13,14). For example, Charrois *et al.* demonstrate that the drug doxorubicin was released more rapidly and to a greater extent for liposomes consisting of lower transition temperature lipids, although similar pharmacokinetics of the liposome lipids themselves were observed (13). It was considered that we may be able to perform a similar study here to find the best formulation for delivering ACP. Hence, this study was focused on three different liposomal formulations of ACP containing different major lipid constituents (DMPC, DPPC and DSPC) varying by carbon chain length and transition temperature (T_m). A method for the preparation and delivery of ACP with liposomes is described. A passive loading approach was used to load ACP (rather than CDDP) into liposomes, taking advantage of the increased solubility of ACP to achieve improved loading (i.e., increased drug-to-lipid ratio). The drug release, pharmacokinetics, biodistribution and antitumor efficacy was then assessed for each formulation.

MATERIALS AND METHODS

Materials

1, 2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG_{2000}) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol (CHOL) was obtained from Sigma Aldrich (Oakville, ON). Cis-diamminedichloroplatinum (II) (CDDP) was purchased from Chem-Impex International, Inc. (Wood Dale, IL) without further purification. All other reagents were of analytical grade.

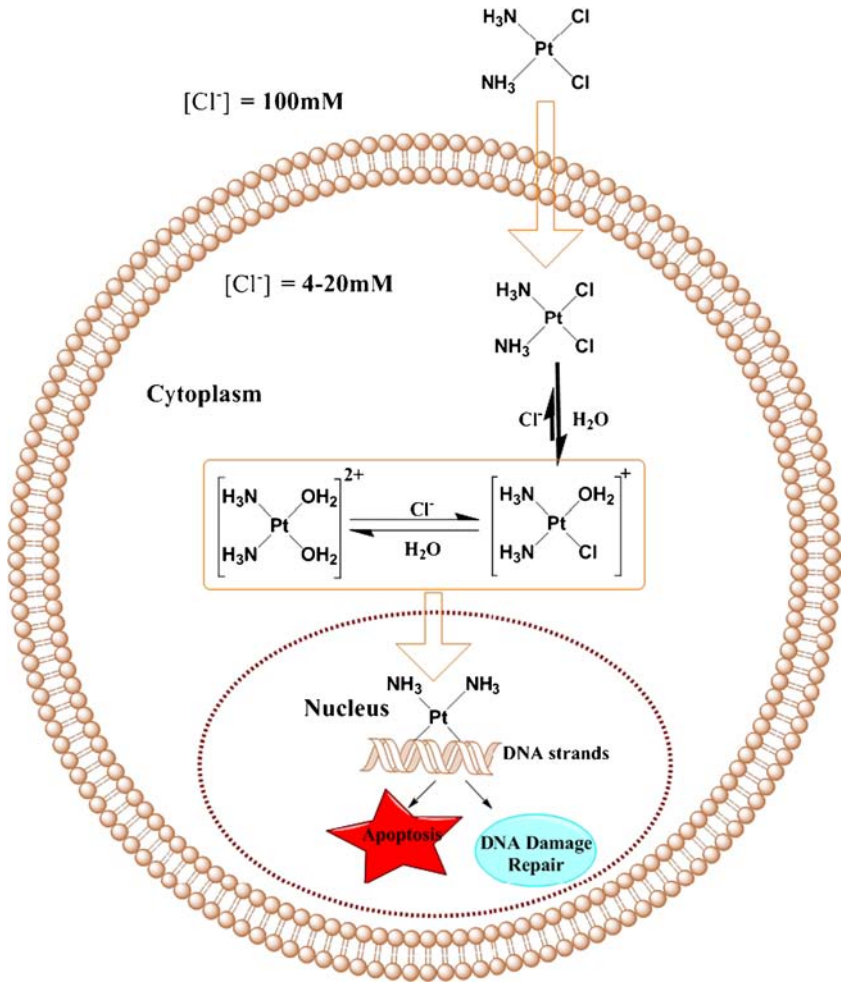

Fig. 1 Mechanism of action of cisplatin.

Preparation of Aquated Cisplatin (ACP)

Cisplatin (30 mg, 0.1 mmol Pt) was dissolved in milliQ water (1.2 mL) and heated with a water bath (50°C) to form a yellow suspension. Silver nitrate (34 mg, 0.2 mmol) was added with stirring, generating a white precipitate and a colorless solution. This solution was centrifuged at 16,000×*g* for 5 min, the supernatant was removed and this process was repeated (total of 3 times), to yield a solution of ACP (0.1 mmol Pt).

Preparation of ACP-Loaded Long Circulating Liposomes (LCL)

Each of the lipid components (DMPC, DPPC, and DSPC) was mixed with CHOL and DSPE-PEG_{2000} at a mole ratio of 56.4:38.3:5.3. Solvent was evaporated at 65°C under a stream of nitrogen gas, followed by further drying under vacuum overnight. The thin-film was then hydrated with an aqueous solution of drug (1 mL, aquated cisplatin in milliQ water at 0.1 mmol) at 60°C, to form multi-lamellar vesicles (MLVs). MLVs were extruded 21 times through polycarbonate filters (Avanti Polar Lipids, Alabaster, AL) of 0.2 μm pore size first, and then repeated with 0.1 and 0.08 μm pore size, each at 65°C, to adjust liposome size to around 85 nm. Formulations were then transferred to a dialysis cassette (Slide–A-Lyzer®10 kDa MWCO, Thermo Scientific, Rockford, IL) and dialyzed against saline (500 mL) for 0.5 h. Saline was changed and dialyzed again for 1 h, and then this process was repeated against saline (1 L) overnight. The size and zeta potential of the liposomes were measured by a particle analyzer (Malven Instruments, Worcestershire, UK). The loading efficiency was determined as $[Pt]_L/[Pt]_o \times 100\%$, where $[Pt]_L$ is the concentration of Pt in the liposome (mM), and $[Pt]_o$ is the concentration of the original stock solution (mM).

Drug Release Study

Release of Pt from liposomes was performed by measuring Pt concentrations using inductively coupled plasma atomic emission spectroscopy (ICP-AES). The Pt band at 265.945 nm was used for all measurements, as this band had the best signal-to-

noise. The formulation (500 μL) was diluted with saline (4.5 mL) and was injected into a dialysis cassette. The dialysis cassette was then placed into a beaker containing PBS (1 L) at 37°C and was covered with parafilm to minimize evaporation. Aliquots (5 mL) were collected and replaced with fresh PBS (5 mL) at a series of time points over 72 h. All samples were then analyzed with ICP-AES, taking all measurements in triplicate.

Animals and Tumor Cells

Female Balb/c mice (age 6–8 weeks, approximately 20 g) were purchased from Harlan. All animal studies were performed according to protocols approved by the Animal Care Committee of the University Health Network (UHN, Toronto, ON). The multidrug resistant mouse breast carcinoma cell line EMT6-AR1 was a gift from Ian Tannock, Princess Margaret Hospital, Toronto. Cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin.

Antitumor Efficacy Studies

Mice were inoculated s.c. with EMT6-AR1 cells (2×10^4 cells) on their right flank. When tumors reached a volume of ~200 mm^3, the mice were i.v. injected with either saline, CDDP (12.6 mmol Pt/kg) or one of the three liposomal formulations (DMPC, DPPC or DSPC) at 12.6 mmol Pt/kg. Tumor size and body weight of the mice were monitored.

Statistical Analysis

All data are expressed as mean ± SEM. Statistical analysis was conducted with the two-tailed unpaired *t*-test for two-group comparison or one-way ANOVA, followed by the Tukey multiple comparison test by using GraphPad Prism (for three or more groups). A difference with $p<0.05$ was considered to be statistically significant.

RESULTS

Characterization of the Formulations

Three formulations were prepared through hydration of lipid thin-films, followed by membrane extrusion to control the size, and dialysis against saline to remove the unencapsulated drug. This method gave a loading efficiency of between 12–17%. The physical parameters of all three formulations were studied, including the particle size and zeta potential (Table 1). The mean particle sizes for all liposomes were within the range of 80–90 nm with polydispersity indices (PDI) of less than 0.05 indicating that the particle populations were uniform. These physical characteristics were studied over a period of 3 weeks and showed no significant changes during this time (Supplementary Figure 1 and 2). The surface charge of liposomes, represented by their zeta potential, were observed to be within the range of −9 to −16 mV, indicating all liposomes had a slightly negative charge (Table 1). Formulations were also measured for Pt content by ICP-AES. All three formulations showed similar drug loading efficiency and drug-to-lipid weight ratio (12–17%, 0.019–0.024 respectively). All studies were performed with freshly prepared Pt formulations.

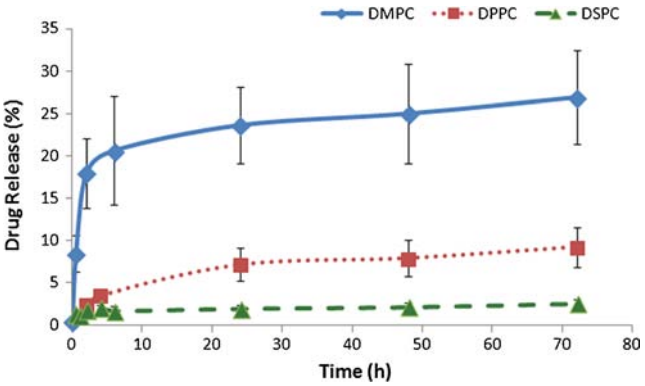

Fig. 2 The *in vitro* drug release profiles of the DMPC, DPPC, and DSPC liposomes in PBS (pH 7.2) incubated at 37°C over 72 h. Aliquots (5 mL) were collected and measured for [Pt] by ICP-AES. All measurements were recorded in triplicate and are represented as a mean ± SEM ($n=3$).

Drug Release Profile

The drug release profile of each formulation in PBS was monitored (Fig. 2). The DMPC formulation showed a rapid drug release (20%) in the first 6 h, and exhibted 25% drug release after 72 h. The DPPC formulation released 7% of drug during the 72 h period and the DSPC formulation showed the slowest drug release over 72 h, with only 2% measured at the end of this period.

Table 1 Summary of formulation parameters

Formulation	Z-average (d.nm)	PDI	Zeta Potential (mV)	Drug Loading (mg/ml)	Loading Efficiency (%)	Drug:Lipid (w/w)
DMPC/CHOL/DSPE-PEG	82.8 ± 0.7	0.041 ± 0.003	−10.0 ± 0.9	4.3 ± 0.2	17.1 ± 0.7	0.024
DPPC/CHOL/DSPE-PEG	85.3 ± 0.5	0.027 ± 0.004	−12.5 ± 0.4	3.5 ± 0.1	14.1 ± 0.8	0.020
DSPC/CHOL/DSPE-PEG	88.3 ± 0.5	0.036 ± 0.005	−15.2 ± 1.0	3.2 ± 0.2	12.1 ± 1.1	0.019

Pharmacokinetics (PK)

The PK of each formulation was studied in Balb/c mice with doses normalized for equal Pt concentrations of 12.6 mmol Pt/kg (Fig. 3). The DMPC, DPPC and DSPC formulations each displayed long-circulating character and significantly increased the area under the curve (AUC) over that observed for the free drug, CDDP (DMPC: 85-fold, DPPC: 117-fold, DSPC: 181-fold increase in AUC) .

Data was then fitted to a one-compartment model and analysed with WinNonLin software to calculate the corresponding PK parameters (Table 2). In general, these values fit well with the observed trend: CDDP << DMPC < DPPC < DSPC, with regards to half life ($t_{1/2}$) and AUC. Meanwhile, the clearance and volume of distribution followed the order: CDDP >> DMPC > DPPC > DSPC. The AUC of the DSPC formulation had an AUC which was 2-fold greater than that for DMPC and 180 times the AUC of the free drug CDDP. Correspondingly, the clearance of the DSPC formulation was almost 4-fold less than that for DMPC and more than 500-fold less than CDDP.

Biodistribution

The biodistribution of all formulations were studied 24 h post-injection, which according to previous studies should allow a significant accumulation of liposomal drug via the EPR-effect (Fig. 4). Low levels of Pt were detected in all of the examined tissues for the free drug group (<5%ID/g), with the highest uptake in the kidney (5%ID/g) and liver (2.5%ID/g). The Pt concentration in the tumor from mice treated with free CDDP was almost undetectable. All liposomal formulations (DMPC, DPPC and DSPC) showed increased tissue uptake, especially in the tumor (4.9±4.9, 7.4±2.2, 20.0±7.4%ID/g, respectively) and spleen (14.7±2.8, 19.1±6.3, 38.6±7.1%ID/g, respectively) relative to CDDP (tumor: 0.2±0.3 and spleen: 0.7±0.3%ID/g). There were also significantly higher levels in the blood for all liposomal formulations (DMPC: 7.2±2.7, DPPC: 11.7±4.7, DSPC: 18.9±2.1%ID/g) relative to CDDP (0.3±0.2%ID/g). These differences between liposomal and free drug were all found to be highly significant ($p<0.0005$). The DSPC formulation displayed a greater than 2-fold increase in tumor uptake ($p<0.001$), relative to the DPPC and DMPC formulations; a similar pattern was also measured in the spleen. Blood levels at 24 h also followed the trend DSPC > DPPC > DMPC, indicating the DSPC formulation retained significantly more than either DPPC or DMPC (1.6 or 2.6-fold, respectively, $p<0.001$). Values obtained for the DPPC and DMPC formulations in the kidney (~10%ID/g) were observed to be twice those determined for the CDDP group (~5%ID/g). The DSPC formulation, on the other hand, did not significantly increase the kidney uptake compared to CDDP.

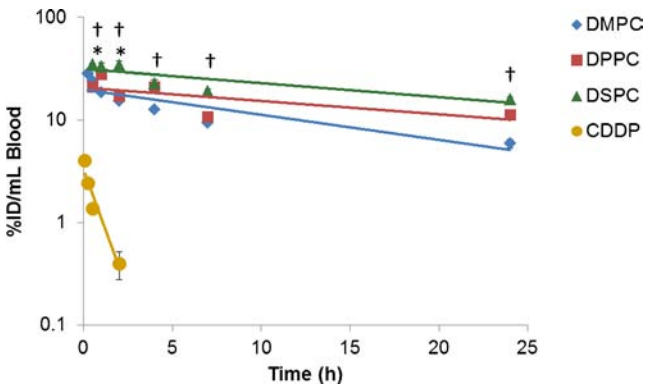

Fig. 3 Pharmacokinetics of different formulations injected at equal Pt dose (12.6 mmol Pt/kg). Data represents mean values ± SEM ($n \geq 3$). Significance ($p < 0.005$) between CDDP and all liposomal formulations are indicated by *. Significance ($p < 0.05$) between the DSPC formulation with the DMPC formulation is indicated by †.

Table 2 Pharmacokinetic parameters of different Pt formulations in Balb/c mice were analyzed by WinNonlin software

	DMPC	DPPC	DSPC	CDDP
$t_{1/2}$ (min)	1215.58	1415.22	2772.28	43.52
AUC (min·μg/mL)	6945.07	9472.03	14668.03	81.09
Cl (mL/min/kg)	0.21	0.12	0.05	26.73
Vss (mL/kg)	343.92	261.82	209.54	1347.67

Antitumor Efficacy

The antitumor efficacy of all formulations was monitored after a single dose at equivalent Pt concentration (12.6 mmol Pt/kg) (Fig. 5). Treatment with the DSPC liposomal formulation showed a significant reduction in tumor growth compared to free CDDP by day 16. Tumors treated with the DMPC formulation showed similar tumor growth to the CDDP group and neither of these, nor even the DPPC formulation, displayed a significant antitumor effect compared to the vehicle control, due in part to the large variation observed in the control tumors of this aggressive cancer.

Toxicological Analysis

An indication of toxicity occurring in the mice treated with the Pt formulations was monitored by measuring changes in body weight. Only mice treated with the DMPC formulation experienced a statistically significant body weight loss at day 7, together with characteristic signs of behavioral weakness such as piloerection and slowness of movement. The DPPC and DSPC formulation groups did not exhibit significant body weight loss (Supplementary Figure 3).

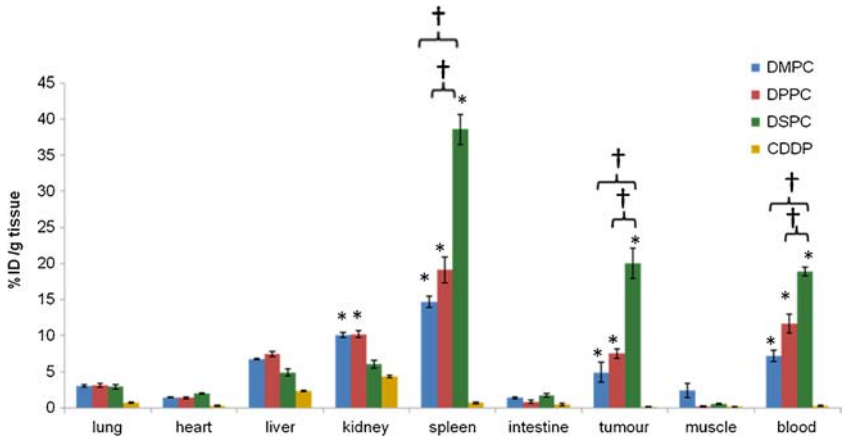

Fig. 4 Biodistribution of different Pt formulations at 24 h post injection (dose = 12.6 mmol Pt/kg). Data represents mean values ± SEM ($n \geq 3$). Significance was observed between CDDP and all three liposomal formulations for values in the blood, tumor and spleen ($p < 0.0005$), which is represented by *. Other significant relationships ($p < 0.001$) are represented by † with an indication of the relationship being referred to.

DISCUSSION

Liposomal drug delivery systems have been widely used to overcome the major side effects of conventional chemotherapy (1). We have investigated the use of three different liposomal formulations (DMPC, DPPC and DSPC) for the delivery of ACP, a highly potent alkylating agent known to promote apoptosis (6). The use of lipid-based NPs as a carrier for antitumor agents can significantly reduce the toxicity and increase the drug delivery to the tumor via the EPR-effect (15,16). However, insufficient drug release in the tumor can be a limitation for liposomal delivery, as found for SPI-077, a liposomal CDDP formulation, that reached phase II trials (10). In an attempt to study the relation between drug release and antitumor effect, we have selected three different lipid formulations to assess the delivery of ACP. ACP was selected because it is the active form of CDDP with increased potency and increased aqueous solubility. However, without proper delivery ACP cannot be used clinically due to its high toxicity. Formulations with different release kinetics were produced using DMPC, DPPC and DSPC lipids, possessing transition temperatures (T_m) below (24°C), around (41°C), and above body temperature (55°C), respectively (17,18). All three formulations formed vesicles of ~85 nm with a PDI <0.05. Preparation of these three formulations through a passive loading method achieved 12–17% drug loading efficiency. All formulations displayed similar physical characteristics and drug-to-lipid ratio (~0.02 weight ratio). There was an increase in the drug:lipid ratio for these three liposomal formulations of ACP compared to SPI-077 (10), due to the improved solubility of ACP compared to CDDP. The DMPC formulation with a T_m at 24°C exhibited the fastest release: 20% release in 6 h and 25% release after 72 h. The DPPC formulation (T_m 41°C) released 7% and the DSPC formulation (T_m 55°C) released around 2% after 72 h. The observed pattern of release kinetics corresponded to the liposomal lipid chain length and the lipid T_m: a shorter chain length led to a decreased T_m, which resulted in increased drug release. Following an i.v. injection, liposomes must retain the drug in the blood circulation and then release it in the target tissue, where it can exert its pharmacological effect. Therefore, the liposomal formulation needs to be carefully designed and optimized to have improved PK, accumulation in the target tissue and drug release, leading to enhanced efficacy. The PK study showed that CDDP was eliminated quickly from the blood circulation, and the drug was undetectable in the blood after 2 h. All of the tested formulations displayed significantly decreased blood clearance compared to the free drug CDDP: DSPC 500-fold, DPPC 200-fold, DMPC 130-fold. Between the three liposomal formulations, the DMPC formulation exhibited the most rapid clearance from the circulation, followed by the DPPC and the DSPC formulations (Pt concentration in blood 24 h post injection: DSPC 15%ID/g, DPPC 12%ID/g, DMPC 9%ID/g). The DSPC formulation had the most long circulating character, exhibiting 2- to 3-fold prolonged half life and 2- to 4-fold reduced clearance relative to the DPPC and DMPC

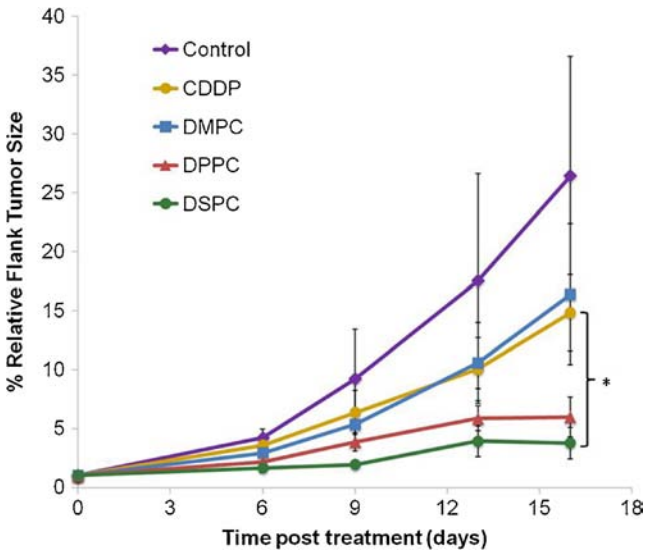

Fig. 5 Efficacy plot for all formulations at equal Pt dose (12.6 mmol Pt/kg). Data represents mean values ± SEM ($n \geq 4$). Significance ($p < 0.05$) is represented by *.

formulations. The data demonstrates that a liposomal formulation with an increased T_m and reduced drug release exhibits prolonged PK.

As represented by the biodistribution data, prolonged PK resulted in increased tumor uptake. The DSPC formulation displayed 2-, 3- and 100- fold enhanced delivery to the tumor compared to the DPPC, DMPC and free drug CDDP formulations, respectively. However, this increased circulation also led to similar increases in spleen uptake. As extravasation of NPs from the blood circulation into the tissue is a slow and cumulative process, extended blood circulation is often a prerequisite for improved tumor delivery and this can lead to increased uptake by the RES. However, it is likely the uptake of the long circulating liposomal drug was mainly mediated by macrophages, which replenish themselves rapidly (19). These results reflect reports for many other long-circulating nanoparticle drug delivery systems (20,21). Interestingly, unlike the increased uptake by the spleen, the delivery of the DSPC formulation to the liver was not enhanced, but the reason for this is yet to be investigated. Additionally, as nephrotoxicity is the dose limiting toxicity of Pt drugs, the approximately 2-fold reduced kidney accumulation observed with the DSPC formulation compared to the DPPC and DMPC formulations, could be advantageous. Highly water soluble Pt that was prematurely released during the blood circulation from these faster-releasing formulations (i.e., the DPPC and DMPC formulations) might be primarily excreted from the kidney, causing significant toxicity through alkylation of off-target cells. The increased toxicity observed for the DMPC formulation (body weight loss, Supplementary Figure 3) could be partially attributed to this increased uptake by the kidney. Previous studies using DSPC, DPPC and DMPC liposomes for the delivery of other drugs have not shown any variation in the biodistribution of liposomes for differing chain length lipids (13,22), providing further reasoning for the premature release of drug from the DMPC formulation being the cause of this increased toxicity.

Multidrug resistant (MDR) cancers are a significant clinical challenge; when an MDR phenotype develops, it halts the effectiveness of a wide range of chemotherapeutic compounds, substantially limiting therapeutic options to patients. Thus far, there has not been an effective therapy for MDR cancer, so in this study the efficacy of each formulation was compared against a murine MDR model, EMT6-AR1. The efficacy data correlates well with the biodistribution data, in which the DSPC formulation with increased tumoral delivery also exhibited enhanced activity in retarding EMT6-AR1 tumor growth compared to the DPPC and DMPC formulations and free CDDP.

CONCLUSION

Our data suggest that ACP can be formulated into liposomes for safe administration *in vivo* for MDR tumor therapy. The DSPC liposomal formulation with an increased T_m and reduced drug release rate exhibited prolonged PK, improved tumor delivery and enhanced antitumor efficacy.

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