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ORIGINAL RESEARCH

Lung Cancer Combination Treatment: Evaluation of the Synergistic Effect of Cisplatin Prodrug, Vinorelbine and Retinoic Acid When Co-Encapsulated in a Multi-Layered Nano-Platform

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Purpose: Lung cancer remains the leading cancer-associated deaths worldwide. Cisplatin (CIS) was often used in combination with other drugs for the treatment of non-small cell lung cancer (NSCLC). Prodrug is an effective strategy to improve the efficiency of drugs and reduce the toxicity. The aim of this study was to prepare and characterize CIS prodrug, vinorelbine (VNR), and all-trans retinoic acid (ATRA) co-delivered multi-layered nano-platform, evaluating their antitumor activity in vitro and in vivo.

Methods: Cisplatin prodrug (CISP) was synthesized. A multi-layered nano-platform contained CISP, VNR and ATRA were prepared and named CISP/VNR/ATRA MLNP. The physicochemical properties of CISP/VNR/ATRA MLNP were investigated. In vitro cytotoxicity against CIS-resistant NSCLC cells (A549/CIS cells) and Human normal lung epithelial cells (BEAS-2B cells) was investigated, and in vivo anti-tumor efficiency was evaluated on mice bearing A549/CIS cells xenografts.

Results: CISP/VNR/ATRA MLNP were spherical particles with particle size and zeta potential of 158 nm and 12.3 mV. CISP/VNR/ATRA MLNP (81.36%) was uptake by cancer cells in vitro. CISP/VNR/ATRA MLNP could significantly inhibit the in vivo antitumor growth and suspended the tumor volume from 1440 mm^3 to 220 mm^3.

Conclusion: It could be concluded that the CISP/VNR/ATRA MLNP may be used as a promising system for lung cancer combination treatment.

Keywords: lung cancer, combination treatment, synergistic effect, cisplatin prodrug, multi-layered nano-platform

Introduction

Lung cancer is the leading cause of cancer death worldwide.[1] In 2019, one-quarter of all cancer deaths are due to lung cancer in the USA.[2] There are only 18% of all patients with lung cancer live 5 years or more after diagnosis.[3] Non-Small Cell Lung Cancer (NSCLC), the most common type of lung cancer, accounts for more than 84%.[2,4] Based on the NCCN Guidelines, surgery, radiotherapy, and systemic therapy are the three modalities most commonly used therapeutic regimens for NSCLC patients. Among them, chemotherapy is recommended for patients with stage IV (advanced) NSCLC and preoperative or postoperative chemotherapy for patients with NSCLC at stage IB-IIIA, and all aforementioned patients account for about 80% of NSCLC cases.[4–7]

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Platinum-based chemotherapy (cisplatin, carboplatin, and their combination regimens) is recommended and beneficial for patients with stage IV NSCLC and preoperative and postoperative therapy in the NCCN guidelines.[8–10] The main mechanism of antitumor effect for cisplatin (CIS) is to form CIS-genomic DNA interstrand and intrastrand cross-links, and hinder transcription and/or DNA replication, then lead to cell death. However, it is severe for the adverse reactions of CIS including nephrotoxicity, myelosuppression, and some patients are not able to tolerate it.[11,12] Therefore, various researchers have devoted to new strategies to overcome these obstacles, including producing CIS prodrug, using nanotechnology, applying combination therapy, etc.[13–15] The current standard combination chemotherapy of most patients with NSCLC remains platinum-based strategy containing vinorelbine (VNR), gemcitabine, paclitaxel, docetaxel, and pemetrexed.[16]

Combinations of two or more therapeutic agents or strategies have been explored for its unique advantages, such as improved drug efficacy, reduced adverse reactions, and multidrug resistance.[17,18] All-trans retinoic acid (ATRA), the biological active metabolites of vitamin A, has been proven to have anticancer activity including lung cancer, leukemias, head/neck cancer, etc.[19–22] Recent studies have investigated combination therapy of ATRA and CIS for lung cancer; results have shown that the combination brings enhancing therapeutic efficacy, and decreasing nephrotoxicity and drug resistance.[23–25] However, the low water solubility of ATRA hinders its clinical application.

Nanocarriers have emerged as platforms for cancer therapy, because they can offer advantages over free drugs including improved intracellular penetration, enhanced distribution of drugs into a selected tissue, extended circulating half-life and long shelf life.[26] Multi-layered nanoparticle platforms have been applied for combination drugs delivery to tumors.[27,28] In this study, a novel CIS prodrug (CISP) was synthesized and a multi-layered nano-platform was constructed for the delivery of CIS, VNR, and ATRA. In vitro and in vivo performances were evaluated, summarized, and discussed.

Materials and Methods
Materials
Poly (D, L-lactic-co-glycolic) (PLGA, 50:50, MW 10,000) was provided by Beijing TopScience Bio-technology Co., Ltd (Beijing, China). Carbopol 940 (CBP, MW 1000,000) was obtained from Shanghai Renmin Pharmaceutical Factory (Beijing, China). Injectable soya lecithin (ISL) was obtained from Nanjing Well Pharmaceutical Co., Ltd (Beijing, China). Hyaluronic acid (HA, 300 kDa) was kindly provided by Bloomage Biotechnology Co., Ltd (Jinan, China). CIS, VNR, ATRA, EDC·HCl, NHS, dimethyldioctadecylammoniumbromide (DDAB) were obtained from Sigma-Aldrich China (Shanghai, China). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from Invitrogen Corporation (Carlsbad, CA).

Synthesis of Cisplatin Prodrug
Cisplatin prodrug (CISP) was synthesized by conjugating the amino group of tryptophan (TRP) with the carboxylated CIS (Figure 1).[29] Briefly, H_2O_2 was added into CIS (300 mg, dissolved with DMSO) to form $CIS-(OH)_2$, in which succinic anhydride (SA, 100 mg) was added and stirred for 10 h to obtain $CIS-(COOH)_2$ (CIS-SA). EDC·HCl (50 mg) and NHS (30 mg) were dissolved in CIS-SA with stirring, in which TRP (50 mg, dissolved in water) was added and stirred for 24 h. Finally, the solution was dialyzed against water for another 24 h and lyophilized to obtain CISP (CIS-SA-TRP). ESI-MS m/z calculated for $[C_{15}H_{22}Cl_2N_4O_6Pt]^+$: 619.06 $[M + H]^+$; found: 619.37. ^{1}H-NMR analysis (DMSO-d6, 300 mHz, ppm): peaks at 2.03 belong to CIS, the signals attributed to TRP are at 3.06, 7.18, and 10.08, the formation of carboxy and amino groups was confirmed by the peaks at 2.65, 2.41, and 4.85, respectively.

Preparation of Multi-Layered Nano-Platform
A multi-layered nano-platform contained CISP, VNR, and ATRA were prepared and named CISP/VNR/ATRA MLNP (Figure 2). Firstly, CISP and VNR loaded inner core was constructed by a solvent displacement technique.[30] Briefly, CISP (20 mg), VNR (20 mg), and PLGA (100 mg) was dissolved in acetone (5 mL) and added drop-wise into 20 mL of water (contained 1% CBP, w/v) which was stirred (300 rpm) at room temperature for 6 h to form solution-1. Secondly, lipid mixed with ATRA as the middle layer was produced by a nanoprecipitation technique.[31] Briefly, ATRA (20 mg), ISL (100 mg), and DDAB (1%, w/v) were dissolved in acetone (5 mL), then added dropwise to the solution-1 which was stirred (400 rpm) at room temperature for 6 h to get solution-2. Finally, positively charged
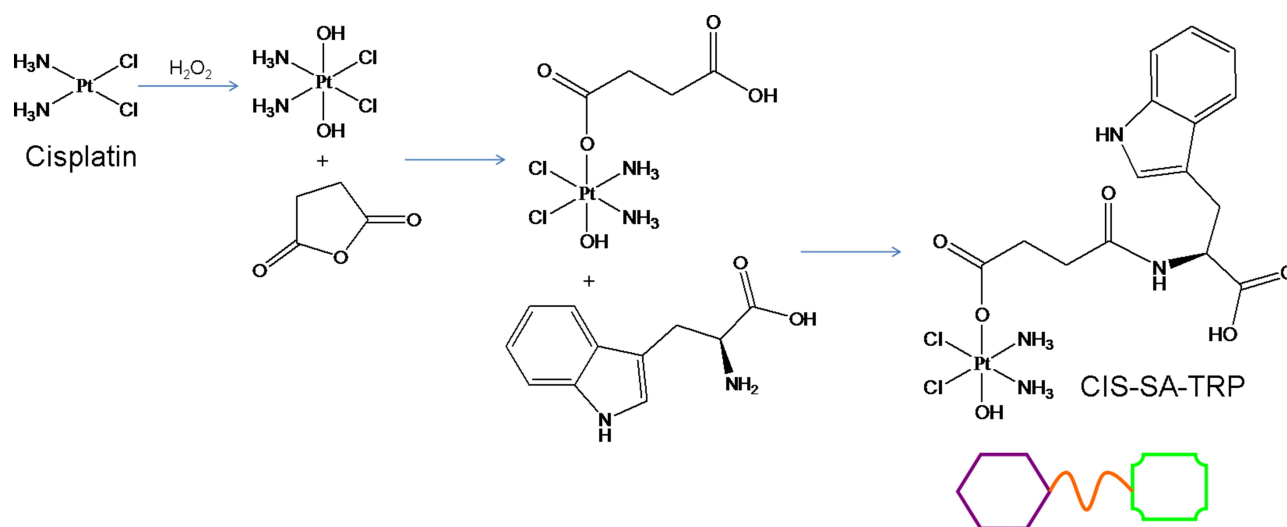
Figure 1 Synthesis of cisplatin prodrug. Cisplatin prodrug (CISP) was synthesized by conjugating the amino group of tryptophan (TRP) with the carboxylated CIS.
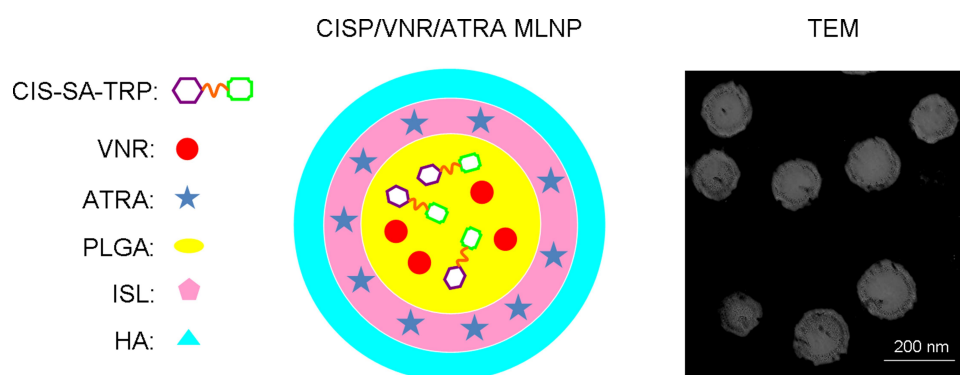
Figure 2 A multi-layered nano-platform contained CISP, VNR and ATRA were prepared and named CISP/VNR/ATRA MLNP. The TEM image showed that the CISP/VNR/ATRA MLNP was spherical particles with inner cores and coats on the outer surface.

HA (the surface layer) was coated onto the negatively charged middle layer.[32] HA (100 mg) was dissolved in water and added to solution-2 which was stirred (400 rpm) at room temperature for 4 h to produce CISP/VNR/ATRA MLNP.

A multi-layered nano-platform contained CIS (not prodrug), VNR and ATRA were prepared by the similar method using CIS instead of CISP, named CIS/VNR/ATRA MLNP. A multi-layered nano-platform contained CISP and VNR was prepared and by the similar method without using ATRA, named CISP/VNR MLNP. Multi-layered nano-platforms contained single CISP, VNR or ATRA were prepared and by the similar method using CISP, VNR or ATRA only (without other drugs), named CISP MLNP, VNR MLNP, or ATRA MLNP. Blank multi-layered nano-platform was prepared and by the similar method without using any drugs, named Blank MLNP. A

nano-platform contained CISP, VNR and ATRA but do not contain HA was prepared by the similar method without using HA as the outer layer, named CISP/VNR/ATRA NP. HA CISP, VNR and ATRA mixed solution were prepared by dissolving the three drugs in water (contained 1% Pluronic F68, w/v), named Free CISP/VNR/ATRA.

Characterization of Multi-Layered Nano-Platform

The morphology of the CISP/VNR/ATRA MLNP was observed by transmission electron microscopy (TEM) using an H-7650 transmission electron microscope (Hitachi, Tokyo, Japan).[33] Average particle size, polydispersity index (PDI) and zeta potential of all the MLNPs were determined by dynamic light scattering using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, U.K.).[34]

Drug Encapsulation and Loading Efficiency

Drugs were isolated from the MLNPs were measured by redispersed the formulations in 0.1% Tween 80 and centrifuged (20,000 rpm, 15 min) and the supernatant was collected.[35] The CIS content in MLNPs was determined by atomic absorption spectroscopy.[13] The VNR and ATRA content were determined using HPLC at 269 nm and 340 nm.[35,36] The drug encapsulation efficiency (EE) and loading efficiency (LE) of drugs loaded MLNPs were calculated by equations: EE (%) = The amount of drugs encapsulated in MLNPs/Total amount of drugs added×100%; LE (%) = The amount of drugs encapsulated in MLNPs/The amount of MLNPs×100%.

Serum Stability

The serum stability of MLNPs was evaluated in PBS mixed with FBS (1:1, v/v).[37] MLNP formulations were incubated with 50% FBS (v/v) solution for 72 h. At 1, 2, 4, 8, 24, 48, and 72 h, 1 mL of formulation was taken out and the particle size and PDI were measured by the same methods described in the "Characterization of multi-layered nano-platform" section.

In Vitro Drug Release

The release study was conducted using a dialysis method.[38] Briefly, MLNP formulations (2 mL each) were sealed into separate dialysis bags, which were immersed into PBS mixed with FBS (1:1, v/v) solutions (48 mL each, 37°C) under shaking (100 rpm). Aliquots (2 mL) were taken out from the solution periodically, and the amount of drugs released from MLNP was analyzed by the same methods described in the "Drug encapsulation and loading efficiency" section.

Cells

The CIS-resistant NSCLC cells (A549/CIS cells) and Human normal lung epithelial cells (BEAS-2B cells) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured (37°C, 5% CO_2) in DMEM medium (10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin).

Cellular Uptake

The uptake MLNP by A549/CIS cells was tested using coumarin 6 (C6) as an indicator.[39] Briefly, C6 (40 mg) was dissolved along with PLGA (in acetone) in the preparation section. C6 contained CISP/VNR/ATRA MLNP and CISP/

VNR/ATRA NP were added to A549/CIS cells which were previously incubated in a 24-well plate (1×10^5 cells/well, 24 h). The medium containing the formulations was discarded and the cells were washed three times with D-Hank's solution and photographed by fluorescence microscopy. To quantify the uptake efficiency, cells were analyzed by a flow cytometer (BD Biosciences, San Jose, CA).

Cytotoxicity

The cytotoxicity of MLNP was evaluated on A549/CIS and BEAS-2B cells by MTT assay.[40] Briefly, all kinds of MLNPs, CISP/VNR/ATRA NP and Free CISP/VNR/ATRA at different concentrations were added to A549/CIS or BEAS-2B cells which were previously incubated in 96-well plates (3×10^4 cells/well, 24 h). After removal of the medium, MTT solution (20 μL, 5 mg/mL in PBS) was added to each well. DMSO (200 μL) was added 4 h later and the absorbance at 570 nm was recorded.

Synergistic Effect

The synergistic effect of CISP/VNR/ATRA MLNP was evaluated by Chou-Talalay method.[41] Combination index (CI) is applied for the evaluation of synergy or antagonism, which is calculated by the equation: $CI = \sum_{i=1}^{n} (C)i/(Cx)i$.[42] $(C)_i$: the concentrations of drugs in the combination; $(C_x)_i$: the concentrations of drugs used alone. CI < 1 indicated synergy, closer to zero means higher synergy efficiency; CI > 1 represented antagonism.

Animals

BALB/c nude mice (6–8 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) and the mice are maintained and treated in compliance with the guidance of the National Institutes of Health for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and approved by the Animal Care and Use Committee of Peking University Cancer Hospital & Institute. A549/CIS cells ($1×10^7$) were injected into the abdominal cavity of mice to produce NSCLC xenograft mice.

In Vivo Tumor Inhibition

NSCLC xenograft mice were randomized into 10 groups (6 mice/group). CISP/VNR/ATRA MLNP, CIS/VNR/ATRA MLNP, CISP/VNR MLNP, CISP MLNP, VNR MLNP, ATRA MLNP, CISP/VNR/ATRA NP, Free CISP/VNR/ATRA, Blank MLNP, and 0.9% normal saline (NS)

was intravenously injected every 3 days at an equivalent dose of CISP, VNR, and/or ATRA (2 mg/kg).[43] The tumor volumes (V) of mice were measured every 3 days and calculated by the equation: V = length × width^2/2. The tumor inhibition efficiency (TRE) was evaluated by the equation: TIE (%) = (Tumor weight of the mice treated with NS group - Tumor weight of the mice treated with formulations/Tumor weight of the mice treated with NS group × 100. At the end of study (day 18), the macroscopic images of tumor tissues were presented.

In Vivo Tissue Distribution

After intravenously injected with CISP/VNR/ATRA MLNP, CIS/VNR/ATRA MLNP, CISP/VNR/ATRA NP, and Free CISP/VNR/ATRA as the "In vivo tumor inhibition" section, mice were sacrificed at 1 h and 48 h.[44] Major tissues (tumor, lung, heart, kidney, liver, and spleen) were harvested, weighed, and dissolved in the concentrated nitric acid by heating up and evaporated to dryness, and then re-dissolved in 0.1 N HCl for tissue distribution analysis by the same methods described in the "Drug encapsulation and loading efficiency" section.

Systemic Toxicity

The systemic toxicity of the formulations were evaluated by the changes of body weights and the variations of alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and blood urea nitrogen (BUN).[45] The body weights of mice were measured every 3 days and recorded. At the end of the study, blood was collected and centrifuged to obtain the serum. ALT, LDH, and

BUN levels were assayed as indicators of renal and hepatic function.

Statistical Analysis

The non-parametric data were analyzed using Friedman test (n=6). *$P < 0.05$ was considered statistically significant.

Results

## Characterization of Multi-Layered Nano-Platform

The TEM image showed that the CISP/VNR/ATRA MLNP was spherical particles with inner cores and coats on the outer surface (Figure 2). Particle sizes, PDIs, and zeta potentials were summarized (Figure 3). The size, PDI, and zeta potential of CISP/VNR/ATRA MLNP were about 158 nm, 0.11, and 12.3 mV. Similar results were found in other MLNPs, which illustrated the loading of drugs did not change the size and surface charge of the particles. The size of CISP/VNR/ATRA NP (127 nm) was smaller than CISP/VNR/ATRA MLNP, with a reversed surface charge (−13.5 mV). The EE and LE of drugs loaded MLNP were around 90% and 5%, respectively (Figure 4). The sizes and PDIs of MLNPs remained unchanged during the serum stability test, indicating the MLNPs may be stable in the plasma when administrated in vivo.
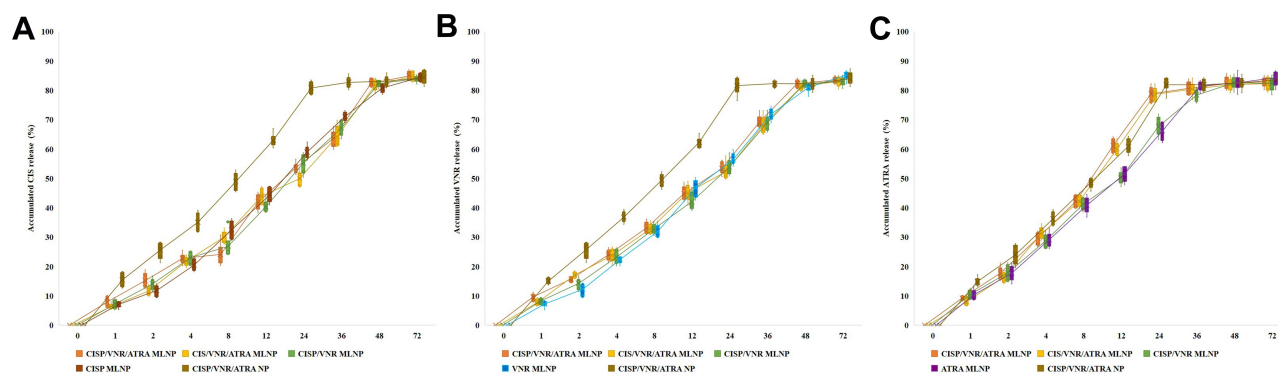
In Vitro Drug Release

All the three drugs showed sustained release from MLNPs. CISP and VNR exhibited similar release profiles, showing complete release at 48 h (Figure 5). However, ATRA were released from MLNPs in different manners compared with
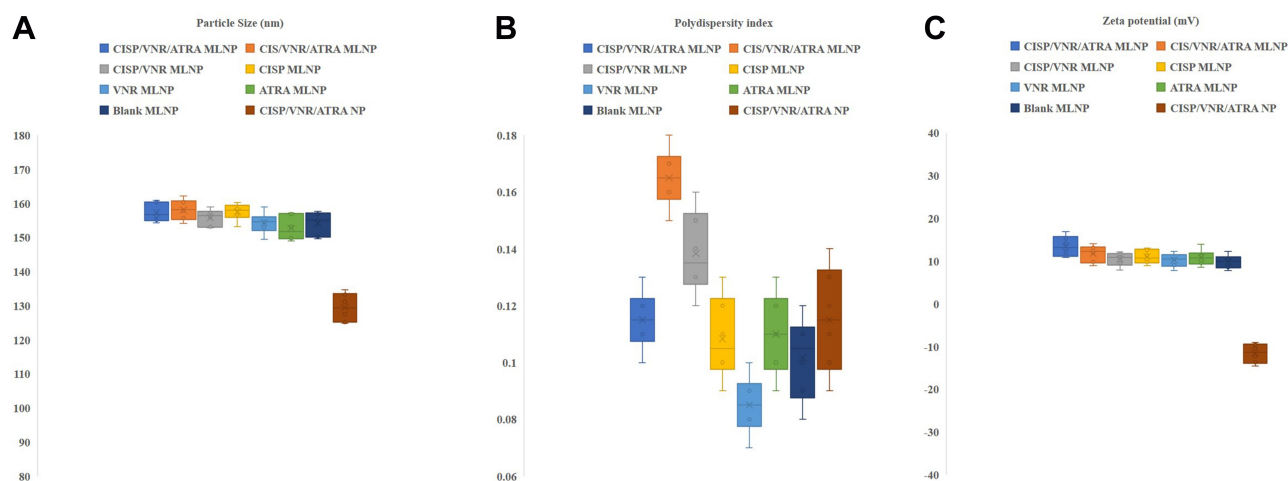
Figure 3 Particle sizes (**A**), PDIs (**B**), and zeta potentials (**C**) of the particles.
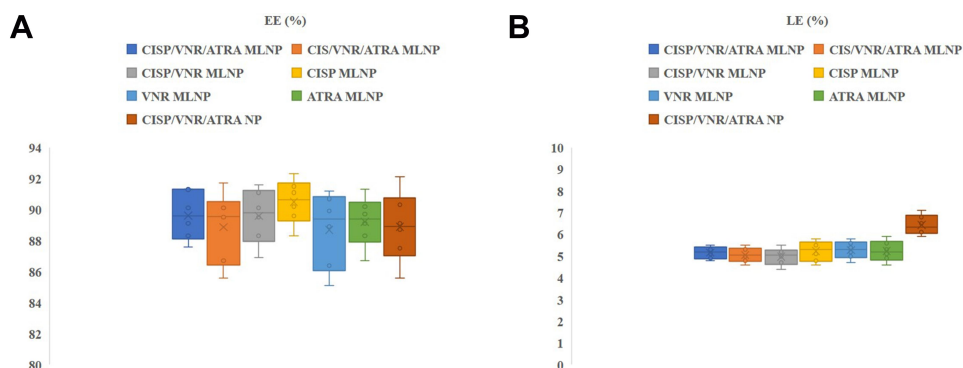
Figure 4 The EE (**A**) and LE (**B**) of drugs loaded particles.

Figure 5 In vitro drug release profiles of CISP (**A**), VNR (**B**) and ATRA (**C**). All the three drugs showed sustained release from MLNPs. CISP and VNR exhibited similar release profiles, but ATRA were released from MLNPs in different manners compared with CISP and VNR.

CISP and VNR. Faster release was observed by ATRA, it took 36 h to totally release from MLNPs. CISP/VNR/ATRA MLNP showed slower drug release than that of non-HA coating CISP/VNR/ATRA NP.

Cellular Uptake

The cellular uptake efficiency of CISP/VNR/ATRA MLNP and non-HA coating CISP/VNR/ATRA NP was compared by the images and quantitative results (Figure 6). The result clearly showed that more CISP/VNR/ATRA MLNP (81.36%) were uptake by cancer cells in comparison with CISP/VNR/ATRA NP (43.33%) (P < 0.01). Remarkably higher uptake efficiency may be explained by the HA coating that improved the uptake of the particles, which was going to be discussed later.

Synergistic Cytotoxicity

Concentration deepened manners were found in all drugs contained formulations on both A549/CIS and BEAS-2B cells (Figure 7). On A549/CIS cells, CISP/VNR/ATRA MLNP exhibited higher cytotoxicity than CIS/VNR/ATRA

MLNP and CISP/VNR/ATRA NP (P < 0.05), while no significant difference was found on BEAS-2B cells. The cell viability was more remarkably inhibited by drugs loaded MLNPs and CISP/VNR/ATRA NP formulations than Free CISP/VNR/ATRA on A549/CIS cells (P < 0.01). However, BEAS-2B cells viability differences among the formulas were not that obvious. The synergistic cytotoxicity was evaluated by CI values on the bases of IC_{50} values of different formulations. The effect of three-drug combination treatment was compared with the effect of treatment with each drug alone. Figure 8 shows the CI values were <1 when the fraction of affected cells at the intervals of 20%-80%. CI values closer to 0 means higher synergy effects. More remarkable combination effect was exhibited by CISP/VNR/ATRA MLNP than that of CISP/VNR MLNP, indicating the better efficiency brought by ATRA.

In Vivo Tumor Inhibition

In vivo tumor inhibition ability of CISP/VNR/ATRA MLNP was better than CISP/VNR/ATRA NP (P < 0.01), which could be the evidence of the tumor-targeted ability of the
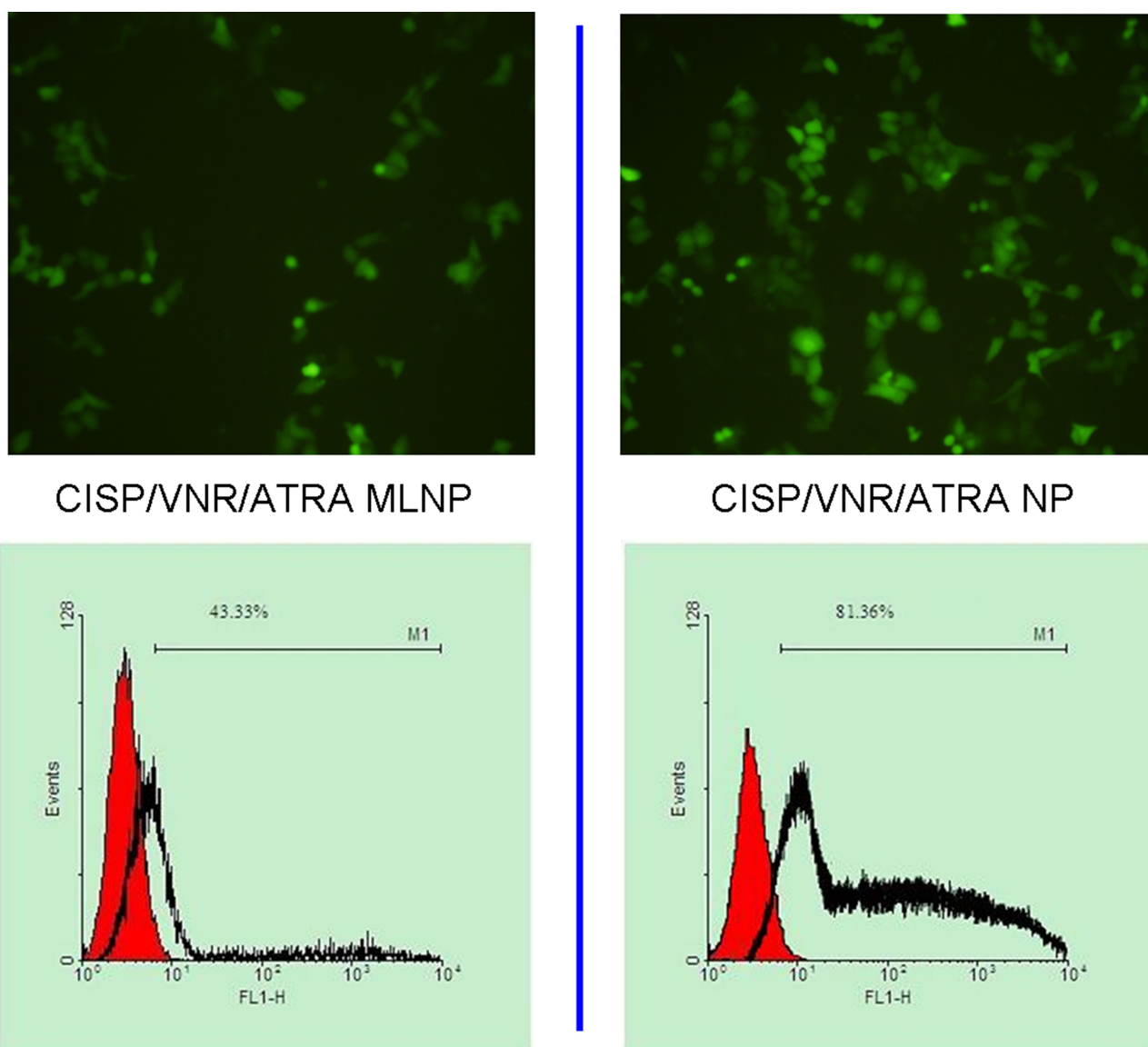
Figure 6 The cellular uptake efficiency of CISP/VNR/ATRA MLNP and non HA coating CISP/VNR/ATRA NP were compared by the images and quantitative results.

multi-layered nano-platform with an HA coat (Figure 9). CISP/VNR/ATRA MLNP also exhibited higher tumor inhibition efficiency than double drugs and single drug-loaded MLNPs ($P < 0.01$), this may attribute to the synergy effects of the three agents. Compare CISP/VNR/ATRA MLNP with CIS/VNR/ATRA MLNP, more significant antitumor efficacy was found by prodrug-based CISP/VNR/ATRA MLNP ($P < 0.05$). Drugs loaded nanoparticles illustrated stronger in vivo tumor inhibition effects than Free CISP/VNR/ATRA ($P < 0.05$).

In Vivo Tissue Distribution

Higher tumor accumulation was found on MLNP formulations in comparison with CISP/VNR/ATRA NP and Free CISP/VNR/ATRA (Figure 10) ($P < 0.05$). In the meantime, drugs distribution in kidney and heart was lower for MLNP and CISP/VNR/ATRA NP formulations than the Free CISP/VNR/ATRA ($P < 0.05$). These results may influence the systemic toxicity of these systems when delivered in vivo.

Systemic Toxicity

No significant change of body weight was found on drugs loaded nanoparticles groups (Figure 11A). However, free CISP/VNR/ATRA exhibited obvious body weight lost, which may indicate the systemic toxicity. ALT, LDH, and BUN levels illustrated that drug-loaded nanoparticle groups did not change the parameters compared with
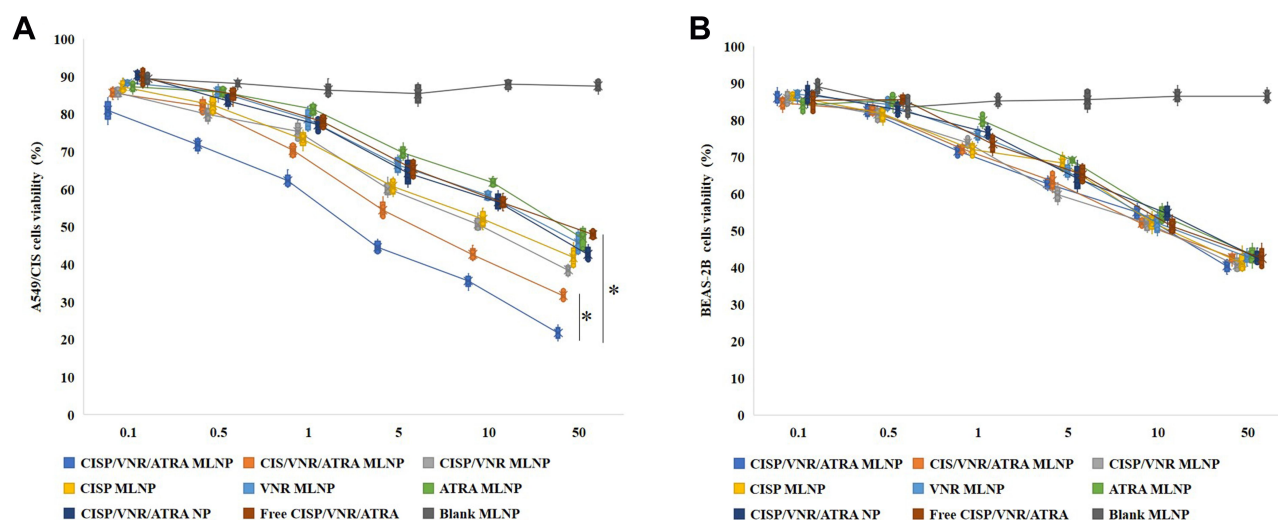
Figure 7 In vitro cytotoxicity. Concentration deepened manners were found in all drugs contained formulations on both A549/CIS (**A**) and BEAS-2B cells (**B**). *P < 0.05.
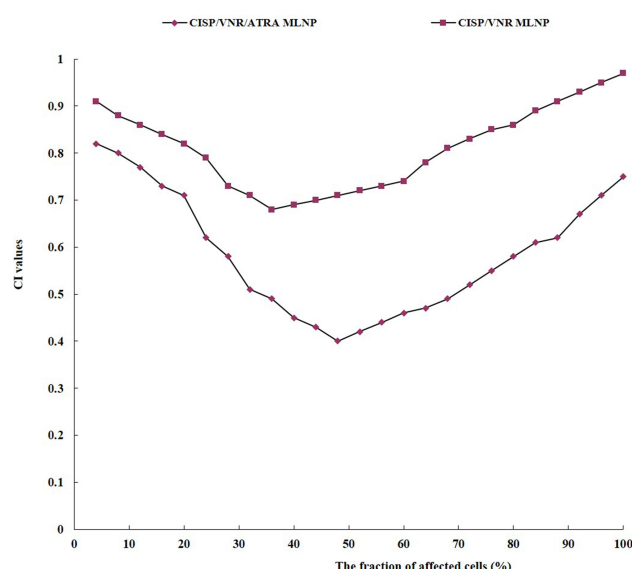
Figure 8 The synergistic cytotoxicity was evaluated by CI values on the bases of IC$_{50}$ values of different formulations. The effect of three-drug combination treatment was compared with the effect of treatment with each drug alone.

control groups, while free drugs caused significant increase of these data (Figure 11B–D) (P < 0.05).

Discussion

In this study, CIS prodrug (CISP) was synthesized and used as an antitumor agent, which was loaded in a multi-layered nano-platform. CIS prodrugs were widely used to overcome the main problems that limit the clinical use of cisplatin include acquired resistance and serious side effects such as neurotoxicity and nephrotoxicity, what is closely related to the lack of tumor selectivity of cisplatin.[46] Examples included that Ling

et al, produced a cisplatin conjugated 1.2-distearoyl-*sn*glycero-3-phosphoethanolamine-N-mPEG$_{3000}$ to reverse cisplatin resistance in ovarian cancer.[47] As the start of the research, a CIS prodrug: CIS-SA-TRP was synthesized using an amino acid: tryptophan. Tryptophan is the precursor of serotonin, an important neurotransmitter, and one of the essential amino acids in human body.[48] It could be used as nutrition supplements and anticonvulsant. As a tranquilizer, it can regulate mental rhythm and improve sleep.[49] Tryptophan was used to produce CIS prodrug here and the in vivo effects will be discussed later.

A multi-layered nano-platform CISP/VNR/ATRA MLNP was constructed by CISP and VNR loaded polymeric core, ATRA contained middle layer, and HA-coated outer layer. TEM image of CISP/VNR/ATRA MLNP was spherical particles with inner cores and coats on the outer surface. The size and zeta potential of CISP/VNR/ATRA MLNP and other MLNPs are similar, which illustrated the loading of drugs did not change the size and surface charge of the particles.[50] CISP/VNR/ATRA NP showed a smaller size and reversed negative surface charge than CISP/VNR/ATRA MLNP, indicating the HA coating enlarged the size and bring negative surface charge.[34] HA is reported to prevent unnecessary protein adsorption and opsonization in the blood stream and also has targeting ability to the CD44 overexpressing cancer cell line.[51,52] The sizes and PDIs of MLNPs remained unchanged during the serum stability test, indicating the MLNPs may have good stability when administrated in vivo. This was in accordance with the founding of Li et al, who concluded that no significant size changes means the proteins in the
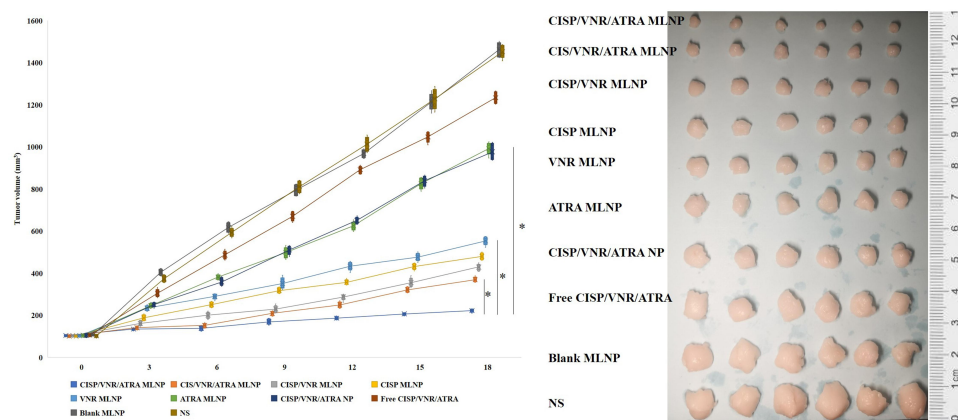
Figure 9 In vivo tumor inhibition ability of CISP/VNR/ATRA MLNP, CISP/VNR/ATRA NP and Free CISP/VNR/ATRA. *P < 0.05.
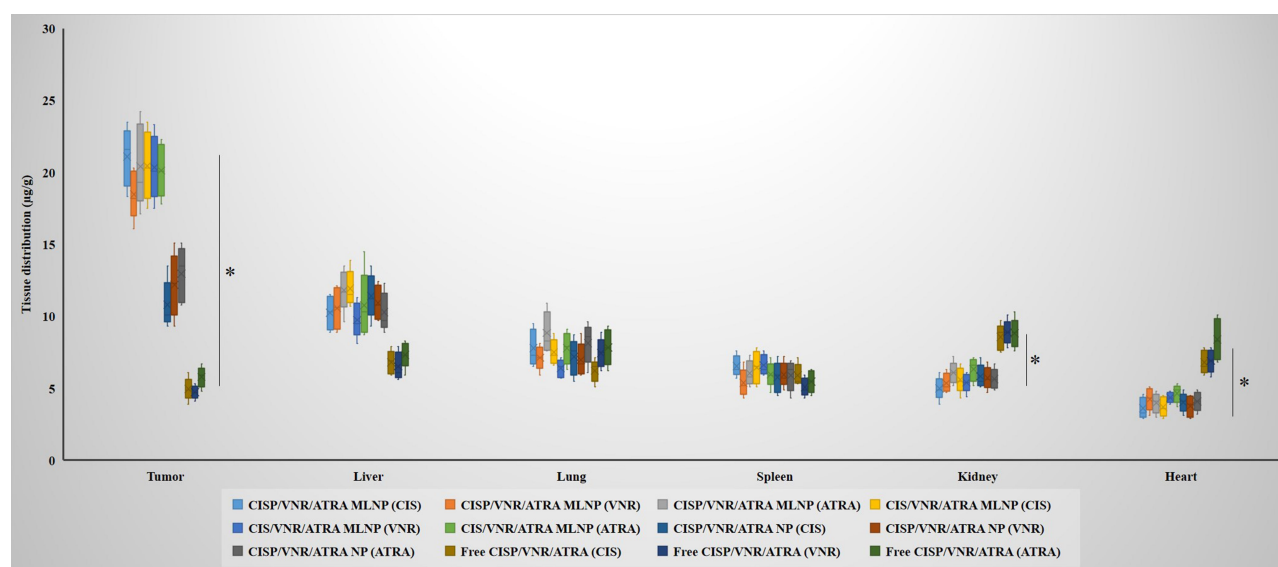

Figure 10 In vivo tissue distribution of CISP/VNR/ATRA MLNP, CISP/VNR/ATRA NP and Free CISP/VNR/ATRA. *P < 0.05.

serum did not adsorb on the nanoparticles and did not cause aggregation, thus leading to increase in particle size.[37]

The sustained release of drugs from nanocarriers may be due to homogenous entrapment of the drugs, and the anticancer drugs could maintain continuous efficiency.[53] In the section, drugs showed sustained release from MLNPs. Faster release was observed by ATRA when compared with CISP and VNR, this may be explained by the different position of drugs in the multi-layered carriers. CISP and VNR are encapsulated in the inner core, while ATRA are loaded in the middle layer. The depth of drugs loaded in the system can affect the release behavior.[54] Drugs in the outer layers may release more quickly than the inner layers. So the effective and specific release manner of

drugs in the presence of serum suggests that this multi-layered nano-platform may be more effective as an anti-tumor agent with sustained release and excellent stability in the blood.[55]

The cellular uptake efficiency of CISP/VNR/ATRA MLNP and non-HA coating CISP/VNR/ATRA NP was evaluated on A549/CIS cells, which have been reported to overexpress CD44 receptors on cell surface.[56] Remarkably higher uptake efficiency of CISP/VNR/ATRA than CISP/VNR/ATRA NP. This phenomenon clearly demonstrates the role of HA-CD44 interaction in terms of enhancing cellular uptake.[57] HA-coated multi-layered nano-platform may penetrate high overexpressed CD44 cells (A549 cells) through a receptor-mediated endocytosis.[57] The penetration of drugs can be improved
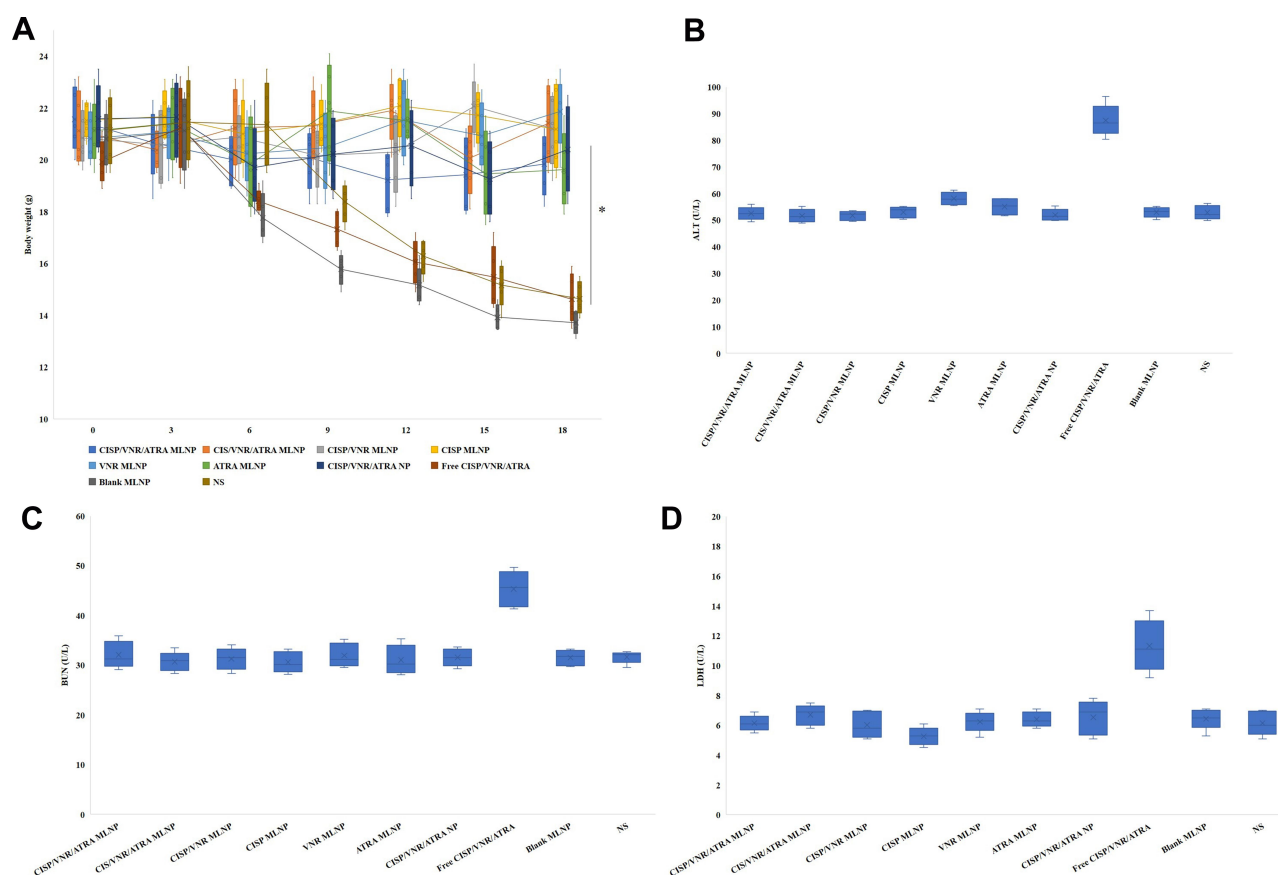
Figure 11 Drugs loaded nanoparticles groups presented no significant change of body weight (**A**). ALT (**B**), LDH (**C**), and BUN (**D**) levels illustrated that drugs loaded nanoparticles groups did not change the parameters compared with control groups, while free drugs caused significant increase of these data. *P < 0.05.

by HA surface layer thus could reduce the drug dose, attenuate side effect, and overcome drug resistance.[58]

Combination therapy using nanocarriers containing CIS has received much interest in cancer therapy to overcome CIS-resistant.[59] For example, Cheng et al developed a CIS and Curcumin co-loaded liposomal delivery system to deliver into hepatocellular carcinoma cells.[60] CIS and Curcumin co-encapsulated lipid-polymer hybrid nanoparticles were prepared by Li et al which showed and higher cytotoxicity than free drugs.[61] Our study evaluated three agents encapsulated in one multi-layered nano-platform. The cell viability was more remarkably inhibited by CIS/VNR/ATRA MLNP than CISP/VNR/ATRA NP on A549/CIS cells but not BEAS-2B cells. These were in line with the cellular uptake results that HA-coated multi-layered nano-platform may penetrate high overexpressed CD44 cells (A549 cells) and exhibited more prominent cytotoxicity. To further determine the synergistic cytotoxicity, CI values were evaluated by on the bases of IC$_{50}$ values of different formulations. More remarkable combination effect was exhibited by CISP/VNR/ATRA MLNP than

that of CISP/VNR MLNP, indicating the better efficiency brought by ATRA.[62]

In vivo tumor inhibition ability of CISP/VNR/ATRA MLNP was better tumor than CISP/VNR/ATRA NP, which suggests the multi-layered nano-platform with an HA coat may increase the stability and the concentration of drugs in the blood and tumor.[63] The results were summarized by Liu L et al that multi-layered nano-platform can also promote the apoptosis of lung cancer cells in vivo, thereby promoting subsequent apoptotic signaling and cell apoptosis, which is consistent with that in vitro.[64] The in vivo distribution of drugs loaded in MLNPs followed the order: tumor > liver > lung > spleen > kidney > heart, which is in accordance with the conclusion drawn by Liu J et al.[65] They argued that these results were attributed to the efficient accumulation of MLNPs in the tumors through HA targeting to the CD44 receptor. Body weight, ALT, LDH, and BUN parameters of MLNPs remained stable, which could be the evidence of the side effects were successfully alleviated.[45] Our future study will devote to the application of this nano-platform from

bench to bedside. Our group has devoted to the design and evaluation of these nano-platforms, which will be eventually used to treat cancer patients.

Conclusion

A multi-layered nano-platform CISP/VNR/ATRA MLNP was prepared. It has layered morphology with particle size and zeta potential of 158 nm, and 12.3 mV. As high as 81.36% of CISP/VNR/ATRA MLNP was uptake by cancer cells in vitro. CISP/VNR/ATRA MLNP could significantly inhibit the in vivo antitumor growth and suspended the tumor volume from 1440 mm^3 to 220 mm^3. CISP/VNR/ATRA MLNP may be used as a promising system for lung cancer combination treatment.

Disclosure

The authors report no conflicts of interest for this work.

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