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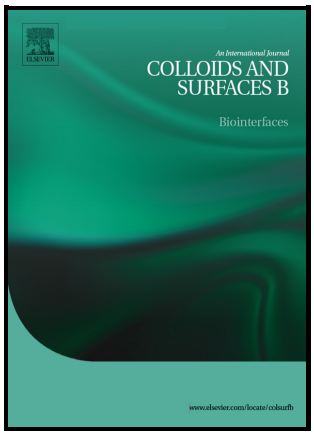

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Liposomal delivery of organoselenium-cisplatin complex as a novel therapeutic approach for colon cancer therapy

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Abstract

Cisplatin is a widely-used chemotherapeutic agent for the treatment of various solid neoplasms including colon cancer. Cisplatin-induced DNA damage is restricted due to dose-related adverse reactions as well as primary resistance mechanisms. Therefore, it is imperative to utilize novel therapeutic approaches to circumvent cisplatin limitations and attenuate its normal tissues toxicity. In this study, we exploited a novel PEGylated liposomes with greater efficiency to treat colon cancer. For this, an organoselenium compound (diselanediylbis decanoic acid (DDA)) was synthesized, and liposomes composed of Egg PC or HSPC, as well as DOPE, $mPEG_{2000}$-DSPE, cholesterol and DDA at varying molar ratios were prepared by using thin-film method. Cisplatin loading was performed through incubation with liposomes. Characterization of nanoliposomes indicated a favarable size range of 91 to 122 nm and negative zeta potential of -9 to -22 mv. The organoselenium compound significantly improved cisplatin loading efficiency

within the liposomes (83.4%). Results also revealed an efficient bioactivity of cisplatin liposome on C26 cells compared to the normal cells. Further, DDA bearing liposomes significantly improved drug residence time in circulation, reduced toxicity associated with the normal tissues, and enhanced drug accumulation within the oxidative tumor microenvironment. Collectively, results indicated that cisplatin encasement within liposomes by using this method could significantly improve the therapeutic efficacy *in vivo*, and merits further investigations.

Keywords:

Cisplatin; Liposome; Organoselenium; Oxidative TME; Colon cancer

Introduction

Cancer statistics demonstrated 19.3 million new cancer cases and almost 10.0 million cancer-related deaths worldwide in 2020. According to these data, lung cancer remained the most commonly diagnosed cancer with 1.8 million deaths, followed by colorectal cancer as the second cause of death, as well as liver, stomach and female breast cancers as other leading causes of cancer death globally [1]. Unfortunately, despite attempts for mitigating risk factors, the prevalence of cancer is continuing to increase. Current common therapies including radiotherapy, chemotherapy and/or surgical resection possess significant adverse effects [2]. Cisplatin (cis-diamminedichloro platinum[II], CDDP) is a highly effective and widely-used anticancer drug in the treatment of varying solid cancers including colon carcinoma, ovarian, breast, endometrial, metastatic testicular, bladder, cervix as well as non-small cell lung cancers (NSCLC) [3, 4]. Despite therapeutic benefits, CDDP application is restricted due to its high binding affinity to the plasma proteins and bio-components, low bioavailability and severe toxicities including nephro- and neurotoxicity [5].

Nanotechnology has advanced cancer therapy by providing smart drug delivery approaches to supply potent therapeutics to the tumor site. Nanocarriers offer tremendous advantages including enhancing drug solubility, extending drug circulation in blood and improving drug accumulation within the tumor through the enhanced permeability and retention (EPR) effect, which subsequently reduce normal tissue toxicity [6]. Various nano-particluate systems have shown to enhance CDDP tumoral delivery including polymeric nanoparticles, gelatin based nanoparticles, dendrimers, liposomes, transferosomes, micelles, albumin based nano-formulations, cubosomes, as well as carbon-based nanoformulations [7-10]. Among different nanoparticles, liposomes of spherical geometry and capability of enclosing both lipophilic and hydrophilic drugs have proven to be safe, effective and functional carriers [11-14]. So far, several CDDP liposomal formulations entered clinical trials, yet none has received FDA approval due to an insufficient therapeutic potential [9, 15, 16].

Cisplatin liposomal formulation faces certain challenges due to low aqueous solubility of drug molecule leading to a very low efficiency of encapsulation, low drug/lipid ratio, and an insufficient cytotoxicity. Further, in case of liposomal premature drug release, drug may be irreversibly inactivated and lose its functionality due to the high binding affinity of CDDP to the bio-components in circulation [15]. Lipoplatin®, Liplacis®, and SPI-077 are among clinical CDDP liposomal formulations that have been extensively discussed in literature. In all cases, preclinical studies demonstrated their potentials in increasing blood circulation time, and enhancing patient tolerance. A phase I study of SPI-077 reported that high doses of CDDP liposomes could be administered safely, and a phase I-II study indicated that treatment with this formulation was well-tolerated without any significant toxicities related to the kidneys, nerves,

blood, or liver. Despite initial successful results, a phase II clinical study suggested that this formulation lacks enough efficacy in treating non-small cell lung cancer (NSCLC) [17, 18].

The lack of specific and controllable drug release could severely hinder nanoparticle biomedical applications. Drug release from the conventional liposomal formulation could occur through diffusion, partitioning, and dissolution [19]. With the huge progress in bio-materials chemistry and the advent of stimuli-responsive drug delivery systems, nanocarriers could control the spatio-temporal release of the entrapped cargo by responding to different internal/external stimuli [20, 21]. It has been shown that redox-responsive moieties are prone to structural modifications when exposed to the oxidative tumor microenvironment (TME). Diselenide bonds are among the widely-used redox-responsive linkers in cancer drug delivery approaches [22, 23]. Thus, implementation of these agents within the nano-particluate systems could ameliorate the drug release pattern and ultimately improves the therapeutic efficacy at the target-site [24-26]. In this case, redox-responsive nanocarriers fabricated to react with the high concentrations of reactive oxygen species (ROS) within the tumor sites have shown promising results [27]. Recently, we have also shown that tumor ROS could oxidize diselenide bonds within the liposomes, causing it to break down and release the anti-tumor agents contained within this nano-particulate system [28].

Most of the recent reports focused on the release properties of CDDP from nano-particulate systems by designing stimuli-responsive drug carriers [29-31]. Considering the two major challenges associated with CDDP liposomes including the efficacy of drug encapsulation as well as the release issue, herein, we introduced a novel PEGylated CDDP liposomal formulation by using a redox-responsive organoselenium compound (diselanediylbis decanoic acid (DDA)) for the treatment of colorectal carcinoma. We hypothesized that this novel system could offer

advantages in enhancing CDDP entrapment within liposomes due to the possible complex formation between DDA and CDDP [32-35]. Further to the enhanced drug retention in circulation, triggering CDDP release through the cleavage of the diselenide bond in the oxidative TME of the cancerous site could improve the anti-tumor efficacy of the liposomes. Collectively, this approach could offer a promising strategy for cancer targeting by enabling specific delivery of the anti-tumor agent to the cancerous cells, while sparing healthy cells.

2. Methods and materials

2.1 Materials

Hydrogenated soy phosphatidylcholine (HSPC), Egg L-α-phosphatidylcholine (EggPC), Methoxy-polyethylene glycol (MW 2000) distearoylphosphatidylcholine ($mPEG_{2000}$-DSPE) (Molecular weight: 2780.38) and Cholesterol, all were purchased from Avanti Polar Lipids (Alabaster, AL) and all were utilized in the preparation of liposomes. Selenium powder (Se 200 mesh), Sodium borohydride ($NaBH_4$), 10-Bromodecanoic acid, Magnesium sulfate ($MgSO_4$), Dimethylformamide (DMF), Diethyl ether, Dimethyl sulfoxide (DMSO) and Ethanol 98% were obtained from Sigma-Aldrich (Taufkirchen, Germany), all of which were used in the procedure of DDA synthesis. Trypan blue, as a dye was used to indicate dead cells that have lost their membrane integrity, causing them to appear darker than viable cells, was purchased from Merck (Darmstadt, Germany). MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Promega (Madison, WI). C26 cell line (murine colon carcinoma cells) and NIH-3T3 cell line (mouse embryonic fibroblast cells) were purchased from Cell Lines Service (Eppelheim, Germany), RPMI 1640 and DMEM culture media and Fetal bovine serum (FBS) were purchased

from Gibco (Carlsbad, CA). CDDP powder was obtained from Tocris Bioscience (USA). All other solvents and reagents were used as chemical grade.

2.2 Preparation of liposomes

Synthesis of 10, 10'-diselanediylbis decanoic acid (DDA) was performed as previously described [36]. Liposomal formulations were prepared by using the thin film method [37]. Lipids including HSPC, EggPC, mPEG$_{2000}$-DSPE, DOPE, cholesterol as well as DDA were mixed at different ratios (Table 1) in a round-bottom flask. The organic solvent (Chloroform) was then removed by using rotary evaporator (Heidolph, Germany), followed by a two-hour freeze-drying (VD-800F, Taitech, Japan) to exclude the solvent traces. The thin film layers were hydrated by using normal saline solution (0.9%, pH=6.5) at 65 °C. Then, liposomes were turned into nano-sized particles by using 10-minute sonication and eleven rounds of extrusion (Lipex extruder, USA) through polycarbonate membranes (200, 100, and 50 nm, respectively). For CDDP loading, liposomes were incubated with CDDP (5 mg of powder per one milliliter of each liposomal formulations) at 65 °C for 2 hours followed by a 48 h incubation at 37 °C. Finally, liposomes were dialyzed (5% dextrose, pH=6.5) with dialysis membrane (12-14 kDa molecular weight cut-off (MWCO), Spectrum, Houston, TX, USA) to remove the free drugs.

2.3 Characterization of CDDP liposomes

Dynamic Light Scattering instrument (Nano-ZS; Malvern, UK) was used to determine the mean diameter, polydispersity index (PDI) and zeta potential of liposomes. Particle size, polydispersity index and zeta potentials were reported as means ± standard deviation (SD) (n = 3). CDDP quantification was done by using atomic absorption spectroscopy (AAS) (model: 240FS. Varian Inc., Australia) and graphite tube atomizer (GTA 120) against a standard curve.

Measurements were operated at the wavelength of 265.9 nm with a slit band width of 0.2 nm [38]. CDDP concentration was determined by using the following formula:

% encapsulated = CDDP concentration after dialysis / CDDP concentration before dialysis × 100

2.4 Cytotoxicity assay

C26 and NIH-3T3 cells were purchased from Cell Lines Service (Eppelheim, Germany) and were cultured at 37 °C in a 5% CO_2/95% air humidified atmosphere in RPMI 1640 and DMEM media, respectively. The media were supplemented with 10% FCS and 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, UK). The cytotoxicity of liposomal preparations containing CDDP was determined by using MTT assay [39]. C26 and NIH-3T3 cells were seeded at a density of 5000 cells/100 µL complete medium/ well in 96-well plates and were cultured overnight. Consequently, the medium was replaced with FCS-free medium containing 1:2 serial dilutions of free CDDP and different CDDP liposomal formulations added triplicate in each well. After a 48h incubation at 37 ºC, cells were washed with pre-warmed PBS and MTT (with the ratio of 1:10, MTT/media to each well). Media (100 µL/well) was added to each wells and incubation continued for 4 h at 37 ºC. The absorbance of MTT was then measured by using a microplate reader (SpectraMax, M5, Sunnyvale, CA, USA) at 545 nm. Relative cell death was calculated as follows:

$$\text{Relativecelldeath} = 1 - \frac{\text{A (sample)\_ A (blank)}}{\text{A (control)\_ A(blank)}}$$

A (sample) and A (control) were the absorbance of the cells treated with the sample solutions and the culture medium (negative control), respectively. A (blank) was the absorbance

of cell free wells. IC_{50} values were calculated using CalcuSyn software based on the linear dose-effect curve (BIOSOFT, UK).

2.5 Blood hemolysis assay

To assess the effect of DDA liposomes on the erythrocyte, the blood hemolysis assay was performed. To do this, heparinized human blood sample was centrifuged at 1500g for 10 min, and the plasma was separated. Red blood cells (RBCs) were washed two times with PBS and the RBCs pellet was diluted with PBS (pH 7.4) at 1:10 ratio. The assay was performed by using V-bottom 96-well plates. So, diluted blood samples (50 µL) were treated with different concentrations of liposomes bearing cisplatin (3.125–100 µg/ml) and free liposomes (equivalent dilutions) and incubated for 2 h at 37 ◦C in a shaker incubator with gentle shaking at 100 rpm. Then, samples were centrifuged at 2000g for 5 min and to quantify cell lysis the absorbance of hemoglobin in the supernatant was measured at 405 nm by using a microplate reader (Tecan Group Ltd., M¨annedorf, Switzerland). The negative control included 100 µL of physiological saline (PBS) and 100 µL of red blood cell suspension, while the positive control comprised of 100 µL Distilled water and 100 µL of red blood cell suspension [40]. The hemolysis percentage was calculated based on the following equation, where OD_{sample} is the absorbance value of the liposome, OD_{Neg} is the absorbance value of the negative control group, and OD_{Pos} is the absorbance value of the positive control.

$$\text{Hemolysis (\%)} = (OD_{sample} - OD_{Neg}) / (OD_{Pos} - O\,OD_{Neg}) \times 100\%$$

2.6 *In vivo* study

BALB/c mice (aged 6–8 weeks, 18–20 g) were purchased from Pasteur Institute (Tehran, Iran). All animal experiments were approved by the Institutional Ethical Committee and Research Advisory Committee of Mashhad University of Medical Sciences guidelines (Ethical number: 990974). Every animal experiments and methods were carried out in accordance with the relevant guidelines and regulations approved by the ethical committee and ARRIVE guidelines.

2.7 Liposomal CDDP kinetic in plasma and accumulation in the tissues

Plasma elimination of CDDP was assessed in BALB/c mice (n=3). Mice received a single bolus tail vein injection of various liposomal formulations as well as free CDDP (3 mg/kg CDDP equivalent). Blood samples were collected at 1, 4, 24 and 96 h after injection into EDTA (ethylene diamine tetra acetic acid)-coated tubes, and were immediately centrifuged at 4 ºC for 15 minutes (4000 * g) to obtain plasma. Plasma samples were then transferred to Eppendorf cups and stored at -80 ºC until further analysis. All the samples were then diluted with 0.1% Triton X-100 and 0.2% nitric acid to fit into the dynamic range of the calibration curve. Calibration standards were made by spiking platinum into the plasma of control mice diluted with 0.1% Triton X-100 and 0.2% nitric acid (37.5 to 500 µg/mL platinum equivalent) [41].

To investigate CDDP tissue distribution, BALB/C mice were subcutaneously injected with 3.5×10^5 C26 cells in the right hind flank. Once tumors reached ~200 mm3, the animals were randomized into 7 groups of three mice each, including liposome formulations, free CDDP and control buffer (PBS). On the day of dosing, mice received a single dose of all formulations at 3 mg/kg CDDP by tail vein injection. Animals were sacrificed 24 h later and consequently tissues including liver, spleen, kidney, lung, heart and tumor were removed, washed with saline, blotted dry and weighted. Organ samples were dissolved in nitric acid and hydrogen peroxide and

subjected to AAS analysis for Pt determination [42]. Samples were stored at -80 ºC until further analysis.

2.8 Tumor growth study and histopathological analysis

After tumor inoculation (as previously mentioned) and when mice had palpable tumors (9 days), animals were randomly classified into 7 groups (n = 4) including liposomal formulations, free CDDP, and control group (PBS). All groups except control, received free CDDP and liposomal formulations (3 mg/kg CDDP equivalent) via tail vein injections (three doses at one-week interval) and control mice received 200 µL PBS. The animals' weight, tumor volume, and overall health were monitored three times a week for 50 days. Tumor volume was calculated with calipers in three dimensions using the following formula:

Tumor volume (mm3) = (height*length*width) *0.52

Considering ethical consideration, the exclusion criteria were as follows: tumor enlargement (more than 1 cm in one dimension), body weight loss of more than 20% of the initial mass or becoming sick or lethargic. The survival results were determined by Kaplan-Meier analysis. The time to reach the end-point (TTE) for each mouse was calculated from the line equation obtained by logarithmic regression of the tumor growth curve. The percent of tumor growth delay (%TGD) was calculated from the following formula [43].

%TGD = [(mean TTE of treatment group – mean TTE of control group)/ mean TTE of control group] × 100

For histopathological study, BALB/c mice treated with liposomal formulations, PBS and free drug at three doses of 3 mg/kg of CDDP on days 0, 7 and 14. Mice were euthanized on day 21 and blood samples were collected. Then, kidney, liver and lung tissues were extracted, fixed

with 10% formalin for 24 hours and stained with hematoxylin and eosin for 5 minutes. Samples were then embedded in paraffin wax and cut into sections to for microscopical examinations (×40) [44].

2.9 Statistical analysis

Statistical analysis was performed by using GraphPad Prism version 8 (GraphPad Software, San Diego, CA). Survival data were analyzed by the log-rank test. A one-way ANOVA statistical test was used to assess the significance of the differences among various groups for MTT and biodistribution tests. A p value of < 0.05 was considered statistically significant in all cases.

3. Results

3.1 Physicochemical characterization of liposomes

The average diameters of various liposomes after CDDP loading were approximately 91 to ~159 nm. Nanoparticles showed narrow size distribution with polydispersity index (PDI) ranging from 0.1 to ~0.25. All liposomes demonstrated negative zeta potential (-9 to -22 mV). Encapsulation efficiency (EE %) of CDDP within Egg PC liposomes improved by adding cholesterol ratios from S1 to S3 (up to 20%) as represented in Table 1. Regarding HSPC bearing formulations, S_4 (HSPC, Chol 0%, DOPE 37.5%) showed a considerable levels of CDDP entrapment efficiency (~34%). Other formulations including S_5 (HSPC, Chol 10%, DOPE 27.5%) and S_6 (HSPC, Chol 20%, DOPE 17.5%) were stable before CDDP loading, however showed instability and drug precipitate 24 hours following dialysis despite several attempts. So, these formulations were excluded from further studies.

3.2 Cytotoxicity of liposomal formulations on C26 and NIH-3T3 cell lines

To assess the bioactivity of CDDP entrapped within liposomes and also evaluate the release kinetic of the formulations, time-dependent MTT analysis was performed at 48 and 72 h incubation on C26 colon cancer and NIH-3T3 normal cells. As shown in **Figure 1A to C**, after 48 h exposure, the half maximal inhibitory concentrations (IC_{50}) of S_1, S_2 and S_3 were significantly higher compared to free CDDP (5.30, 6.13, 11.93 µg/mL versus 0.75 µg/mL, respectively), while S_4 and S_7 showed a remarkable toxicity to C26 cells comparable to that of free drug (0.89, 0.009 µg/mL versus 0.75 µg/mL, respectively). At 72 h exposure, the toxicity was significantly improved for S_4 and S_7 formulations, and was greater against C26 compared to other formulations. All liposomal formulations demonstrated less toxicity to NIH-3T3 cells compared to C26 cells (**Figure 1D to F**).

3.3 Blood hemolysis assessment

The hemolysis test was performed to assess the effect of free and cisplatin-loaded liposomes on the erythrocyte. The hemolysis rate of free liposomes at varying dilutions was found to be only 2 to 10 % after 2 h. The hemolysis rate of cisplatin bearing liposomes showed a dose-dependent pattern. For example at the highest concentration of cisplatin, the hemolysis increased up to 20%. The rate of hemolysis with distilled water as a positive control was significantly higher compared to either free or drug-loaded liposomes ($p<0.0001$). Collectively, the liposomes demonstrated an acceptable blood compatibility for intravenous administration (**Fig. 2A**).

3.4 *In vivo* plasma CDDP concentrations

CDDP concentrations in BALB/c mice plasma samples were measured following i.v. administration of liposomal formulations and free drug (3 mg/kg equivalent to CDDP). In general,

most of the liposomal formulations showed higher plasma CDDP levels 30 min following administration compared to the free drug. Among various liposomal formulation, S_4 bearing HSPC and DDA, showed the highest CDDP plasma levels following the first hour of administration (**Figure 2B**). We have observed that the mean residence time (MRT) of HSPC formulations (S_4 and S_7) were significantly higher compared to their EggPC counterparts. In the next following hours, though there was a steep decrease in the CDDP concentrations of almost all liposomal formulations, S_4 could preserve the drug plasma levels which was significantly higher compared to other liposomes. The pharmacokinetic parameters indicated that the AUC of S_4 was significantly greater compared to free CDDP and other liposomal formulations (**Table 2**).

3.5 Biodistribution study

Tissue distribution of free drug and liposomal formulations in C26 tumor bearing mice was investigated 24 hours after a single i.v. dose administration of each formulations (3 mg/kg equivalent to CDDP). As shown in **Figure 3**, the accumulation of S_1 within the liver was significantly higher compared to free CDDP ($p<0.01$), however, it was not significantly different compared to other liposomal formulations. The splenic CDDP concentrations of S_2 and S_3 was significantly lower compared to other groups, while S_4 and S_7 showed no difference compared to free drug. CDDP accumulation within the lung of animals was lower following S_4 and S_7 administration as compared to S_1, S_2 and S_3, and no significant differences were observed between liposomal formulations and CDDP in the heart tissue. In the tumor site, the highest accumulation of the drug was observed with S_4, while S_1, S_2 and S_3 exhibited significantly lower drug accumulation compared to free CDDP ($p<0.0001$, $p<0.001$, $p<0.001$ and <0.05, respectively). Results also indicated that liposomal CDDP could protect the kidney as one of the most critical

target tissue of the free drug. We have observed that the lowest drug accumulation was reported with S_3 ($p<0.0001$), S_4 and S_7 ($p<0.001$ and $p<0.0$, respectively) compared to CDDP. No significant difference was observed in the other groups.

3.6 Histopathological and hematological study

The histological photomicrographs of liposomal formulations, free drug and PBS treatments are shown in **Figure 4**. Kidney lesions, tissue necrosis and degeneration, atrophy, bleeding around the renal tubes, severe edema and inflammation with mononuclear predominance (macrophages and lymphocytes) was observed with the free drug, while tissue pathology was less severe with the liposomal platforms. We have also observed severe necrosis, degeneration and atrophy of hepatocytes with a scattered pattern, hyperemia and inflammation with mononuclear predominance (macrophages and lymphocytes) and increased sinusoidal space within the liver of CDDP -treated animals, while treatment with liposomal formulations resulted in less severe lesions with the same pattern. In the examined lung, the lesions were limited and included mild edema, hyperemia, mild necrosis and degeneration in the alveoli and airways with a pattern similar in all treatment groups. Finally, investigation of the hematological parameters (**supplementary Table 1s**) demonstrated that both CDDP and liposomal formulations significantly reduced the counts of neutrophil, lymphocytes, monocytes, eosinophils and basophils, while increased the white blood cell counts compared to PBS. Other blood factors did not change significantly.

3.7 Anti-tumor efficacy study

BALB/c mice were treated with PBS, free CDDP and liposomal formulations once weekly for three consecutive weeks, and the efficacy of treatment was investigated for almost two months.

The tumor growth curve as represented in **Figure 5A**, showed a significant difference between liposomal formulations (S_1, S_2, S_3, S_4 and S_7 (**Figure 5B**)) and PBS control up to day 18 post tumor inoculations ($P<0.05$). Data also indicates a considerable delay in the tumor growth with S_4 and S_7 formulations compared to PBS and free drug ($p<0.01$ and $p<0.001$ for S_4 and S_7 compared to PBS, respectively). For S_3 treated animals, the tumor growth rate was also slower compared to free CDDP and control PBS ($p<0.001$, $p<0.001$ and $p<0.05$, respectively). **Figure 5C** exhibits the survival time of all treatment groups as determined by the Kaplan–Meier method. Data shows that S_4 significantly prolonged the survival of animals, while other liposomal formulations including S_1, S_3 and S_7 have also shown to improve the life span of animals compared to free CDDP ($p<0.05$, $p<0.05$ and $p<0.01$, respectively). **Table 3** compared the survival factors including TTE, %TGD, MST and ILS of all treatments. S_4 showed the highest TTE and MST of 34.52 ±1.9 and 34.77 days, respectively. As it is shown in **Figure 5D**, no significant difference was observed in the weight of animals treated with liposomal formulations and control groups; however, S_4 and S_7 treatment showed the lowest fluctuation.

4. Discussion

Various nano-formulations of CDDP including Aroplatin (L-NDDP), AP5280 (HPMA conjugated CDDP), and polymeric micelles of CDDP conjugated PEG-P(Glu) (NC-6004 or Nanoplatin™) have been investigated with promising results [45]. Among varying drug delivery systems (DDS), liposomes have paved the way to the clinic due to the highlighted biocompatibility and the capability to improve the drug pharmacokinetics [46, 47]. It has been frequently reported that by reducing the size of liposomes to less than 200 nm, chemotherapeutics could preferentially target the TME through the EPR mechanism [57, 58]. Despite several clinical trials on liposomal

formulations of CDDP, enormous challenges exist including low drug/lipid ratio as well as low release rate at the tumor site, resulting in an inefficient cytotoxicity and even the induction of CDDP resistance in the tumor cells. Indeed, though liposomal CDDP have not caused major intolerability in clinical studies, suboptimal drug delivery to the tumors is still a significant concern [46, 48].

The revolutionary liposomal systems has brought attention to various physico-chemical aspects of these carriers [49]. For instance, complex formation has shown to exceed CDDP selectivity against varying types of cancers including colon, lung, liver as well as breast cancer [50]. Larasati et al. has extensively reviewed the higher anticancer potentials of several cisplatin-derived Pt (IV) prodrugs synthesized through the incorporation of varying bioactive ligands [51]. Recently, Salehi et al. has also reported on the utilization of an organic ligand (N(4)-phenyl-2-formylpyridine thiosemicarbazone (HTSC)) to develop a four-coordinated Pt complex to attenuate CDDP-associated kidney damage [52]. Despite numerous investigations on the promising platinum compound complex with higher anticancer potentials, protecting these prodrugs from circulation and efficient delivery of the chemotherapeutics to the cancerous tissue is a challenge. Thus, suitable carriers including liposomes were exploited to improve their features for more efficacious tumoral drug delivery [53]. For example, it's been shown that the complex formation of positive CDDP species with negative phospholipids could improve the encapsulation efficiency of drug within liposomes [54]. Vhora et al. used an emulsification solvent evaporation technique to load CDDP complexed caprylic acid within liposomes, and they have shown a pH-dependent release pattern within the cancerous tissue [34]. Wang et al. used CDDP-alginate conjugate as a highly soluble derivative with enhanced incorporation within liposomes for the treatment of epidermal growth factor receptor-positive ovarian carcinoma with promising results [55].

Alginate-CDDP coordination complex was also used by Ruttala et al. to develop a transferrin targeted hybrid liposome co-loaded with both CDDP and doxorubicin through lipid film and extrusion technique with improved loading and enhanced anti-tumor effects [56]. Coordination nanocomplex of 3-octadecylcarbamoylacrylicacid-cisplatin encapsulation within liposomes through reverse phase evaporation has shown an improved loading and anti-tumor efficacy [57]. Similarly, bile acid–cisplatin complex encapsulated within liposomes with high encapsulation efficiency has shown to target the liver tumor cells and dominate resistance in Hepa 1-6/10R cells [58]. The role of leaving groups configuration of DACH platinum complexes bearing linear alkyl carboxylato moieties (5–18 carbons) on liposomal stability showed a loading efficiency of greater than 90% which was independent of the length of the leaving group and the lipid composition [59]. Han et al. developed a stable lipophilic (DACH)Pt(II) complexes by replacing the two monocarboxylate ligands in the parent lipophilic platinum drugs with dicarboxylic malonate derivatives, and used a lyophilization-rehydration method to obtain liposomal platinum complex with high efficiency of loading [60].

Considering the two major challenges associated with CDDP liposomes including an inadequate loading efficiency as well as poor release properties, carriers with controllable drug release is still required [53]. In the present investigation, an organoselenium compound was used to offer a dual-functional role. It was hypothesized that by improving the loading efficiency of CDDP within liposomes, and further enhancing the drug release within the TME, the therapeutic efficiency of the resulting liposomes could be improved [36].

Reactive oxygen species (ROS) are crucial in the signal transduction pathways and metabolism. Their overexpression within the cells and the tissues induces an oxidative stress state that has implication in varying diseases including cancer, atherosclerosis, aging and inflammation

[61]. TME-targeted therapy thus makes novel opportunities for the treatment of cancer by more accurately controlling the drug release into the tumor area [62]. Spurred by the newly developed DDSs [63], ROS-responsive moieties have come to attention due to their drug release capabilities through various mechanisms including ROS-induced carrier solubility change, prodrug linker cleavage or carrier cleavage [64]. Numerous scholars have employed disulfide containing nanocarriers to enhance the bioavailability of the therapeutic agents [65-67]. Low-bond energy of diselenide facilitates its breakage, thus making it a superior moiety to be applied in elaborating DDS [68, 69]. Diselenide-bearing liposomes encapsulating dehydroascorbate (DHA) have shown to enhance drug release and has led to a greater potential in treating cancer [70]. Further, diselenide were applied to prepare polymeric micelles [71] and have also been used in other diseased models including ischemia-reperfusion injury [72].

Previously, we have shown that the carboxylic groups within the aqueous core of liposomes could facilitate cisplatin entrapment due to the formation of a coordination complex [41]. Herein, the novel liposomal formulations were developed based on the presence of the organo-selenium compound within the liposome bilayer. The developed formulations possessed varying ratios of HSPC or Egg PC, DOPE, cholesterol, $mPEG_{2000}$-DSPE and DDA. The essential features of liposomes including drug encapsulation efficiency, release kinetic, surface charge, pharmacokinetics, *in vivo* clearance rate, half-life time, biodistribution, etc., rely on the tuning of the lipid composition [73]. Further, the stability, and permeability of the liposomes is affected by their phospholipid concentrations and compositions [74]. Some liposomal formulations were developed by using EggPC as a phospholipid bearing low phase transition temperature (Tm < 0 °C) (S_1, S_2 and S_3), and others were formed by using HSPC as a higher Tm lipid (55 °C) (S_4, S_5 and S_6). Previous reports addressed the choice of the lipids within the liposomal bilayers which

could affect nanoparticles behavior. For example, by using phospholipids with higher Tm, liposomal integrity could be maintained and premature drug leakage in the bloodstream could be reduced [38, 75]. DOPE, a zwitterionic phospholipid, displays a pH-dependent hexagonal phase transition that is expected to perturb the liposomal membrane upon exposure to the acidic TME due to its non-bilayer forming characteristics [76]. It has been reported that incorporating HSPC could increase the stability of redox-responsive formulations compared to Egg PC [77]. Further, the shielding effect of $mPEG_{2000}$-DSPE against opsonin recognition could reduce opsonization and liposome phagocytosis. Lastly, cholesterol incorporation has proven to strengthen the membrane properties of liposomes including rigidity and stability thus, contributing to the optimization of the release features [78].

In this study, the CDDP-loaded liposomal formulations demonstrated a particle size of less than 200 nm, a PDI of less than 0.3 and a negative zeta potential ranging from -9 to -22 mV, which is expected to improve the steric hindrance and colloidal stability of the nanoparticles [79]. Data revealed that the incorporation of DDA organoselenium compound at an optimum ratio of 2.5% could improve drug encasement within liposomes. The encapsulation efficiency (EE) of CDDP also showed a significant increase by raising the cholesterol ratio in Egg-PC formulations (54 and 84% entrapment efficiency for S_1 and S_3 formulations, respectively). Contrary to EggPC formulations, drug precipitation was observed with HSPC liposomes (S_5 and S_6, 10 and 20% cholesterol contents), except for S4 formulation devoid of cholesterol which was stable following drug loading. Thus, we hypothesized that the simultaneous incorporation of both DDA and cholesterol in HSPC formulations could lead to some degree of instability. Despite several attempts, HSPC formulations bearing 2.5% DDA and 10 and 20% cholesterol were excluded due to the formation of drug precipitate (S_5 and S_6). To prove our hypothesis, we have developed

another HSPC formulation (S_7) without DDA contents (0%) but with 22.5% cholesterol, for which no drug precipitate was observed. Collectively, the entrapment efficiency of both Egg-PC and HSPC formulations (34.20% for S4) was significantly improved in the presence of organoselenium compound.

The study indicated a significant toxicity of the organoselenium liposomes on the C26 cancerous cells compared to the normal cells. Considering the role of lipid ingredients on the toxicity profile, we observed that with increasing the cholesterol contents up to 20% in formulations bearing Egg PC (S_3), the toxicity was less significant on C26 cells compared to S1 (devoid of cholesterol), presumably due to the improving bilayer stability conferred by cholesterol. Among DDA formulations, S_4 HSPC liposomes showed a significantly greater toxicity compared to Egg PC (S_1 to S_3) formulations which might be relevant to the higher uptake of this formulation by the cancerous cells. Further studies regarding the plasma elimination of drug demonstrated that the greater stability of HSPC liposome could ameliorate its toxicity potential and efficacy in therapy. In this regard, animal study revealed a significant improvement in the kinetic behavior of cisplatin entrapped within HSPC liposomes (S_4), compared to their EggPC counterparts. S_4 demonstrated a significantly greater cisplatin concentration at 1 and 4 h following liposome administration compared to the less stable Egg PC formulations (S_1 to S_3). Further, the cholesterol contents of the Egg PC liposomes has shown to influence the liposome integrity and drug release in plasma within the first 4 h following administration.

As mentioned earlier, animal study revealed a significant improvement in the kinetic behavior of cisplatin entrapped within HSPC liposomes (S_4) compared to their EggPC counterparts. Considering that all the liposomal formulations had a particle size of less than 200 nm and negative surface charge, the stability of these formulations in the bloodstream seems to be

due to the higher Tm of HSPC [36]. We have previously demonstrated that the low Tm lipids could reduce the bilayer rigidity and improve the release kinetics of the entrapped drug [80]. *In vivo* biodistribution study also indicated a significant difference between tumor accumulation of EggPC and HSPC liposomes. While S_4 showed the highest tumor drug accumulation, it significantly reduced drug distribution to the kidney, as the main target of CDDP. Further, S_4 exhibited the greatest efficacy in decreasing the tumor size, consistent with the plasma concentration of drug. On the whole, HSPC formulation with DDA moiety (S_4) significantly improved CDDP entrapment efficiency and effectively improved the values of TTE and MST survival factors in animals. Histopathology study also indicated severe necrosis and degeneration of the liver and kidney with the free drug, while the lesions in the liposome-treated animals was less severe.

Conclusion

Complex nanomaterials have extensively been explored to develop smart nanomedicine however, there are concerns about the detrimental toxicity issues associated with each and every added moieties to confer multi-functionality. In the current study, a novel CDDP liposome was developed which exerts dual roles through improving the drug loading efficiency as well as enhancing the drug release. We found that this liposome is capable of preserving the drug bioactivity on colon cancer cells. Further, the liposomal drug stability in blood circulation and accumulation within the tumor area highly depends on the phospholipid choice. The bi-functional liposomes of rigid bilayers reduced tumor burden and improved the animals' survival which makes it a promising nominee for the efficient therapy of varying solid tumors. Future investigations however, should focus on the optimization of the release behavior in a way to balance circulation stability and tumor site triggered release properties.

Figure captions

Figure 1. *In vitro* cytotoxicity of CDDP-loaded liposomes against C26 (A, B, C) and NIH-3T3 (D, E, F) cells. The IC_{50} was calculated at 48h and 72 hours after exposure with MTT assay (A and B). The relative cytotoxicity of different concentrations of CDDP-loaded formulations on C26 (B,C) and NIH-3T3 (E,F) cells are shown following 48 and 72 h incubation. The data represented as the means ± SD (n = 3). Samples was compared with CDDP (one-way ANOVA).

Figure 2. Hemolysis assay of free and CDDP loaded liposomes (3.125–100 μg/ml) incubated for 2 h at 37 ◦C. Data are means ± SD (n = 3). Free liposomal formulations dilution was identical to CDDP liposomes (equivalent to 3.125–100 μg/ml CDDP concentration) (A). Concentration–time profile of CDDP in plasma following administration of free drug and liposomal formulations (3 mg/kg equivalent to CDDP). Data are means ± SD (n = 3) (B).

Figure 3. *In vivo* biodistribution of liposomal formulations and free CDDP at 24h in the heart, kidney, lung, liver, spleen and tumor in BALB/c mice bearing C26 tumor after a single dose of formulations administered i.v. (3 mg/kg of CDDP) after the tumor inoculation. Data are means ± SD (n = 3).

Figure 4. Photomicrographs of the tissues from BALB/c mice treated with liposomal formulations, PBS and free CDDP at 3 doses of (3 mg/kg of cisplatin) on days 0, 7 and 14. Mice were euthanized on day 21 and the liver, lung and kidney of each mouse were removed, washed and fixed in 10% (v/v) buffered formalin. Then, tissues were embedded in paraffin blocks, sectioned at 5 mm thick and placed onto glass slides. Hematoxylin eosin staining was performed

to prepare samples for observation using light microscopy (400 X, Scale bar is 100 μm). Data are means ± SD (n = 3).

Figure 5. *In vivo* anti-tumor efficacy of liposomal formulations, PBS and free CDDP in BALB/c mice. On day 9 of tumor inoculation, mice received free and liposomal CDDP, (3 mg/kg of CDDP) as well as PBS, via lateral tail vein once weekly for 3 weeks. (A) Tumor growth curves (B) Tumor inhibition rates of animals in each treated groups (S_1, S_2, S_3, S_4, S_7, CDDP and PBS) (C) Survival of animals treated (D) percentage change in animal body weight, Data represented as mean ± SD, (n = 4).

Table 1. Liposomes characterization

Formulations	HSPC/Egg PC/DOPE/ $mPEG_{2000}$-DSPE/Cholesterol/DDA	Z-Average (nm±SD)	PDI[2]	ZP[3] (mV)	EE[4]%
S_1	0/55/37.5/5/0/2.5%	122/1±3/81	136±0/004	/0±0/65 -12	3/376
S_2	0/55/27.5/5/10/2.5%	116/1±3/56	/256±0/010	/9±0/05 -14	5/743
S_3	0/55/17.5/5/20/2.5%	91/24±2/14	246±0/008	/0±0/03 -13	8/853
S_4	55/0/37.5/5/0/2.5%	102/4±1/88	241±0/007	/3±0/16 -10	3/204
S_5	55/0/27.5/5/10/2.5%	±3/78 159/7	±0/004 0/253	/4±1/03 -10	Drug precipitate
S_6	55/0/17.5/5/20/2.5%	±2/68 150/9	±0/008 0/184	22.±1.2 -6	Drug precipitate
S_7	55/0/17.5/5/22.5/0%	94/6±3/06	218±0/005	3±0/09 -9/5	1/485

1. Standard deviation
2. Poly dispersity index
3. Zeta potential
4. Encapsulation efficiency

Table 2. Pharmacokinetic parameters of CDDP following i.v. administration of liposomal formulations and free drug (3 mg/kg)

Parameters	CDDP	S_1	S_2	S_3	S_4	S_7
AUC[1] (µg/ml*h)l	110.50	122.40	105.20	101.56	238.78	158.20
MRT[2] (h)	264.45	126.75	120.59	69.42	133.29	206.19
Cl[3] (mg)/(µg/ml)/h	0.0082	0.0128	0.0156	0.0214	0.0062	0.0069
$T_{1/2}$ [4](h)	183.66	90.18	87.57	50.53	91.76	141.80

1. Total area under the plasma concentration-time curve
2. Mean residence time
3. Clearance
4. Terminal half-life

Table 3. Therapeutic efficacy data of free CDDP, liposomal formulations in BALB/c mice bearing C26 tumor

Treatments	TTE (Days±SD)	TGD	MST (days)	ILS (%)
PBS	24.0±2.1	-	23.5	-
CDDP	26.7±1.7	11.25	26.48	12.68
S1	29.64±5.9	23.49	31.58	34.38
S2	26.67±1.9	11.13	26.36	12.17
S3	31.57±2.0	31.54	31.61	34.51
S4	34.52±1.9	43.83	34.77	47.95
S7	31.35±4.3	30.63	30.18	28.42

Figure 1.

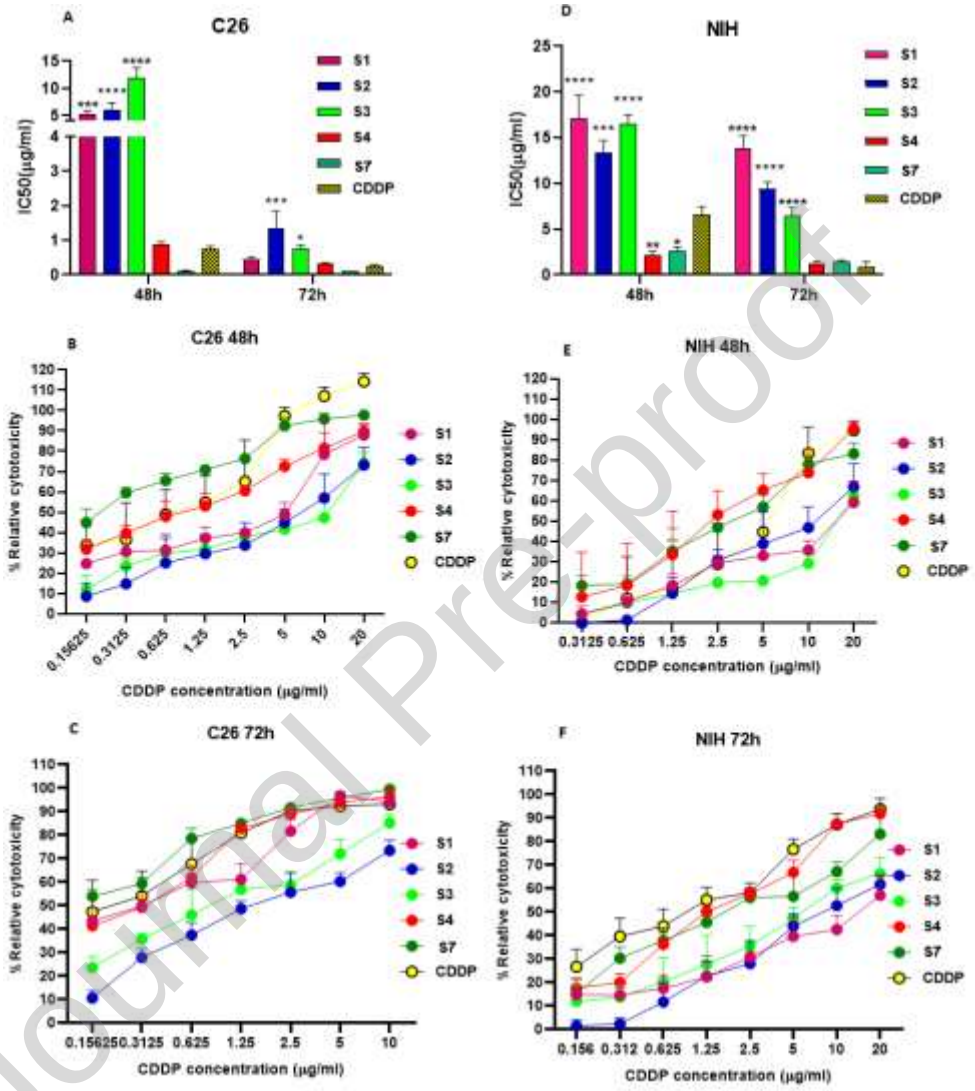


Figure 2.

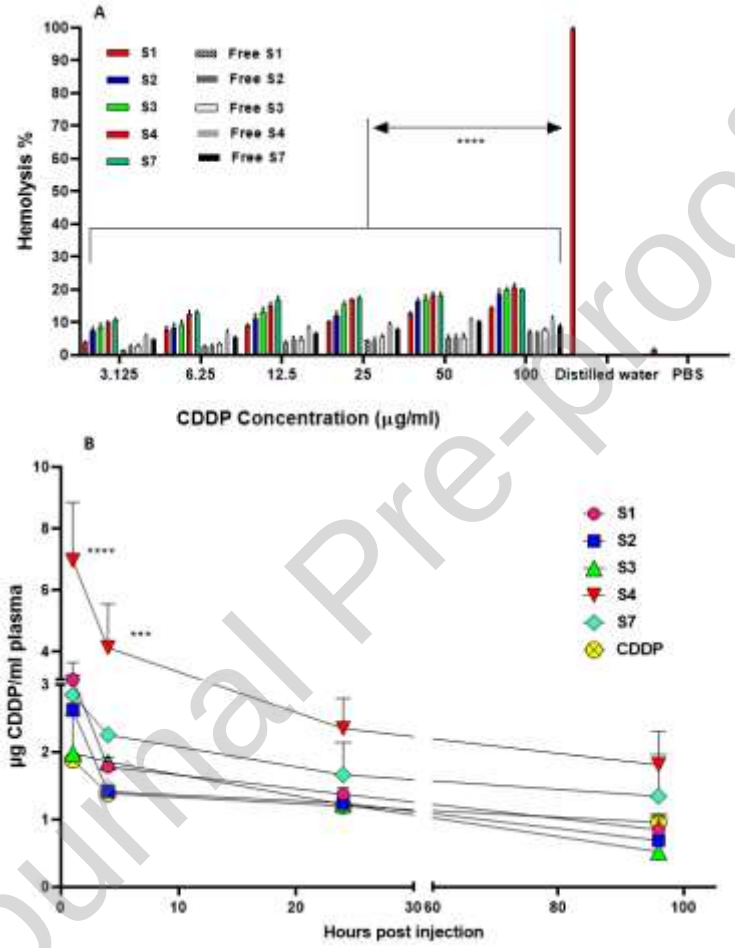


Figure 3.

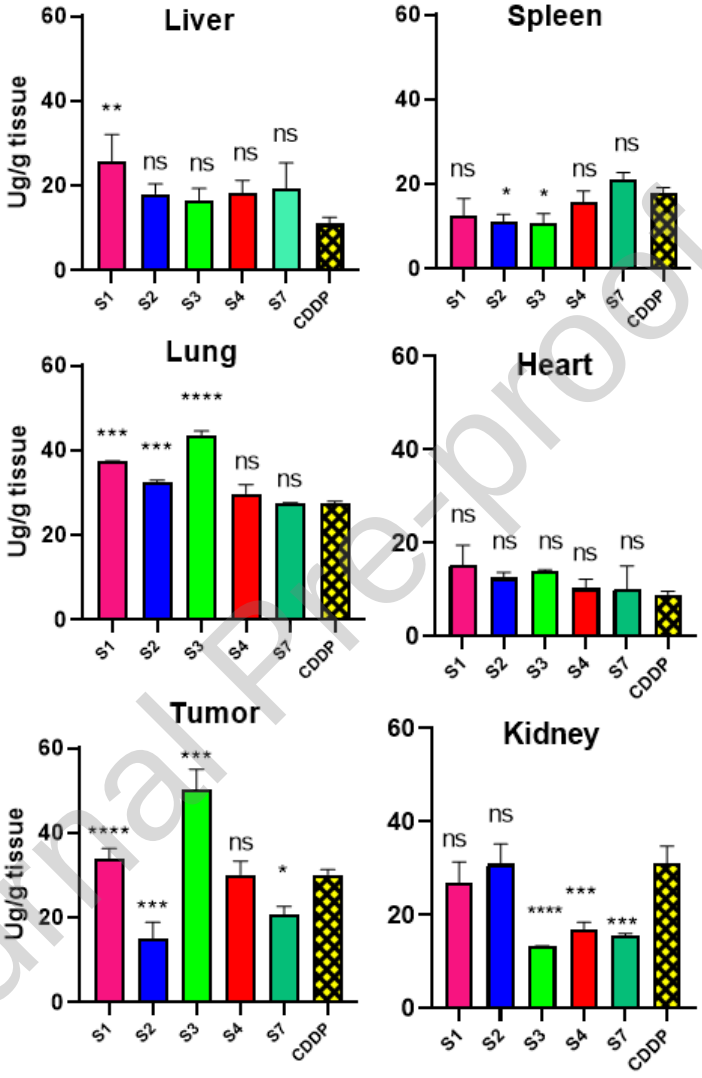


Figure 4.

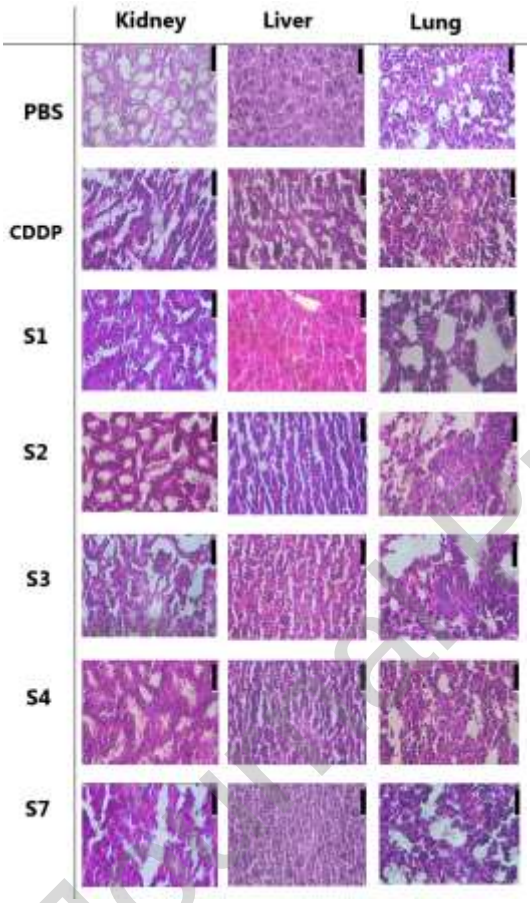
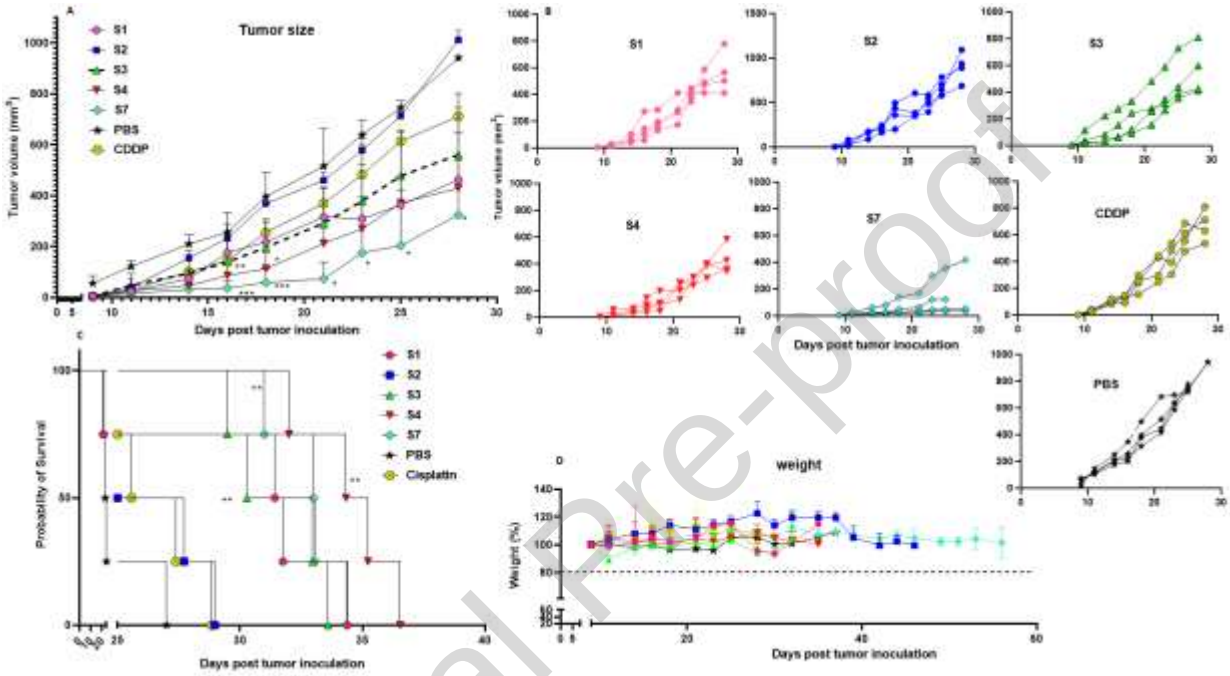


Figure 5.



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Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Highlights

- PEGylated liposomes are promising drug delivery carriers for cancer therapy
- Organoselenium compound (DDA) could improve cisplatin loading within the liposomes
- DDA liposomes significantly improved cisplatin residence time in circulation
- DDA liposomes enhanced cisplatin accumulation within the oxidative tumor area
- Kidney toxicity reduced following treatment with DDA liposomes bearing cisplatin