Precise Engineering of Cisplatin Prodrug Into Supramolecular Nanoparticles: Enhanced on in Vitro Antiproliferative Activity and Treatment and Care of in Vivo Renal Injury

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Research Article

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Abstract

Renal cell carcinoma (RCC) is a widespread type of urological tumor that derives from the highly heterogeneous epithelium of the kidney tissue. For the past decade, the treatment of kidney cancer cells has changed clinical care for RCC. Herein, we present a very easy and cost-effective method that incorporates tumor-specific targeting supramolecular nanoassembly, and therapeutically to overcome the different challenges raised by the distribution of the pharmaceutical potential anticancer drug Cisplatin (CIS-PT). On covalent conjugations of hydrophobic linoleic acid by carboxylic group, the CIS-PT prodrugs were skilled in impulsively nanoassembly into extremely steady nanoparticles size (~100 nm). Electron microscopic techniques have verified the newly synthesized morphology of CIS-PT-NPs. The anticancer properties of CIS-PT and CIS-PT-NPs against Caki-1 and A-498 (renal carcinoma) cancer cell lines have been evaluated after successful synthesis. Other research, such as dual staining acridine orange/ethidium bromide, Hoechst 33344 and flow cytometry study on the apoptosis mechanisms, have shown that proliferation in renal cancer cells is associated with apoptosis. Further the *In vivo* toxicity results displays the CIS-PT-NPs remarkably alleviated the toxicity of the potential anticancer drug CIS-PT *In vivo* while conserving the Pharmaceutical activity. Compared to CIS-PT, CIS-PT-NPs demonstrate excellent *In vitro* and *In vivo* property, this study clarified the CIS-PT-NPs as a healthy and positive RCC care chemotherapeutics technique and deserve further clinical evaluations.

Highlights

1. Fabrication of Cisplatin (CIS-PT) on conjugations of linoleic acid by nanoassembly (CIS-PT-NPs).
2. The *in vitro* antiproliferative activity shows CIS-PT-NPs induced apoptosis in renal cancer cells.
3. The morphological examinations of CIS-PT-NPs have studied AO/EB and nuclear staining methods.
4. The cell death of CIS-PT-NPs was confirmed by flow cytometry analysis.
5. The *In vivo* toxicity results displays the CIS-PT-NPs remarkably alleviated the toxicity compared to CIS-PT.

Introduction

Renal cell carcinoma (RCC) is not a single organism but rather a group of tumors that arise from the highly heterogeneous epithelium of kidney tissue [1–5]. According to the Heidelberg description of renal cell cancer, histopathological subtypes of RCC includes clear cell adenocarcinoma, the most prevalent form of RCC, chromophobe accumulating duct carcinoma, papillary carcinoma, and unclassified carcinoma [6–10]. Among urological tumors, RCC has the highest risk of cancer-specific mortality and the 5-year survival rate for patients with metastatic disease is just 12%. In comparison, RCC reports for about 3.8% of all adult human cancers, with an average rise in incidence. Cancer treatment is one of the key methods of treating cancer; however, its usefulness is hampered by drug resistance [11–14].

Cisplatin is a potent chemotherapy agent that has been commonly used as a standard drug for the treatment of different forms of cancer. However, the therapeutic application of Cisplatin is consistent with dose-limiting toxicity (e.g., nephrotoxicity and myelosuppression) [15–18]. In addition, the endogenous or acquired opioid resistance often greatly impedes its therapeutic benefits. Numerous formulations, including liposomes, polymer mice and albumin, have been explored to completely exploit platinum-based agents. However, owing to the major formulation problems presented by platinum products, current methods are subject to hurdles such as the need for a vast quantity of excipients and complex production processes [19–21]. It is also not concerning that very few nano-medicines have reached clinical trials, although a significant number of platinum-encapsulated nano-devices have been described to date. As described above, the formulation of novel platinum-encapsulated candidates reconstructed with simple structural modifications and self-assembly into supramolecular nanostructures is highly desirable. These activities will advance the therapeutic use of platinum complexes[22–26].

In addition, unperformed modification of CIS-PT to inactive metabolites correspondents may be significantly inhibited after systemic control due to reduced metabolic properties of in the plasma membranes [27–29]. Therefore, the CIS-PT encapsulation prodrug was shown to reduce drugs stimulations and increase the performance of drug loading efficacy by the effects of permeability and retention of enhancement. In this report, we defined simple and superior method of effect that energetically combine with the supramolecular nanoassembly tumor targeting and drug reconstruction therapies of CIS-PT. To effectively targets the objective, hydrophobic linoleic acid (LA) is an integral fatty acids, by carboxylic group formation, was conjugated with the amino moiety of the CIS-PT equivalent. Fruitfully, we discovered in the water solutions the CIS-PT prodrugs of increasing self-modeling into the nanoparticles of nanoassembly. The *In vitro* proliferation of renal cancer cells was investigated by the nanoassembly CIS-PT prodrug and the morphological deviations and cell death examinations of renal cancer cells were identified.

Experimental Section

2.1. Fabrication of CIS-PT NPs

DIEA was combined with CIS-PT (75 mg, 0.20 mol) and hydrophobic linoleic acid (78 mg, 0.20 mol) solutions containing 2 mL of DMF (103 mg, 8.1 mol). The reaction mixture solutions was allowable to stir overnight at 37 °C and then to evaporate to extract the DMF solution. The reaction mixtures that have been absorbed in the DCM. Five percent citric acid, NaCl and aqueous NaHCO₃ is used to wash the organic coating. The DCM solution were removed using vacuum dried with Na₂SO₄ solution. Figure 1 demonstrates the comprehensive fabrication techniques.

2.2. Characterization

The morphology of CIS-PT NPs was tested by transmission electron microscopy (TEM; JEM-1200EX, Japan) ensuing with phosphotungstic acid staining (2%, w/v) for 3 min, and CIS-PT NPs were then

perceived and examined by employing them on films of copper grid-irons. The polydispersity index (PDI), zeta potential and particle size of nanoparticles were examined using the Zetasizer ZEN3600 (Malvern, England).

2.3. Cell culture

Caki-1 and A-498 renal cancer cell lines were cultured in DMEM (Gibco, NY, USA) enclosing 10% fetal calf serum (Hyclone, Logan, UT, USA), 100 µg/mL streptomycin (Life Technology, NY, USA), 100 IU/mL penicillin (Bioscience Technology, NY, USA), 1% L-glutamine (Bioscience Technology), and 1% non-essential amino acids (Bioscience Technology) at 37°C, 95% and 5% for humidity and CO₂ respectively.

2.4. *In vitro* proliferation assay by MTT

According to previously recorded protocols [30–34], a MTT assay was achieved. It is used to measure CIS-PT and CIS-PT-NPs for *In vitro* cytotoxicity. The cells of Caki-1 and A-498 were plated 2×10⁴ cell per well in a 96-well culture plate for the attachment followed by cultured for 24 h at 37°C. 10 µL of the MTT solution was applied to each well after incubation at 37°C for 24 hours, and the 525 nm absorbance was analyzed 2 hours later by Eliza microplate reader (TECAN Sunrise, Switzerland). The percentage of the cell proliferation were calculated by using the graph pad prism software. As seen below, the cell viability (percent) was evaluated using the formula;

$$\% \text{ of cell viability} = \text{OD}_{\text{treated}} / \text{OD}_{\text{control}} \times 100$$

2.5. Cellular Uptake

According to previously recorded protocols, a cellular uptake assay was performed. In 6-well plate at 1 × 10⁴ Caki-1 renal cancer cells were pictured confocal laser scanning microscopy (LSM 510 Meta; Zeiss, Germany). LysoTracker CIS-PT-NPs with Dil (Molecular Probe, USA) stained the cell lysosome (red color), then DAPI stained the nuclei. Images were examined by confocal laser scanning microscopy (CLSM) utilizing the image analyzer software manufacturer's protocol [35–37].

2.6. *In vitro* drug release profiles

The controlled cumulative release of the CIS-PT and CIS-PT-NPs have been assessed using membrane dialysis (Spectrum Laboratory, 14 kDa-molecular weight). In short, 100 µg/mL of CIS-PT-NPs (CIS-PT concentration) is dialyzed at 37°C to 20 mL of phosphate buffer solutions containing Tween 80 (0.2%) at pH 7.4. The releasing medium was obtained by constant mild stirring 37°C in an orbital shaker incubator (100 RPM) at and an equivalent amount of supplemented fresh medium.

2.7. Acridine orange and ethidium bromide (AO/EB) and Hoechst 33344 staining

AO/EB and Hoechst 33344 staining fluorescent dyes which bound strongly to the chromatin and subsequently shine more fluorescent upon bounding. Apoptotic morphological improvements upon treating with IC₅₀ concentrations of CIS-PT and CIS-PT-NPs against Caki-1 and A-498 cancer cell lines

were assessed. After staining, the cells at 20 X magnification were displayed under a fluorescence microscopy (Olympus IX81, Germany) [38–40].

2.8. Flow cytometry techniques

Flow cytometry was completed to study modes of cell death over FITC-Annexin V and propidium iodide (PI) based apoptosis detection assay kit (Invitrogen, Thermo Fischer Scientific, USA) followed by previously reported protocol [41–43]. The Caki-1 and A-498 cells were through the trypsinization to avoid the cell death. Trypsinized the cells and twice washed with PBS. The cells supernatant was removed and the cell pellets was suspended in a 250 μ L 1 $\times$ binding buffer (from the kit) with 5 μ L of FITC Annexin-V and PI was added and incubated with 37°C in dark conditions. After 10 minutes, the samples were subjected to flow cytometry analysis using FACS Canto TM II, BD Biosciences.

2.9. *In vivo* renal toxicity

Normal ICR mice (4–5 weeks old) were assigned to 10 classes (n = 10, 5 males and 5 females in each group) and injected intravenously at varying doses of CIS-PT-NP solution (200 μ L) and CIS-PT solution thrice times every three days. Saline has been used as a control. CIS-PT treatment was given at doses of 5, 10, 20 mg/kg. CIS-PT-NPs were delivered at 5, 10, 20, 30 mg/kg doses (CIS-PT equivalents). Changes in the body weight of mice have been recorded. After having received injections of saline, CIS-PT and CIS-PT-NP, two mice in each group were randomly picked and discarded by inhalation of CO₂. Primary organs, such as kidneys and spleens, were extracted and processed with 4 percent formaldehyde. After that, the specimens were covered in paraffin and sliced into 5- μ m-thick strips. These slices have been stained with hematoxylin and eosin (H&E, Sigma). For the terminal deoxynucleotidyl transferase– controlled dUTP nick end labeled (TUNEL) experiment, the dewaxed and rehydrated kidney parts were incubated with proteinase K at 37°C for 15 minutes, washed with PBS once, and flushed with the TUNEL In Situ Cell Damage Detection Kit as per the manufacturer's procedure (Sigma-Aldrich). TUNEL-stained cells were counter-stained with DAB (DAKO) and imaged by optical microscopy in ten different fields for each class [44–47].

Results And Discussion

3.1. Fabrication and characterisation of CIS-PT

Figure 1 demonstrates the complete synthetic procedure. CIS-PT analogs conjugated with a number of successive groups at the carboxylic terminal ends demonstrate improved stability in cellular membrane due to the dehydrogenation purpose. In addition, LA hydrophilic regulator is used for the development of polymeric prodrugs. In this work, we planned and produced the CIS-PT analogs for the LA polymer chains to render the CIS-PT-LA prodrug (Fig. 1). Conjugation was achieved by coupling CIS-PT to LA under the structure of CIS-PT-LA, and the CIS-PT-LA prodrugs were filtered using thin layer chromatography (TLC) and column chromatography approaches with the high yield (80.5 %). The lipophilic design of the drug delivery systems, which create nano-assemblies, could be synthesized in water by nanoassembly

approaches without the addition of any surfactants. In addition, the organic phases of the CIS-PT-LA prodrugs are absorbed in the solution of DMSO into the DD-H₂O aqueous suspensions. More importantly, the elimination of excess organic solvents through double distilled water from the fabricated nanoparticles rather than prodrug accumulation. In addition, TEM observations were conducted to determine the structure of supramolecular nanoassembly CIS-PT-NPs (Fig. 2A). The results of the supramolecular nanoassembly CIS-PT-NPs showed that the well-structured form with a sphere-shaped was $\sim 80.3 \pm 3.12$ nm (Fig. 2B). In addition, the hydrodynamic parameter of the nanoassembly CIS-PT-NPs was 82.7 ± 2.05 nm with less poly dispersive index (PDI), as shown by the DLS method (Fig. 2C). Admirable findings are associated with the formations of supramolecular nanoassembly CIS-PT-NPs. The stability of the CIS-PT, and CIS-PT-NPs in PBS media was observed by measuring the particle size of the CIS-PT, and CIS-PT-NPs by dynamic light scattering. Polyplexes index (PDI), precisely CIS-PT, and CIS-PT-NPs, at an nanoparticles ratio of 100:1 were organized and incubated for 30 min at 37°C in order to check broad polyplex formations (Fig. 2D-E). All the stability analysis were three times repeated.

3.2. *In vitro* CIS-PT release profiles

As a traditional nucleosides analog, the main shortcomings of CIS-PT as a chemotherapeutic agents contain half-life small plasma and fast cytokine deaminase deactivation. We also developed supramolecular nanoassembly CIS-PT-NPs which could help as a reservoir to keep drugs safe and avoid the release of drugs into the general blood circulation, thereby slowing down CIS-PT authorization from the human body condition. Observation of the controlled release of CIS-PT-NPs by dialyzing against PBS at RT. As seen in Fig. 2F, CIS-PT free was rapidly released from CIS-PT-NPs and plateaued at 90.2 ± 2.9 compound releases after 25 hours of incubation. In the other hand, CIS-PT-NPs demonstrated continuous drug release. Our findings of *In vitro* drug leasing revealed a sluggish inhibition of the release of free CIS-PT kinetics from the CIS-PT-NPs, which is useful to enhance the half-life blood plasma of free CIS-PT and enhancing drug delivery to cancer cells.

3.3. *In vitro* proliferation assay by MTT

After efficient fabrication of CIS-PT-NPs, MTT experiments were conducted to determine the proliferation of CIS-PT and CIS-PT-NPs of renal carcinoma cells, with Caki-1 and A-498 cancer cells. After treating with drugs for 24 hrs, the proliferation of the Caki-1 and A-498 cells was controlled and the dose dependent curvature shows the half inhibitory concentrations (IC_{50}) (Fig. 3A-B). Positively, associated to the free CIS-PT, supramolecular nanoassembly displayed significantly increased cytotoxic effects in both renal cancer cell lines tested. In half-inhibitory concentrations of Caki-1 cells, the IC_{50} is 11.12 ± 5.09 and 6.13 ± 1.29 for CIS-PT and CIS-PT NPs respectively. In A-498 cells, the IC_{50} is 15.87 ± 2.18 , and 5.16 ± 2.80 for Corilagin and Corilagin-NPs, respectively. In addition, we examined the selectivity of the CIS-PT and CIS-PT NPs shows remarkable proliferation against the CaSki and HeLa cells (Fig. 3C). The CIS-PT NPs shows remarkable proliferation against the Caki-1 and A-498 cells which may be due to the presence of the hydrophilic linoleic acid of π -conjugation chain. This may be attributed due to the hydrophobic chain of the linoleic acid which may efficiently penetrate the cell membranes. Several attempts will be made to

establish the small molecule nano-assembled nanoparticles for promising anticancer agents by introducing the various lipophilic spacers on the potential small molecules.

3.4. Cellular uptake efficiency of CIS-PT NPs

We cross-linked that the CIS-PT-NPs make it possible to interact with Caki-1 renal tumor cells and ions, thereby enhancing intracellular absorption into the renal cancer cell. A confocal laser scanning microscopy (LCSM) was used to test the localizations of subcellular of CIS-PT-NPs in the Caki-1 renal cancer cell lines (Fig. 4). In the concentration 20 nM with CIS-PT-NPs with different incubation times (10, 20 and 30 min) were labelled with the Dil fluorescence tracker (red color) for the interpretations, for this comparison the cells lysosome and the nucleus was labeled with the DAPI (blue color). The new yellow color fluorescents were paired with Dil, and DAPI fluorescence in the Caki-1 renal cancer cell line, whereas CIS-PT-NPs could be confined with lysosome during internalization.

3.5. Morphological examination by acridine orange/ethidium bromide (AO/EB)

CIS-PT and CIS-PT-NPs were tested using a fluorescent microscopic examination of acridine orange/ethidium bromide (AO/EB) stained in Caki-1 and A-498 renal cancer cell lines indicative of morphological changes (Fig. 5). Usually nanoparticles causes cell death through the apoptosis and necrosis pathways. Ironically, after 24-hour treatment with their IC_{50} concentration of the CIS-PT and CIS-PT NPs, CIS-PT-NPs display a higher proportion of cell death by apoptosis pathway than free CIS-PT treatments.

3.6. Morphological examination by Hoechst-33258 staining

CIS-PT and CIS-PT-NPs were tested using a fluorescent microscopic examination of Hoechst-33258 staining (nuclear staining) stained in Caki-1 and A-498 renal cancer cell lines indicative of morphological changes (Fig. 6). Usually nanoparticles causes cell death through the apoptosis and necrosis pathways. Ironically, after 24-hour treatment with their IC_{50} concentration of the CIS-PT and CIS-PT NPs, CIS-PT-NPs display a higher proportion of cell death by apoptosis pathway than free CIS-PT treatments.

3.6. Flow cytometry-confirmation of the apoptosis

Apoptosis can be used as a significant impairment to the growth of a tumor cells. The primary qualitative screening methods of the AO/EB and nuclear staining methods clearly shows the supramolecular nanoassembly induce the cell death through apoptosis mode. In this quantitative experiments displays the number of cancer cell death by the flow cytometry via staining of the FITC Annexin-V and propidium iodide (PI) in the Caki-1 and A-498 renal cancer cells. CIS-PT and CIS-PT-NPs were tested using a FITC Annexin-V with the propidium iodide (PI) examination in Caki-1 and A-498 renal cancer cell lines (Fig. 7).

Ironically, after 24-hour treatment with their IC_{50} concentration of the CIS-PT and CIS-PT NPs, CIS-PT-NPs with high percentage of the apoptosis than free CIS-PT.

3.7. *In vivo* renal toxicity

In vivo systemic toxicity induced by CIS-PT has hindered its therapeutic use [48]. To confirm if this CIS-PT-NPs scaffold transforms high toxic CIS-PT to stable nanotherapy, we intravenously delivered stock solution CIS-PT-NPs to normal ICR mice. After injection of CIS-PT-NPs or clinically used CIS-PT solution, we measured the shift in body weight of the mice. Normal ICR mice (4–5 weeks old) were assigned to 10 classes ($n = 10$, 5 males and 5 females in each group) and injected intravenously at varying doses of CIS-PT-NP solution (200 μ L) and CIS-PT solution thrice times every three days. Saline has been used as a control. CIS-PT treatment was given at doses of 5, 10, 20 mg/kg. CIS-PT-NPs were delivered at 5, 10, 20, 30 mg/kg doses (CIS-PT equivalents). The healthy ICR mice ($n = 10$, 5 males and 5 females in each group) and injected intravenously injected with 5, 10, 20, or 30 mg/kg (CIS-PT-equivalents) thrice times every three days. Even 5 mg/kg of CIS-PT was well received by mice ($\sim 4.7\%$ body weight reduction) as larger concentrations (10 and 20 mg/kg) resulted in substantial weight loss (Fig. 8). However, CIS-PT-NPs significantly increased the therapeutic efficacy of the parental CIS-PT. After these three injections of elevated doses of drugs (e.g. 30 mg/kg of CIS-PT-equivalents), no obvious effects on body weight were found, suggesting little or no drug toxicity.

Renal toxicity is a significant adverse side effect of CIS-PT, which has reduced dosage enhancement in clinical practice. We have also checked if this CIS-PT-NPs scaffold is less dangerous to the tissues. Healthy ICR mice were treated with double injections of CIS-PT-NPs (5, 10, 20 and 30 mg/kg with CIS-PT-equivalents), and CIS-PT (5, 10 and 20 mg/kg) was used as a standard. The animals were sacrificed on day 6 of post-injection and the organs were exposed to histological immunohistochemistry (H&E) and TUNEL staining. Lower damage to kidneys and spleen caused by the use of CIS-PT-NPs has also been reported by H&E staining (Fig. 8). As seen in Fig. 9, kidney segments in CIS-PT-treated mice displayed substantial cell death activation, while only marginal nephrotoxicity was identified in mice given CIS-PT-NP at larger concentrations up to 30 mg/kg. Together to, such findings show that CIS-PT-NPs protect the kidney from CIS-PT-induced dysfunction in animals, which may potentially favour humans when evaluating medical properties.

Conclusion

We have effectively engineered and illustrated the lipophilic and fast metabolic CIS-PT prodrugs for a more pharmacologically effective nano prodrug. We find out that supramolecular nanoassembly of CIS-PT nanoparticles display improved controlled drug release and enhanced extracellular environment uptake to possibly resolve cancer cell aggregations by EPR. After a successful development, we tested the MTT of CIS-PT and CIS-PT-NP nanoparticles against Caki-1 and A-498 renal tumor cell lines. In addition, the morphological changes experiments such as acridine orange/ethidium bromide (AO-EB), Hoechst-33258 staining results shows that the supramolecular nanoassembly of CIS-PT nanoparticles

induce apoptosis in renal cancer cells. Further, we have confirmed the apoptosis by flowcytometry techniques. More admirably, CIS-PT-NPs demonstrated decreased toxic effects relative to CIS-PT. As such, the new CIS-PT prodrug-assembled scaffold can be used in patients with compromised renal function. Ultimately, this work offers a simple approach to producing cost-effective and healthy platinum-based nanotherapy and deserves further application to clinical practice.

Declarations

Conflict of the interest

None

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Figures

Figure 1

Graphical description of CIS-PT-NPs affecting renal cancer cells. (a) CIS-PT Structure (CIS-PT). Graphic design of the CIS-PT prodrug formulation preparation process and can be nanoassembly in water by CIS-PT-NPs displaying a possible chemotherapy outcome.

Figure 2

Characteristic assessment of CIS-PT-NP. (A) TEM representation of CIS-PT-NPs. The scale bar is 100 nm. (B) Hydrodynamic parameter CIS-PT-NPs. (C) Hydrodynamic parameter diagram of CIS-PT-NPs. D and E) the Stability of Zeta potential and PDI to be investigated by a DLS method. F) In vitro controlled drug release profiles of CIS-PT drugs from CIS-PT-NPs. The solution contains CIS-PT-NPs which have been dialyzed against PBS at 37°C.

Figure 3

In vitro proliferation of Caki-1 and A-498 renal cancer cell lines. (A and B) MTT assay was performed to examine the proliferation of the cancer cells with different concentrations. (C) MTT assay was examined by the proliferation of the non-cancerous NIH-3T3 cells.

Figure 4

Cellular uptake efficiency of the Caki-1 renal cancer cells. Lysosomes labeled with subcellular localization of CIS-PT-NPs in Caki-1 renal cancer cell lines at different incubation periods. Scale bar = 10 μ m.

Figure 5

Dual AO/EB fluorescent staining of Caki-1 and A-498 renal cancer cell lines after treating with CIS-PT and CIS-PT-NPs with IC50 concentration for 24 hrs.

Figure 6

Nuclear staining of Caki-1 and A-498 renal cancer cell lines after treating with CIS-PT and CIS-PT-NPs with IC50 concentration for 24 hrs.

Figure 7

Flow cytometry examination were performed to confirm the apoptosis of Caki-1 and A-498 renal cancer cells. The Caki-1 and A-498 cells were treated with CIS-PT and CIS-PT-NPs with IC50 concentration for 24 hrs and stained by FITC-Annexin V and PI for flow cytometry examination.

Figure 8

Histological analysis of kidney and spleen of healthy ICR mice. The tissues were excised from mice on day 6 after two intravenous injections of CIS-PT-NPs at days 0 and 3. Saline and CIS-PT were used as controls. The kidney and spleen sections were stained with H&E. Scale bars: 200 (left) and 50 (right) μm .

Figure 9

Representative TUNEL analysis of the excised tumors. A) The issues were excised from mice on day 6 after two intravenous injections of CIS-PT-NPs at days 0 and 3. Saline and CIS-PT were used as controls. The kidney sections were stained with TUNEL analysis. Scale bars: 200 (left) and 50 (right) μm . B) Quantification of TUNEL positive ratio.

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