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Enhanced Anti-tumor Efficacy of Lipid-Modified Platinum Derivatives in Combination with *Survivin* Silencing siRNA in Resistant Non-Small Cell Lung Cancer

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Abstract

Purpose—Cisplatin, is recognized as a first line therapeutic for the treatment of non-small cell lung cancer (NSCLC). Cisplatin resistance is identified as the most detrimental complication during treatment and has been associated with upregulation of several genes, such as the anti-apoptotic gene *survivin*. In this study, we have evaluated the cytotoxic activity of lipid (C6 and C8)-modified platinum compounds in combination with a *survivin*-silencing siRNA against cisplatin resistant tumors.

Methods—We synthesized and characterized several lipid-modified platinum compounds and evaluated their cytotoxic activity alone or in combination with *survivin*-silencing siRNA *in vitro* and *in vivo* against A549^{DDP} cells and *in vivo* in tumor xenograft model.

Results—The lipid-modified compounds exhibited significantly stronger cytotoxic activity *in vitro* compared to cisplatin, with CDDP-C6 and CDDP-C8 producing the most pronounced effect, in both A549 and A549^{DDP} cells. Pre-treatment of the A549^{DDP} cells with *survivin*-silencing siRNA enhanced the cytotoxic activity of these compounds. *In vivo*, the co-treatment of the *survivin*-silencing siRNA and CDDP-C8 produced the strongest tumor growth inhibition effect (64.5%, $p < 0.05$) on a cancer mouse model of chemoresistant lung cancer. In contrast, cisplatin treatment exhibited no significant tumor growth inhibition (4.5%, no p).

Conclusion—Co-treatment of lipid-modified compounds and *survivin*-silencing siRNA can constitute a reliable alternative to cisplatin treatment for cisplatin-resistant lung tumors that merit further evaluation.

Keywords

Non-small cell lung cancer; lipid-modified platinates; *survivin*; small interfering RNA; chemoresistance

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1. INTRODUCTION

Lung cancer is the leading cause of cancer related deaths in the United States of America, with a 5-year survival rate of <20% (1). Currently, our front line therapy includes platinum derivatives, such as cisplatin and carboplatin, but their use has been associated with significant side effects, such as nephrotoxicity, cardiotoxicity, nausea and neurotoxicity (2, 3). Furthermore, the most detrimental complication for platinum-based therapies is the development of chemoresistance from the tumor cells. This results in reduction of the drug's effectiveness and in significant disease relapse, characterized by a more aggressive outlook (4). Interestingly, the cisplatin resistant tumors demonstrate resistance to other subsequent drugs that may have different structure or mode of action, with this phenomenon described as multi-drug resistance (4).

The development of resistance by the tumors cells has been associated with epigenetic changes in the tumor cells that result in alterations in the expression of several proteins, surface receptors, microRNAs and other (5). In our previous work using A549^{DDP} cells, the cisplatin resistant phenotype of the A549 lung epithelial carcinoma cell line, we determined that the acquired chemoresistance was associated with transcriptional upregulation of a number of genes, such as survivin, BCL2 and *mdr1* (6).

RNA interference (RNAi) has emerged as a valuable tool for better comprehending mechanistic intracellular functions by selectively silencing specific gene expressions. Short interfering RNAs, with the assistance of several proteins participating in the RNA interference silence complex (RISC), identify specific mRNAs, on which they attach leading to cleavage and subsequent degradation of mRNAs, thus downregulating the respective protein expression (7). A number of researcher attempt to use RNAi to complement chemotherapy in order to enhance its therapeutic potential, overcome chemoresistance and inhibit metastasis (8–13). In this respect, we previously developed siRNAs specifically targeting the survivin and BCL2 mRNAs. Using these siRNAs, a significant reduction in the levels of the corresponding mRNAs was observed *in vitro*. This effect was accompanied with enhancing the cytotoxic activity of cisplatin against the chemoresistant lung cancer cells, suggesting a potential approach for reversing or inhibiting chemoresistance (6). We further demonstrated the potential of this approach in an subcutaneous animal model of lung cancer, with the co-treatment of the survivin siRNA enhancing the therapeutic outcome of cisplatin treatment (6).

Alternative to the platinum (Pt(II))-based molecule of cisplatin, Pt(IV)-based molecules have attracted significant attention, due to their ability to be *in-situ* reduced to the active Pt(II) inside the cell, and target the DNA (14). Pt(IV) provides ease in its chemical modification and a large number of such derivatives have been produced (15). On our previous work, we chemically modified Pt(IV) molecules by attaching fatty acids of different chain-lengths, making products of high purity and increased lipophilicity. We have previously evaluated some of these compounds *in vitro* and *in vivo* against wild-type (A549) and cisplatin resistant (A549^{DDP}) non-small cell lung cancer cells using a chitosan based drug delivery system and we determined that the lipid-modified platinum derivatives demonstrate superior cytotoxic activity relative to cisplatin in both wild-type and resistant cells (16).

In this study, we present some Pt(IV) molecules modified with fatty acids and report their efficacy in wild-type (wt) and resistant A549 cells, alone or in combination with the *survivin*-silencing siRNA, against the wild type and chemoresistant A549 lung cancer cells. As we demonstrate, the lipid-modified platinum derivatives exhibit strong antitumor activity against A549 lung cancer cells. Furthermore, their cytotoxic activity was significantly higher as compared to their parent compound, cisplatin, in both the wild-type A549 and the cisplatin-resistant A549^{DDP} cells and was enhanced by pretreatment of the cells with siRNA against the pro-apoptotic gene *survivin*. Finally, the lipid-modified platinum derivatives exhibited strong antitumor activity *in vivo*, at relatively low doses, which was significantly augmented by co-treatment with the *survivin*-silencing siRNA. In more detail, the compound CDDP-C8 demonstrated the strongest tumor growth inhibition *in vivo* when co-treatment of the *survivin*-silencing siRNA was included (64.5%, $p < 0.05$).

2. MATERIALS AND METHODS

2.1 Materials

Cis-diammine-dichloro-platinum (cisplatin or CDDP) was purchased from Sigma-Aldrich Inc. (St. Louis, MO). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), butanoic (C4) anhydride, hexanoic (C6) anhydride, octanoic (C8) anhydride, and decanoic (C10) anhydride were purchased from Thermo Fisher Scientific (Waltham, MA). Highly purified pharmaceutical grade sodium hyaluronate of average molecular weight of 20 kDa was purchased from Lifecore Biomedical (Chaska, MN). Branched poly(ethyleneimine) (bPEI, 10,000 Da) was obtained from Polysciences Inc. (Warrington, PA). Dulbecco's modified Eagle medium (DMEM) was purchased from CellGro (Manassas, VA) and fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Norcross, GA).

2.2 Synthesis and Characterization of Lipid-Modified Platinum Derivatives

The overall experimental approach for the synthesis of the lipid (C4 to C10)-modified platinum molecules is presented in Scheme 1, and was according to previously established protocols (16, 17). Briefly, we prepared an aqueous solution of cisplatin (**1**) and hydrogen peroxide and heated this mixture at 80°C for 4h under reflux in the dark. Subsequently, we concentrated this solution by rotary evaporation and the mixture was placed at 4°C overnight. The product was collected by vacuum filtration, washed with ice-cold water, ethanol, and diethyl ether, and vacuum-dried to yield a bright yellow powder of c,c,t - Pt(NH₃)₂Cl₂(OH)₂ (**2**).

To further modify the derivative (**2**), we dissolved the compound in DMF, followed by the addition of the respective C4 to C10 lipid anhydride and the solution was heated to 50 °C while stirring. The conjugation reaction was allowed to take place overnight, in dark conditions and a nitrogen atmosphere. The DMF was evaporated to yield a concentrated solution of the products and was added drop-wise in 40 ml of diethyl ether under intensive stirring, yielding precipitates of (**3**). The precipitates, purified lipid-modified platinum derivatives, were collected through filtration, washed with diethyl ether and dried under

vacuum. Each of the products was analyzed by ^1H -NMR, HPLC, LC-MS and ICP-MS to determine structure and purity.

2.3 Synthesis, Preparation and Characterization of HA-PEI/HA-PEG/siRNA Nanoparticles

As a transfection system, we synthesized HA-PEI and HA-PEG complexes using a EDC/NHS coupling reaction between HA and PEI or PEG, as previously described (18). Briefly, we dissolved 100 mg of HA and 15 mg of PEI in 10 ml of distilled water containing sodium chloride (0.5 M). A solution containing EDC (35 mg) and NHS (22 mg) dissolved in distilled water was then added to the polymer mixture and the reaction was allowed to take place overnight under mild stirring. The final product was obtained after dialysis (MWCO 12–14k) with consecutive solutions of sodium chloride (0.5 M), followed by distilled water. Similar protocol was followed for the synthesis of HA-PEG, but without the use of sodium chloride in the respective aforementioned steps. Lyophilized samples of HA-PEI and HA-PEG complexes dissolved in D_2O with and without Sodium chloride, respectively, were characterized by 500 MHz ^1H -NMR spectroscopy (Varian Inc., CA).

HA-PEI/ HA-PEG/siRNA complexes were prepared by mixing the HA-PEI and HA-PEG with *survivin*-silencing siRNA at a previously established weight ratio, followed by intense mixing and incubation for 10 min at room temperature (19).

2.4 Wild-type and Resistant NSCLC Cell Lines

Human non-small-cell lung cancer (NSCLC) wild-type cell line A549 was obtained from ATCC (Manassas, VA), and the corresponding platinum-resistant cell line A549^{DDP} was obtained from Massachusetts General Hospital (Boston, MA). Cells were cultured in a humidified environment at 37 °C and 5% CO_2 , in the presence of DMEM/F12 medium from Life Technologies (Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (100 U/mL, Thermo Fisher Scientific, Waltham, MA). A549^{DDP} cells were cultured in medium supplemented with cisplatin at a final concentration of 2 $\mu\text{g/mL}$, to maintain drug resistance.

2.5 In Vitro Evaluation of Cellular Uptake

For the uptake studies, we chemically modified the HA-PEI polymer by attaching a fluorescent probe, Sulfo-Cyanine-5 (Cy5) (Lumiprobe, Life Sciences, FL). Briefly, 20 mg of HA-PEI were dissolved in phosphate buffer (pH 7.4) and 1mg of Sulfo-Cyanine5-NHS ester was added to the solution. The reaction was allowed to take place overnight and the final product was washed by dialysis (MWCO 12–14k) with 1 M sodium chloride for 6 hours and distilled-water overnight. The purified product was used for creating the HA-PEI-Cy5/HA-PEG/siRNA complex, as described above.

The wild-type A549 and cisplatin resistant A549^{DDP} cells were seeded at a density of 5×10^4 cells/well in 8-chambered glass slides. On the following day, the HA-PEI-Cy5/HA-PEG/siRNA complex was prepared, as described above, and the samples were placed for transfection into the cell chambers for predetermined incubation periods (0.5, 1, 2, 4, 6h) at an siRNA concentration of 10 nM. At the end of the incubation period, the transfection solution was gently removed from the respective chamber and the cells were washed twice

with PBS and were fixed with formalin for 15 minutes. Subsequently, the chamber structure was removed and mounting media containing DAPI (0.01% w/v) was applied on top of the cell samples, followed by a glass cover slip. The cells were visualized using a Zeiss confocal microscope (LSM 700, Germany) at 650 nm excitation and capturing emission above 670 nm for Cy5 and at 360nm excitation and 460 nm emission for DAPI.

2.6 *In Vitro* Evaluations of Cytotoxicity, Transfection, and Combination Therapy in NSCLC Cell Lines

For the transfection studies, A549^{DDP} cells were seeded at a density of 10^5 cells/well in a 6-well tissue culture plate. HA-PEI/HA-PEG/siRNA against survivin complexes were prepared as described above and were used for transfecting the cells. The targeted concentration of 10 nM of siRNA and the transfection protocol were according to previously published work from our group on the same siRNA (20). For comparison, cells were treated with naked siRNA, scramble siRNA complexed with HA-PEI/HA-PEG and polymers alone. As positive control, we used lipofectamine RNAimax (Invitrogen, Carlsbad, CA), following the manufacturer's instructions.

At 24, 48 and 72 h post transfection, cells were harvested, washed thrice with PBS and stored at -80°C until further analysis. We isolated RNA from the cell samples using the Quick-RNA mini-prep kit (Zymo Research, CA) and synthesized cDNA by reverse transcription using the verso cDNA synthesis kit (Thermo Scientific, Cambridge, MA). The expression of survivin in each sample was quantified through qPCR using the LightCycler 480 SYBR green I master kit (Roche) and custom made primers, according to manufacturer's protocol. B-actin was used as a housekeeping gene. Relative expression to the control samples was quantified using the LightCycler 480 PCR system.

The cytotoxicity of cisplatin and lipid-modified platinum derivatives, alone or in combination with siRNA against survivin was evaluated 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay, where the clear tetrazole dye is converted to colored formazan crystals in viable cells. Briefly, A549 or A549^{DDP} cells were seeded at a density of 10,000 cells/well in 96-well tissue culture plates. Following the same transfection conditions as above, cells were incubated in the presence of the HA-PEI/HA-PEG/siRNA against survivin complexes. 24 h post transfection initiation, solutions of cisplatin or lipid-modified platinum derivatives were added at different concentration ranging between 0.1–1000 μM for each compound. The cells were incubated in the presence of the compounds for 24, 48 and 72h, and at the end of the respective incubation periods, a solution of MTT was added to each well. After 3 hour of incubation, the formazan crystals in each well were dissolved and the absorption from each concentration group was used to determine the drug concentrations that induced 50% cell death (IC_{50}) for each compound in comparison to control samples.

2.7 Pharmacokinetic Analysis of Lipid-Modified Platinum Derivatives in Naïve Mice

A single dose of CDDP-C6 and CDDP-C8 at equimolar dose (1.76 mg/kg and 1.95 mg/kg respectively) was injected to mice (n=3/time point) by intravenous injection. We chose these two compounds for any further analysis, due to the higher cytotoxic activity, as

demonstrated by the *in vitro* data in both the A549 and A549^{DDP} cells. Due to the lipophilic nature of the platinum derivatives, the compounds were solubilized using the solvent displacement method in a surfactant solution. Briefly, 2 mg of each compound were dissolved in 1 ml of acetone, and the solution was added drop-wise into 4 ml of 10% Tween-80 in PBS. The procedure took place inside a bath sonicator and under intensive stirring. Subsequently, the acetone was evaporated from the solution using low pressure. After treatment, the animals were sacrificed at designated time points, and tissue and blood samples were harvested, and snap-frozen in liquid nitrogen. The platinum was extracted by incubation in concentrated nitric acid and its concentration for each tissue was determined using inductively coupled plasma mass spectroscopy (ICP-MS) (Agilent 7500cx). Pharmacokinetic parameters were determined with PK solver, a Microsoft Excel Plug-in. For the blood samples analysis, a two-compartment intravascular model provided the best fitting, with the drug clearance demonstrating a biphasic profile. The derived parameters are: area-under-the-curve between 0 and 24 hours ((AUC)_{0-24h}); distribution half-life ($t_{1/2\alpha}$); elimination half-life ($t_{1/2\beta}$); and total body clearance (CL).

2.8 *In Vivo* Antitumor Efficacy of Platinum Derivatives in Combination with *Survivin* Gene Silencing siRNA

To evaluate the anti-tumor activity of the novel platinum derivatives, we used a subcutaneous tumor mouse model of cisplatin-resistant lung cancer. We injected A549^{DDP} in both flanks on the back of female nude mice (4–6 weeks old, n=3–4/ per group, Charles River, MA). Following the tumor engraftment (tumors per group: 4–8) and when tumors reached an average volume of 100–150 mm³, the animals were divided in the following treatment groups: 1) Untreated; 2) CDDP (1 mg/kg); 3) CDDP-C6 (1.76 mg/kg); 4) CDDP-C8 (1.95 mg/kg); 5) siRNA + CDDP-C6, and; 6) siRNA + CDDP-C8. All dosages for the platinum compounds/cisplatin represent equimolar amounts. The siRNA against *survivin* was administered to the animals at a dose of 0.5 mg/kg, where applicable. The weekly treatment administration was of 3 doses of HA-PEI/HA-PEG/siRNA against survivin complexes, at two day intervals between each injection, with a subsequent injection of the respective platinum compound on the following day after the last siRNA treatment. The administered cisplatin samples were prepared in PBS, while the lipid-modified platinum derivatives were solubilized in 10% tween-80 in PBS, as described above. Throughout the treatment period, tumor growth was monitored twice per week. The health of the animals was regularly monitored and their body weight was recorded for determining signs of toxicity. At the completion of the third week, animals were sacrificed. Tumors, blood and liver were harvested from each animal and tumor weight was determined.

The tumor samples were analyzed for the levels of siRNA presence, by homogenizing them using a tissue homogenizer (Fisher Scientific) in the presence of Lysis buffer to prepare tissue lysates, as previously described (19). The primers used for the analysis were:

Survivin siRNA: Sense:

5'GGmCGmUAAGAmUGAmUGGAmUmUmUmUmU3'

Antisense: 5'AAAUCmCAUmCAUCUmUACGCCmUmU3'

Primers Reverse: GGAAGCCGACAAGGCGTAA

Forward: /56-FAM/ACTCCCTCCCTCGATTT AAATCCATCATCT

Anti-primer: AAATCGAGGGAGGGAG/3BHQ_1/

One step qPCR reaction protocol was followed using the AgPath-ID One step RT-PCR kit (ThermoScientific), as previously described (19). The siRNA levels were quantitated by using a standard curve of known siRNA concentration spiked in untreated mouse tissue.

Liver tissue samples were used for histological evaluation, by fixing them in formalin, followed by a standard paraffinization, sectioning and Hematoxylin/Eosin (H&E) staining procedure. ALT/AST liver enzymes in the blood were determined for identifying signs of liver toxicity, using the AST or ALT detection kits from Pointe Scientific, Inc. (Canton, MI) according to manufacturer's protocol. Animal body weight was monitored throughout the studied period.

2.9 Statistical Data Analysis

Statistical analysis was performed using Microsoft Excel t-test to determine any significant difference between treatment groups. The two-tailed unpaired t-test was used to compare the mean values $\pm$ standard errors; p values < 0.05 were considered statistically significant.

3. RESULTS

3.1 Synthesis and Characterization of Lipid-Modified Pt(IV) Derivatives, HA-PEI, HA-PEG Conjugates, and siRNA Complexes

Pt(IV) molecules were synthesized with cisplatin as the starting material. Chemical attachment of the carboxylic acids from the corresponding aliphatic anhydrides onto the Pt(IV) substrate in DMF yielded clear solution of the final product. The subsequent purification in diethyl ether resulted in products of high purity as determined by HPLC (Figure 1), LC-MS and $^1\text{H-NMR}$ (Supplementary Figures). In detail, analysis of each of the molecules through HPLC (Figure 1) and LC-MS demonstrated a single peak in the chromatograms, with the corresponding peak for each compound being delayed in correlation to the increasing lipophilic chain length/lipophilicity of the corresponding aliphatic acid attached onto the Pt(IV) molecules. It is important to note that the HPLC absorption peaks originate to UV detectable products, which in our case originate from the cisplatin core of each molecule. Mass spectrometry demonstrated highly pure products (supplementary figures), with the desired molecular weights constituting the majority of the relative abundance. $\text{cis-Pt}(\text{NH}_3)_2\text{Cl}_2$ or $\text{cis,cis,trans-[Pt}(\text{NH}_3)_2\text{Cl}_2(\text{OH})_2]$ were not detected in any of the final products. Inductively couple CP-MS confirmed the existence of Pt molecules in the samples (data not shown). Our analysis with $^1\text{H-NMR}$ produced peaks with chemical shifts that correspond to the appropriate groups, as previously described (16, 17). More importantly, the spectra demonstrate high-purity products with no by-products or reagents present.

To formulate the polymer-siRNA complexes, HA-PEI and HA-PEG were mixed in a 1:1 weight ratio and *survivin-silencing* siRNA was added to the mixture, to a final weight ratio of 27:27:1 for HA-PEI:HA-PEG:siRNA, as was previously identified to be the optimal ratio (19, 20). The mixture of the siRNA and HA-PEI/HA-PEG spontaneously formed nanosized

vectors due to electrostatic interaction between the positively charged PEI and the negatively charged nucleic acids, which were analyzed using a Malvern Instrument's Zetasizer Nano-ZS instrument. We determined their hydrodynamic diameter to be 202 ± 76.2 nm. The surface charge (zeta potential) of the nanoparticles was determined at -34.7 ± 5 mV.

3.2 Cytotoxic Activity of the Lipid-Modified Platinum Compounds in Both A549 and Cisplatin-Resistant A549^{DDP} Cells

We evaluated the cytotoxic activity of the lipid-modified platinum compounds through a standard MTT assay. To this end, we used A549 and, their cisplatin-resistant phenotype counterpart, A549^{DDP} to evaluate the activity of the compounds. As illustrated in Figure 2 and Table 1, the lipid-modified platinum derivatives demonstrated enhanced cytotoxic activity in both cells lines. Among the different compounds, CDDP-C6 and CDDP-C8 demonstrated the strongest cytotoxic activity, as determined by their respective IC₅₀ values, at approximately 0.02 μ M for CDDP-C6 and 0.05 μ M for CDDP-C8 vs. 7.54 μ M for cisplatin, against the A549 cells at the 48h time point. These two compounds also demonstrated potent cytotoxic activity against the cisplatin-resistant A549^{DDP} cells, with IC₅₀ values of 6.05 μ M for CDDP-C6 and 3.32 μ M for CDDP-C8 vs. 66.8 μ M for cisplatin, at the 48 h time point. It is of interest that the demonstrated cytotoxic activity of the two mentioned compounds against the A549^{DDP} was stronger than the activity of the parent compound cisplatin in the non-resistant A549 cells.

3.3 *In Vitro* Uptake of the Complexes by the Tumor Cells and Combinatorial Treatment of Survivin Silencing siRNA and Lipid-Modified Platinum Compounds

Cellular uptake of the polymer with siRNA was determined in A549 and A549^{DDP} cells by confocal microscopy. Bright red signal (Cy5) originating from the modified HA-PEI with sulfo-Cy5 was observed inside both cell lines as early as 2 h after initiating the incubation (Figure 3), with the intensity of the intracellular fluorescence to increase in a time dependent manner, indicating that HA-PEI/HA-PEG/siRNA complexes were effectively internalized.

The successful transfection of the system was also supported after analyzing the survivin mRNA levels by qPCR from cell samples that were incubated with the *survivin*-silencing complex as a function of time. The HA-PEI/HA-PEG/siRNA complex successfully downregulated the expression of survivin (Figure 4), as previously reported (20). The transfection caused a reduction of the survivin mRNA levels by 50.2% at the 24h, by 42.8% at the 48h, while the mRNA levels were normalized by the 72 h time point. In contrast, Lipofectamine, which served as our positive control, produced a stronger effect reducing the survivin mRNA levels by 74.5% at the 24 h, 73.4% at the 48 h, and 45.1% at the 72 h. Scramble RNA, naked RNA and polymers alone were used for comparison and did not demonstrated significant changes in the expression levels of survivin (data not shown).

From the cytotoxicity studies presented above, the lipid-modified compounds demonstrated significantly strong activity against both A549 and A549^{DDP} cells. In an attempt to further enhance the activity of the compounds, we evaluated whether prior treatment of the cisplatin-resistant A549^{DDP} cells with the *survivin*-silencing siRNA can sensitize them to the drug treatment. We transfected A549^{DDP} cells with the HA-PEI/HA-PEG/siRNA

complex at a concentration of 10 μ M, 24h prior to adding the platinum compounds. Subsequently after the drug incubation, we used a standard MTT assay to evaluate the cytotoxic activity of the compounds. Figure 2 and Table 1 show that prior treatment of the A549^{DDP} cells with the survivin- silencing siRNA significantly reduced the IC₅₀ values for the platinum compounds, enhancing their cytotoxic activity. Although the final IC₅₀ values for each compound did not reach their respective low values as observed with the non-resistant A549 cells, the transfection approximately halved the IC₅₀ values for a number of the platinum compounds, producing an IC₅₀ reduction of 49.1% for cisplatin and 49.5% for CDDP-C6, while had less pronounced effect for CDDP-c4, with 26.4% reduction, and for CDDP-c10 with 31.7% reduction. Interestingly, CDDP-C8 demonstrated the strongest enhancement in its cytotoxic activity following transfection, reaching approximately 78% reduction in its IC₅₀ value. The combinatorial treatment arguably enhanced the activity of the lipid-modified compounds, which prompted the subsequent inclusion of the combination in the *in vivo* studies.

3.4 Pharmacokinetic Analysis of Lipid-Modified Platinates in Naïve Mice

Prompted by the strong cytotoxic activity of CDDP-C6 and CDDP-C8, we evaluated their pharmacokinetic profile in nude mice after a single i.v. injection (1.76 mg/kg and 1.95 mg/kg respectively, equimolar, n=3/time point). Both drugs demonstrated a similar pharmacokinetic profile in the blood, characterized by a biphasic response, having a rapid distribution phase during the first 1h hour post injection, followed by a slower elimination phase (Figure 5). As a result, two half-lives were determined for each phase, with t_{1/2 α} of 0.18 h and 0.05 h, and t_{1/2 β} of 38.46 h and 25.45 h for CDDP-C6 and CDDP-C8, respectively. The AUC_{0–24h} for the two compounds was rather comparable, although for the more lipophilic CDDP-C8 was 28% lower (5713 vs. 4223, respectively). This could be associated with the consistently and lower presence of the CDDP-C6 in the liver, while CDDP-C8 appears to be more uptaken by the specific organ, demonstrating overall 6.4-fold higher presence as determined by their respective AUC_{0–24h} (CDDP-C6: 14.5; CDDP-C8: 90.23). In contrast, CDDP-C6 demonstrated slightly higher presences in the kidneys, being uptaken more than CDDP-C8 by 2-fold, as determined by their respective AUC_{0–24h} (CDDP-C6: 23.9, CDDP-C8: 15.9, Figure 5, Table 2).

3.5 *In Vivo* Combination Therapy with *Survivin*-Silencing siRNA and Lipid-Modified Platinum Compounds in Cisplatin-Resistant Models

In our previously studies, we established that the use of a *survivin*-silencing siRNA can enhance the anti-tumor activity of cisplatin against lung cancer *in vivo* (20). Similarly, we used an animal model of resistant lung cancer to evaluate the antitumor activity of the lipid-modified platinum compounds with and without the co-treatment of the *survivin*-silencing siRNA. A549^{DDP} cells were injected subcutaneously on both flanks on the back of female nude mice. When tumors reached an average size of 100–150 mm³, animals were divided into groups and treatment was initiated. For the tumor growth inhibition studies, we did a direct comparison of untreated animals and animals treated with cisplatin, CDDP-C6 or CDDP-C8, which were the two lipid-modified platinum compounds that demonstrated the strongest cytotoxic activity *in vitro*. We also included treatment groups, where animals were injected regularly with the HA-PEI/HA-PEG/siRNA against survivin complexes in

combination with the two lipid-modified compounds. The cisplatin was injected at a dose of 1 mg/kg and the lipid-modified platinum compounds were injected at the equimolar dose of 1.76 mg/kg for CDDP-C6 and 1.95 mg/kg for CDDP-C8.

Figure 6 shows that in the cisplatin-resistant tumor model, the lipid modified compounds demonstrated a relatively enhanced antitumor activity with tumor growth inhibition of 31% and 15% for CDDP-C6 and CDDP-C8, respectively, vs 4% for cisplatin alone (no statistical significance reached). The combinatorial treatment with *survivin*-silencing siRNA resulted in enhancing the activity of the lipid-modified compounds, with CDDP-C6 reaching 46.7% ($p=0.09$) and CDDP-C8 reaching 64.5% ($p<0.05$) tumor growth inhibition, respectively. The enhanced activity of the two compounds is also evident by the reduction of the tumor doubling time, as presented in Table 3. In detail, the untreated group demonstrated a doubling time of 6.44 days, followed closely by cisplatin with doubling time of 6.52 days. The lipid-modified compounds demonstrated 6.63 days for CDDP-C6 and 6.4 days for CDDP-C8 (no statistical significance reached). In contrast, groups treated with the combination of *survivin-silencing* siRNA, exhibited a slower tumor double time, with 7 days for CDDP-C6 (no p significance) and 10.2 days for CDDP-C8 ($p<0.05$).

We attempted to determine the levels of siRNA in the tumor samples that were collected at the end of the experiment, which was 48h post the last injection of the siRNA. For the majority of the animals, the levels detected were below the detection limit, without reaching statistical significance for any of the groups (data not shown). Additionally, we determined the levels of the ALT and AST enzymes in the blood from the animals participating in the tumor efficacy studies, to detect any signs of liver toxicity resulting from the treatments. Although we detected slightly elevated levels of the ALT enzyme in the blood from animals treated with CDDP-C8, no statistical significance was observed for any of the groups when compared to the control animals. No difference in the blood levels of AST or significant body weight changes were detected, indicating for signs of strong toxicity (supplementary figures). The histopathological evaluation of the liver tissues from the untreated and treated animals did not reveal any differences among the groups.

4. DISCUSSION

Platinum compounds are regarded our first-line treatment against lung cancer and our most potent approach for effective controlling tumor growth (2, 3). Although their significant advantages, it has become evident that cisplatin treatment, the most commonly used platinum compound, is associated with significant hurdles, such as nephrotoxicity, cardio toxicity, low solubility in many solvents, and most importantly, the development of chemoresistance (2, 3). Several approaches have been utilized to overcome these limitations, such as combinatorial treatment of cisplatin with other compounds, use of novel drug delivery approaches and chemical modification of cisplatin to produce new derivatives, such as oxaliplatin (21).

In our previous study, we compared the expression levels of key resistant-associated genes in A549 and A549^{DDP} cells, with the latter being the cisplatin-resistant phenotype of the former (20). We showed that cisplatin-induced resistance is associated with differential

expression in a number of genes, with upregulation of anti-apoptotic genes *survivin* and *BCL-2* as being the most pronounced. We developed a custom siRNA against surviving and treated the A549^{DDP} cells, resulting in sensitizing the cells to the cisplatin treatment, partially reversing the observed resistance.

In the present study, we synthesized lipid-modified platinum compounds to evaluate their activity *in vitro* and *in vivo* alone or in combination with the *survivin-silencing* siRNA. As we show above, the Pt(IV) compounds were synthesized from cisplatin, generating products with high purity as determined by HPLC, LC-MS, ICP-MS and NMR. Furthermore, these compounds demonstrated enhanced cytotoxic activity, when compared to cisplatin, in both A549 and A549^{DDP} cells. In more detail and focusing primarily on the A549^{DDP}, the chemoresistant phenotype of the lung cancer cells, we observed a 3.6-fold reduction for CDDP-c4, 11-fold reduction for CDDP-C6, 20.1-fold reduction for CDDP-C8 and 9.6-fold reduction for CDDP-c10 in their respective IC₅₀ values, when compared to the cisplatin treatment for the 48h incubation period. In addition, further enhancement of the cytotoxic activity of these compounds was demonstrated with prior treatment of the A549^{DDP} cells with the *survivin*-silencing siRNA, when compared to the non-transfected cells. It is of interest that transfection with the siRNA produced a more pronounced effect for CDDP-C8, with a 4.5-fold reduction of its IC₅₀ value in the A549^{DDP} cells compared to non-transfected cells. The other compounds demonstrated a smaller and similar among them effect, with a 1.3-fold reduction for CDDP-C4, 1.9-fold reduction for CDDP-C6 and 1.46-fold reduction for CDDP-C10 in their respective IC₅₀ values, when compared to non-transfected cells.

Prompted by these results, we chose two of the lipid-modified platinum compounds with the strongest cytotoxic activity *in vitro*, CDDP-C6 and CDDP-C8, to evaluate their ability to inhibit tumor growth *in vivo* alone or in combination with *survivin*-silencing siRNA. We compared their effect with cisplatin and untreated animals. We established a sub-cutaneous mouse model of resistant lung cancer, by injecting the A549^{DDP} cells in the back of nude mice. It is important to point out that the cisplatin dose used was 1 mg/kg, which is a relatively low dose as compared to the doses commonly used in other studies (e.g., up to 5–10 mg/kg), and the lipid-modified compounds were administered in equimolar doses. The siRNA was administered at a dose of 0.5 mg/kg, where applicable. Both lipid-modified compounds inhibited tumor growth more potently than cisplatin, although statistical significance was not reached. In contrast, co-treatment with the siRNA further enhanced their antitumor activity, with CDDP-C8 demonstrating the strongest effect, reaching approximately 65% of tumor growth inhibition (p<0.05). It is of interest, that without siRNA, CDDP-C6 had a slight stronger effect than CDDP-C8 in inhibiting tumor growth, the opposite of what was observed when the siRNA co-treatment was included. This may correlate with the *in vitro* data, where the CDDP-C8 showed the strongest enhancement in its cytotoxicity, when cells were co-treated with the *survivin*-silencing siRNA. Arguably, these data support the notion that the lipid-modified compounds present a strong alternative to cisplatin for resistant lung cancer and combinatorial treatment with the *survivin-silencing* siRNA can provide superior benefits in controlling tumor growth. In addition, the therapeutic benefits are also demonstrated by the increased doubling time for the

combinatorial treatment of siRNA and CDDP-C8 (6.4 for untreated animals versus 10.2 for siRNA+CDDP-C8, $p<0.05$).

Finally, all the treatments appeared to be safe, without any adverse effects as observed by the changes in animal body weight, plasma levels of liver enzymes (ALT and AST) and histopathological evaluation of the liver tissues (Figure 7).

5. CONCLUSIONS

In this study, we synthesized, characterized and evaluated lipid-modified platinum compounds for their antitumor activity *in vitro* and *in vivo*. The lipid-modified compounds demonstrated strong cytotoxic activity against A549 and their cisplatin-resistant counterparts, A549^{DDP}, which were more potent than cisplatin. Furthermore, a combinatorial treatment with siRNA against *survivin* sensitized the cisplatin-resistant A549^{DDP} cells against the platinum compounds. Subsequently, we evaluated the activity of two of the platinum compounds *in vivo* in a subcutaneous animal model of resistant lung cancer, and the compound CDDP-C8 demonstrated approximately 65% ($p<0.05$) tumor growth inhibition, when in combination with the *survivin*-silencing siRNA, vs. 4% tumor growth inhibition by cisplatin alone. These results justify further evaluation of these promising compounds for the treatment of chemo-resistant lung cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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LIST OF ABBREVIATIONS

ALT	Alanine transaminase
AST	Aspartate transaminase
CDDP	Cis-diammine-dichloro-platinum (or cisplatin)
DAPI	4',6-diamidino-2-phenylindole
DDP	Cisplatin resistant
DMF	Dimethylformamide
EDC	1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride
HA	Hyaluronic acid

HPLC	High pressure liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance spectroscopy
NSCLC	Non-small cell lung cancer
PEI	Poly(ethyleneimine)
PEG	Poly(ethylene glycol)
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNAi	RNA interference
siRNA	Small interfering
RNA Wt	Wild-type

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Figure 1. HPLC characterization and structure of lipid-modified compounds

a) The HPLC chromatograms of the platinum derivatives. Increasing lipophilicity due to the increasing length of the aliphatic chain causes for delayed retention time. No cisplatin or other UV detectable products were detected, b) the chemical structure of platinum (IV) derivatives

Figure 2. Cytotoxicity of the different platinum derivatives in A549 and A549^{DDP} cells with and without siRNA transfection

The cytotoxicity of the platinum derivatives was significantly higher when compared to cisplatin in both wild type and cisplatin resistant cells. The compounds CDDP-C6 and CDDP-C8 demonstrated the strongest cytotoxic activity among the compounds in both cell lines. Pretreatment of the cells with survivin-silencing siRNA significantly enhanced drugs' cytotoxicity.

Figure 3. Uptake studies of siRNA/HA-PEI-Cy5 nanocarriers in A549^{DDP} cells
Significant uptake of the complexes is observed after 2h of incubation, steadily increasing up to the 6h time point. Red corresponds to the Cy5 dye and blue to DAPI-stained nuclei.

Figure 4. Suppression of *survivin* expression in A549^{DDP} cells
Survivin mRNA levels were significantly reduced following *in vitro* transfection with 10 nM of survivin-silencing siRNA using HA-PEI or lipofectamine, as determined by qPCR. Negative controls included scramble siRNA and HA-PEI alone.

Figure 5. Pharmacokinetic profiles for CDDP-C6 and CDDP-C8

Animals were injected i.v. in equimolar concentration. Both drugs exhibited a rapid distribution phase, followed by a slower elimination phase in the blood.

Figure 6. Tumor growth inhibition study and tumor weights at the end of the experimental period

A rapid tumor growth was observed for the untreated groups. In contrast, the groups treated with the lipid-modified compounds demonstrated a relatively slower tumor growth inhibition, without reaching statistical significance. Cisplatin treatment had negligible benefits. The combination of *survivin*-silencing siRNA and CDDP-C8 exhibited the strongest effect in inhibiting tumor growth ($p < 0.05$), as observed by the tumor growth curves (left), and the tumor weights at the end of the experiment (right).

Figure 7. H&E staining of liver tissues from the different groups in the *in vivo* tumor growth studies

Hematoxylin and Eosin (H&E) stained liver tissues were observed for any acute toxicity of the formulations. Based on the analysis no differences were observed among the groups.

Scheme 1. Synthetic approach for the development of lipid-modified platinum compounds

Using cisplatin as starting material, we oxidized the Pt(II) to Pt(IV) in the presence of hydrogen peroxide and the final product was reacted with fatty acid anhydrides, yielding the lipid-modified platinum compounds.

Table 1

The IC₅₀ values of lipid-modified platinum derivatives as compared to cisplatin in wild-type A549 and cisplatin resistant A549^{DDP} cells and after transfection with *survivin* silencing siRNA after 48 hours.

Group	A549 Non transfected	A549 ^{DDP} Non transfected	A549 ^{DDP} Transfected with 10nM siRNA
CDDP	7.54 ± 1.41 *	66.84 ± 6.26	34.06 ± 6.52
CDDP-C4	4.16 ± 1.11	18.26 ± 4.02	13.46 ± 4.03
CDDP-C6	0.02 ± 0.01	6.05 ± 5.09	3.05 ± 3.75
CDDP-C8	0.05 ± 0.03	3.32 ± 2.265	0.73 ± 0.82
CDDP-C10	1.14 ± 0.85	6.94 ± 11.62	4.74 ± 3.03

* mean +/- S.D. (n = 4)

Table 2

Pharmacokinetic parameters of the two lipid-modified compounds CDDP-C6 and CDDP-C8 in nude mice (n=3/time point) after i.v. injection in equimolar dosage.

	Pharmacokinetic parameter	CDDP-C6	CDDP-C8
Blood	t1/2Alpha, h	0.186	0.053
	t1/2Beta, h	38.4	25.4
	CL, (mg)/(ng/ml)/h	1.87E-06	3.47E-06
	CL2,(mg)/(ng/ml)/h	8.74E-05	1.56E-04
	AUC 0–24h,ng/ml*h	5713	4223
Liver	AUC 0–24h,ng/mg*h	14.5	90.23
Kidney	AUC 0–24h,ng/mg*h	23.9	15.9

Table 3

Tumor volume doubling time for each of the studied groups, as determined by the tumor growth of each animal (**: $p < 0.05$)

Group	Tumor volume doubling time (days)
Control	6.4 ± 0.49
CDDP	6.5 ± 0.39
CDDP-C6	6.6 ± 0.37
CDDP-C8	6.4 ± 0.4
si+CDDP-C6	7 ± 0.49
si+CDDP-C8	$10.2 \pm 2^{**}$