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Original Research Article

Co-delivery of combretastatin A4 and docetaxel with pegylated nanostructured lipid carriers in tumor cells

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Abstract

Purpose: *To investigate a novel co-delivery system using nanostructured lipid carriers (NLCs) for simultaneous administration of two potent anti-cancer drugs, combretastatin A-4 (CA-4) and docetaxel (DTX), against tumor cells and vasculature.*

Methods: *The CA-4 and DTX co-loaded NLCs (C-D-NLC) were formulated and investigated for physical properties, stability, and drug release. Safety and efficacy of C-D-NLC were investigated on Lewis Lung Carcinoma (LLC) tumor cells in vitro and in vivo using cytotoxicity and anti-tumor assays. The pharmacokinetics of CA-4 and DTX in rats after intravenous injection of C-D-NLC were also studied to evaluate potential drug interactions.*

Results: *The C-D-NLC was successfully prepared with a spherical shape, mean size of 130 nm, negative charge, high encapsulation efficiency and drug loading of 94.89, 88.16, 2.44, and 4.52 for DTX and CA-4, respectively. Also, C-D-NLC had a significant inhibitory effect on LLC cells, superior to a single drug or solution group. Combretastatin A4 did not affect the pharmacokinetics of DTX, but combretastatin–docetaxel nanostructured lipid carriers (C-D-NLC) reduced plasma clearance of CA-4 and DTX, prolonged half-life, mean residence time, and increased area under concentration curves (AUC) values. Furthermore, combretastatin–docetaxel nanostructured lipid carriers (C-D-NLC) inhibited the growth of LLC tumors in mice and reduced drug toxicity.*

Conclusion: *Combretastatin–docetaxel nanostructured lipid carriers (C-D-NLC) sustain drug release and enhance tumor growth inhibition of CA-4 and DTX by targeting both tumor cells and vasculature. The co-delivery system prolongs drug circulation compared to solution administration. Thus, nanostructured lipid carriers (NLCs) with dual drug loading may be a promising strategy for clinical combination chemotherapy in future.*

Keywords: *Combination therapy, Angiogenesis, Nanostructured lipid carrier, Combretastatin A-4, Docetaxel*

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INTRODUCTION

Anti-angiogenesis therapy is a key strategy to inhibit tumor growth and metastasis [1]. However, tumor angiogenesis involves multiple pathways that may limit efficacy of single antiangiogenic agents [2]. Moreover, these agents may induce hypoxia and drug

resistance in tumor cells [3]. Therefore, combining anti-angiogenesis drugs with conventional cytotoxic agents may enhance therapeutic effects and reduce toxicity [4].

Combretastatin A-4 (CA-4) is a potent antiangiogenic drug that binds to tubulin and disrupts cytoskeleton of endothelial cells, leading to vascular collapse and tumor cell death [5]. Docetaxel (DTX) is a cytotoxic agent that inhibits microtubule depolymerization and induces apoptosis in cancer cells [6]. Nanotechnology can overcome multidrug resistance by delivering drugs to tumor tissues using nanocarriers that enhance permeability and retention (EPR) effect [7]. Nanostructured lipid carriers (NLCS) are promising nanocarriers that have high drug-loading capacity, controlled drug release profiles, and low drug expulsion [8]. Polyethylene glycol (PEG) prolongs circulation time of nanoparticles and increases passive accumulation in tumor tissues [9].

In this study, physicochemical, pharmacokinetic properties and synergistic antitumor effects of pegylated NLC co-loaded with CA-4 and DTX (C-D-NLC) were investigated on rats bearing Lewis Lung Carcinoma (Figure 1).

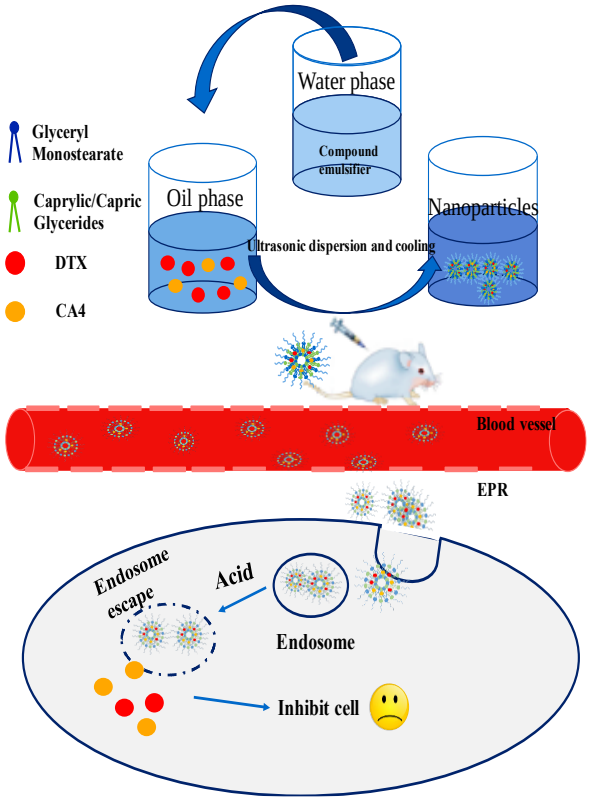


Figure 1: Schematic diagram of preparation and systematic evaluation of nanoparticles

EXPERIMENTAL

Cells and animals

Lewis lung carcinoma (LLC) (Shenyang Pharmaceutical University) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10 % fetal bovine serum (FBS) in a humid incubator (5 % co_2 at 37 ºc). Cells were thereafter detached from trypsin and rinsed in non-supplemented media before inoculation. Male Sprague Dawley (SD) rats (200 ± 20 g, 12 weeks old) and male C57BL/6 mice (20 ± 2 g, 6 – 8 weeks old) (Animal Center, Hebei Medical University) were acclimated for 1 – 2 weeks before experimentation, fed a standard diet, and given unlimited access to water. The animal study was done at Animal Center of Fourth Hospital of Hebei Medical University. Ethics committee of the hospital reviewed and approved all procedures on the animals (Approval No. SYXK(JI)2018-001) which also complied with international guidelines for animal care and use.

Preparation of NLC co-loaded with combretastatin A-4 and docetaxel

Formulation was prepared according to the method of Zhang *et al* [10]. Drug-loaded NLCS of combretastatin A-4 (Hangzhou Great Forest Biomedical Ltd) and docetaxel (Shanghai Jinhe Bio-Technology Co., Ltd) were prepared using melt-emulsification method. Monostearin (300 mg), 25 % w/w miglyol®812 and 1 - 10 % w/w of the drugs relative to lipid were melted and mixed at 75 °c. The molten mixture in 10 ml of a 2.5 % w/w surfactant solution (SPC/SOLUTOL HS15®/PEG-40 stearate at a 1:1:3 ratio) was preheated to 75 °C, using a mechanical stirrer to obtain a preliminary emulsion which underwent laboratory ultrasonic cell disruptor at 400 watts for 5 mins to form a mini-emulsion. Preparation was cooled quickly by immersing in an iced water bath at 0 °C, continuously stirred and thereafter filtered through a 0.22 mm membrane to obtain a uniform dispersion of NLCS.

Particle size and zeta potential analysis

Average volume diameter of drug-loaded NLCS was determined with laser diffractometry (LD) using a Coulter® LS 230 at room temperature. Particle size and dimension with a zeta potential analyzer (DELSA 440SX). Measurements were done in triplicates and averages were taken.

Determination of combretastatin A-4 and docetaxel incorporated into NLC

Amounts of combretastatin A-4 and docetaxel in NLC were determined using high-performance liquid chromatography (HPLC) (HITACHI 2000 series LC system with a Topsiltm C18 column at a flow rate of 1.0 ml/min and detected at a wavelength of 228 nm). Combretastatin A-4 and docetaxel NLCS were dissolved in absolute methanol and injected into HPLC. Mobile phase was acetonitirle (50/50, v/v). Calibration curves of peak area against concentration of combretastatin A-4 and docetaxel (µg/ml) were shown respectively in Eqs 1 and 2.

$Y = 86827X - 1346.0$ ($R^2 = 0.9999$) ..... (1)

$Y = 22619X + 634.5$ ($R^2 = 0.9995$) ..... (2)

Where y is drug concentration (µg/ml) and x is peak area. Linearities of methods were studied in the range of 0.1 - 10 µg/ml [11].

Drug entrapment efficiency and drug loading capacity

The suspension (0.5 ml) of C-D-NLC was eluted with distilled water in a SEPHADEX-G50 column, and opalescence part of the eluate was collected. Concentrations of combretastatin A-4 and docetaxel in collected eluate and suspensions were investigated using HPLC after dilution with absolute methanol. Entrapment efficiency (EE) and drug loading capacity (LC) were determined using Eqs 3 and 4.

$EE\ (\%) = (W_S/W_{total})100$ ..... (3)

$LC\ (\%) = (W_S/W_{lipid})100$ ..... (4)

Where W_S is amount of combretastatin A-4/docetaxel encapsulated in NLCS, W_{total} is amount of combretastatin A-4/docetaxel in nanoparticle suspensions, and W_{lipid} is weight of vehicle.

Transmission electron microscopy (TEM)

Morphology of C-D-NLC was determined using a TEM (JEM-1200). Nanoparticle dispersion was applied to a copper grid coated with carbon film and air-dried. Grids were sprayed with 2 % (w/v) phosphotungstic acid (PTA). Transmission electron microscopy (TEM) determinations were done after negative staining and air-drying samples at room temperature [12].

***In vitro* release studies**

Volume of 1 ml C-D-NLC was fed into a dialysis bag with a cutoff molecular mass of 14 000 da, and placed in 100 ml of release medium containing 0.5 % tween-80 phosphate buffer (pH 7.4) at 37 °c, 100 rpm. Sample solutions (1 ml each) were collected at 0.5, 1, 2, 4, 8, 12, 24, 36, 48, and 72 h, and supplemented with 1 ml of fresh release medium at same time. The samples were filtered through a 0.45 µm microporous membrane and concentration of DTX and CA-4 in release medium was determined using HPLC and percentage of drug cumulative release was computed.

Differential scanning calorimetry (DSC)

Differential scanning calorimeter (dsc) analysis was done using a Mettler DSC apparatus. A total of 3.5 g of samples was transferred into an aluminum pan, which was then sealed. Temperature was increased from 20 to 250 °c at a heating rate of 10 °c/min with a nitrogen purge using an empty aluminum pan as reference. Measurements were carried out on the following samples: (a) lyophilized C-D-NLC; (b) lyophilized drug-free NLC; (c) combretastatin A-4; (d) docetaxel; (e) mixture of combretastatin A-4 and docetaxel; (f) physical mixture of combretastatin A-4, docetaxel and mixed lipid; and (g) mixed lipid composed of monostearin and 25 % Miglyol®812 [13].

***In vitro* cytotoxicity studies**

Cytotoxicity of blank and drug-loaded NLCS was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) assay. Lewis lung carcinoma (LLC) cells (1×10^4 cells/well) were seeded in 96-well plates and cultured in a humidified atmosphere with 5 % CO_2 at 37 °c overnight. The cells were treated with nanoparticles for 24 h, and then 5 mg/ml MTT (in phosphate-buffered saline) was added to each well when they had reached 70 – 80 % confluence. Furthermore, dimethyl sulfoxide (DMSO) was used to dissolve formazan crystals formed in live cells after incubating for 4 h. Absorbance at 490 nm was recorded as a percentage of control cell viability. Experiments were done in triplicate [14].

Pharmacokinetic studies in rats

A total of 30 rats were randomly divided into six groups (5 per group): group 1 (DTX solution); group 2 (CA-4 solution), group 3 (DTX and CA-4 solution), group 4 (DTX loaded NLC (D-NLC)); group 5 (CA-4 loaded NLC (C-NLC)); and group 6 (C-D-NLC). The dosage of DTX and CA-4 were 10 and 20 mg/kg, respectively. Rats were fasted overnight but given water before administration, and drug preparations were administered intravenously via the tail vein. Blood samples were collected from retro-orbital plexus at different times after injection into microtubes with sodium heparin. Plasma extracts were prepared by mixing plasma with methyl tert-butyl ether. Internal standard solution (10 µg/mL) was added to each serum sample and further extracted with methyl tert-butyl ether on a vortex mixer for 60 s. It was then centrifuged at 10,000 rpm for 10 min, and organic layer was transferred to a clean test tube which was later evaporated under nitrogen at 40 °C. The residue was reconstituted with 100 L of acetonitrile, mixed with a vortex mixer for 60 s, and injected into HPLC (Diamond C18 column) for analysis. The mobile phase was acetonitrile, methanol and water (46: 3: 52, v/v/v) with 1 mM phosphoric acid at a flow rate of 1.0 mL/min, detection wavelengths of 228 nm, and column temperature of 25 °C.

Major pharmacokinetic parameters were calculated using statistical moment method with DAS 2.0 software. Non-compartmental pharmacokinetic parameters such as mean residence time (MRT) and clearance (CL) were also computed.

Determination of *in vivo* antitumor activity

Two hundred and fifty liters of serum-free medium were used to inoculate LLC cells into six to eight-week-old C57BL6 male mice subcutaneously on their right flanks. A drug administration schedule was initiated when an average tumor size of 50 mm^3 was reached. Tumor-bearing mice were randomly sorted into seven groups (8 mice/group), and each group was treated by tail vein injection every 48 h at drug doses of DTX 5 mg/kg and CA-4 20 mg/kg respectively. Treatments included one of the following formulations: physiological saline (control); CA-4 dissolved solution (CA-4-SOL); DTX dissolved solution (DTX-SOL); CA-4 and DTX dissolved solution (C-D-SOL); CA-4 loaded NLC (C-NLC); DTX loaded NLC (D-NLC); and CA-4 and DTX co-loaded NLC (C-D-NLC). Tumor size and animal weight were monitored every 2 days until the end of experiment. Measurement of tumor size was performed with a caliper in two dimensions, and individual tumor volumes (V) were calculated using Eq 5.

$$V = \{length \times (width)^2\}/2 \quad \text{..... (5)}$$

At the end of the experiment, 20 µL aliquot of blood from medial canthus was collected for white blood cell counts. Animals were then euthanized by CO_2 asphyxiation followed by cervical dislocation, by AVMA guidelines for euthanasia of animals, and tumors were excised and measured.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical comparisons between two groups were performed using Student's t-test for independent samples. One-way analysis of variance (ANOVA) was utilized to analyze differences in overall therapeutic efficacy of the various treatments on tumor growth. Statistical analyses were conducted employing SPSS software (version 25.0, SPSS Inc, IBM, Armonk, NY, USA).

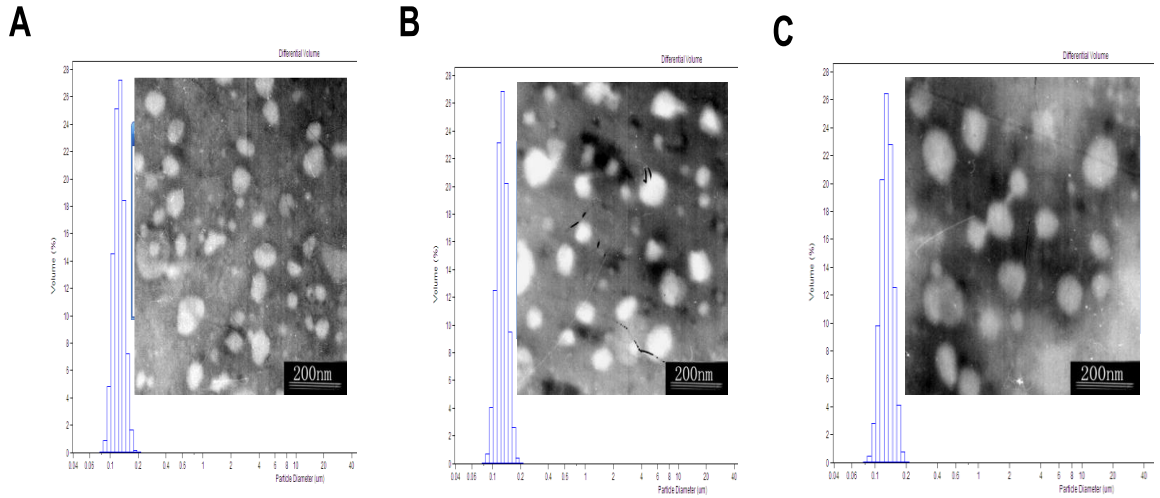
RESULTS

Preparation and characterization of C-D-NLC

Combretastatin and docetaxel nanostructured lipid carriers (C-D-NLC) were transparent liquids with bluish opalescence and spherical shapes with a mean size of about 130 nm (Figure 2 A - C). They also had a negative surface charge of -11.2 mV (Figure 2 D) and high entrapment efficiency of 94.89 and 88.16 % for DTX and CA-4, respectively (Figure 2 E). Drug loading capacities were 2.44 and 4.52 % for DTX and CA-4, respectively (Figure 2 F). These results showed that NLC can encapsulate drugs with no significant differences in the pharmaceutical properties between different groups ($p > 0.05$).

In vitro release

Release of CA-4 from C-D-NLC was faster at 0 – 12 h. Cumulative drug release was almost 60 % within 12 h. However, the release became slower between 12 – 72 h. Cumulative drug release in 72 h was more than 80 %. Similarly, DTX release was rapid between 0 – 8 h (cumulative release 30 % to approximately 40 %), and release became slow between 8 – 72 h. The cumulative drug release within 72 h was 70 – 80 % (Figure 3).



D

Samples	Zeta potential（mV）	RSD (%)
DTX-NLC	-13.2	3.30
CA4-NLC	-12.1	2.48
C-D-NLC	-11.2	4.01

E

Drugs	EE(%)	RSD (%)
DTX	94.89	1.04
CA4	88.16	2.04

F

Drugs	LC (%)	RSD (%)
DTX	2.44	1.31
CA4	4.52	2.03

Figure 2: Characteristics of NLC. (A) Particle size of DTX-NLC (B) Particle size of CA-4-NLC, (C) The particle size of C-D-NLC (D) Zeta potential of DTX-NLC, CA-4-NLC and C-D-NLC (n = 3). (E) Encapsulation efficiency of DTX-NLC, CA-4-NLC and C-D-NLC (n = 3). (F) Drug loading of DTX-NLC, CA-4-NLC and C-D-NLC (n = 3)

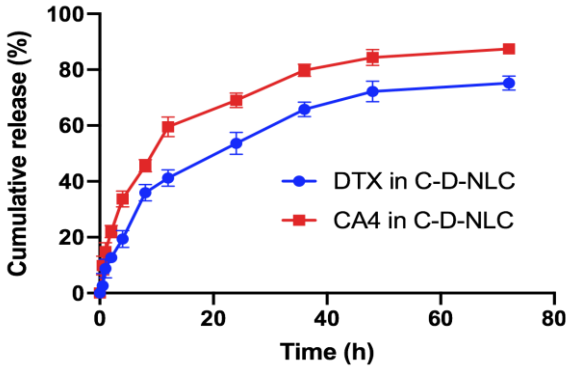


Figure 3: Release rate of DTX and CA-4 from NLC nanoparticles at different time points in pH 7.4 environment (n = 3)

DSC findings

Lyophilized NLC formulations had no drug melting peaks, indicating homogeneous dispersion and no crystallization. Single drugs of CA-4 and DTX had melting peaks at 116.1 and 167.6 °C, respectively (Figure 4). The drug mixture had a single endothermic peak at 113.5 °C, indicating a possible eutectic phenomenon. The drug-lipid mixture had reduced and shifted peak at 105.4 °C, suggesting encapsulation in lipid matrix. An additional peak at 155.5 °C was due to the stabilizers, trehalose and mannitol. Nanostructured lipid carriers (NLCs) had a less ordered lipid matrix and DTX may exist as an amorphous or molecular state.

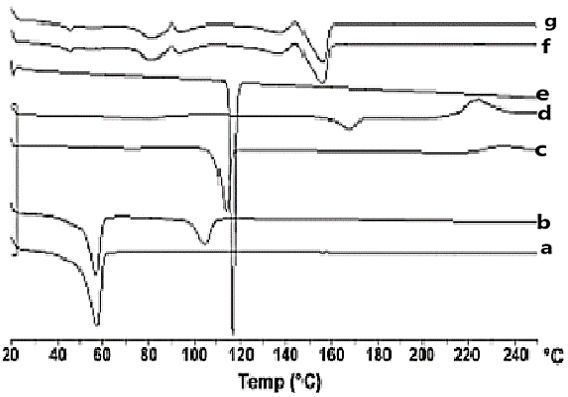


Figure 4: Differential scanning calorimetry for different samples

In vitro cell viability

Cell viability of NLC nanoparticles with different concentrations were all greater than 95 % at 24 h, which means that vectors of NLC had insignificant cytotoxic effects against LLC cells (Figure 5 A). Compared to control, combined

nano-preparation administration significantly (p < 0.001) inhibited cell growth (Figure 5 B). Moreover, compared with free drug group, nano-preparation group further increased inhibitory effect on tumor cells (p < 0.01). These results revealed that combined administration of NLC nanoparticles effectively inhibited growth of tumor cells.

Pharmacokinetic characteristics

The behavior of nanocarriers *in vivo* revealed that DTX and CA-4 solution were quickly eliminated from the blood. Plasma concentrations of docetaxel and CA-4 were below detection limits after 240 and 300 min. But DTX and CA-4 in nanostructured lipid carriers were slowly eliminated. Coadministration of DTX and CA-4 in C-D-NLC did not affect their pharmacokinetics (Figure 6). DTX and CA-4 in nanostructured lipid carriers had significantly lower clearance (CL; p < 0.01), higher area under curve (AUC), MRT, and half-life (T1/2; p < 0.05) than in solutions.

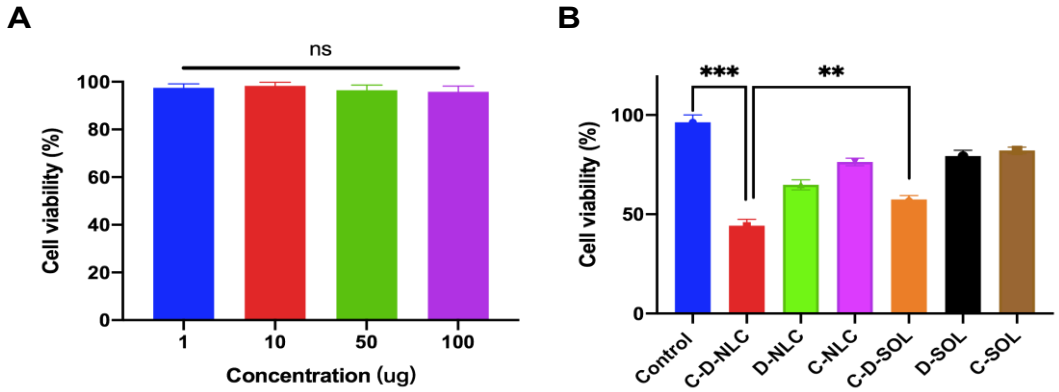


Figure 5: (A): *In vitro* cytotoxicity studies of blank NLC nanoparticles with different concentrations at 24 h (n = 3) (B): *In vitro* cytotoxicity studies of different groups at 24 h. Note: D-SOL, solution of DTX; D-NLC, DTX loaded NLC; C-SOL, solution of CA-4; C-NLC, CA-4 loaded NLC; C-D-SOL, solution of CA-4 and DTX; C-D-NLC, CA-4 and DTX coloaded NLC, (DTX, 10 ng/mL, CA-4, 20 ng/mL). (n = 3, **p < 0.01, ***p < 0.001)

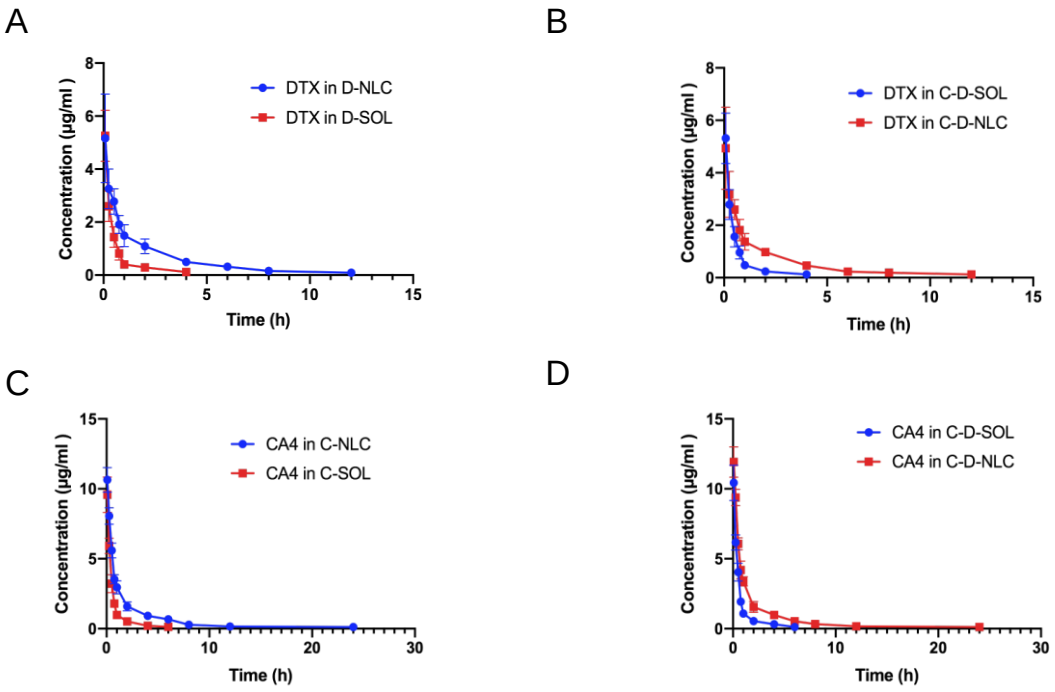


Figure 6: Mean plasma concentration of different formulations after i.v. administration: (A) DTX in D-SOL and D-NLC; (B) DTX in D-NLC and C-D-NLC. (C) CA-4 in C-SOL and C-NLC; (D) CA-4 and DTX in C-D-SOL and C-D-NLC, respectively (n = 5). Note: D-SOL, solution of DTX; D-NLC, DTX loaded NLC; C-SOL, solution of CA-4; C-NLC, CA-4 loaded NLC; C-D-SOL, solution of CA-4 and DTX; C-D-NLC, CA-4 and DTX coloaded NLC

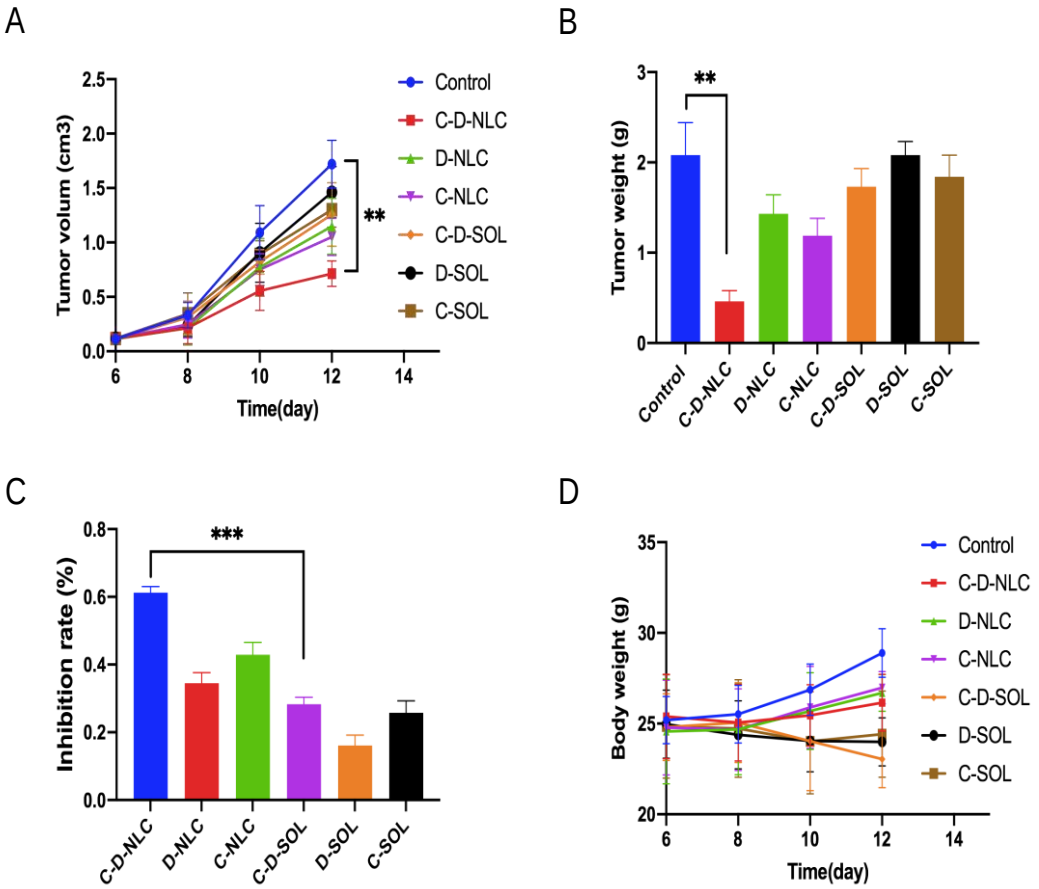


Figure 7: Therapeutic effect by multiple-dose administration of various C-D-NLC at the doses of 20 mg CA-4/kg and 5 mg DTX/kg in C57BL/6 mice bearing LLC tumor with physiological saline as a negative control and docetaxel as a positive control. **Note:** D-SOL, solution of DTX; D-NLC, DTX loaded NLC; C-SOL, solution of CA-4; C-NLC, CA-4 loaded NLC; C-D-SOL, solution of CA-4 and DTX; C-D-NLC, CA-4 and DTX coloaded NLC. (A): Tumor volume; After a 24 h treatment period, tumor growth inhibition was observed in LLC tumor-bearing mice following multi-injections of different formulations. (B): Tumor's weight in each treatment group. (C): Tumor's inhibitory rate in each treatment group. (D): Body weight; Following initial treatment, body weights and tumor size were measured. $^{**}P < 0.01$, $^{***}p < 0.001$)

Antitumor activity

Docetaxel solution barely affected tumor growth, but docetaxel nanostructured lipid carriers shrank tumors. Combretastatin and docetaxel nanostructured lipid carriers (C-D-NLC) group (docetaxel 5 mg/kg and CA-4 20 mg/kg) exhibited strongest antitumor effect, significantly higher than other groups (n = 3). This implies that CA-4 and docetaxel have synergistic effects and nanostructured lipid carriers further improve synergism (Figure 7 A to C). Nanostructured lipid carriers also lowered side effects with no significant reduction in body weight (Figure 7 D).

DISCUSSION

Particle size and surface charge of NLC are important factors that affect biodistribution, cellular uptake, and tumor accumulation. Nanoparticles with sizes smaller than 200 nm have enhanced permeability and retention (EPR) effect and preferentially accumulate in tumor tissues through leaky tumor vasculature [15]. Furthermore, nanoparticles with negative surface charge have reduced nonspecific interactions with plasma proteins and avoid opsonization and phagocytosis by macrophages. Therefore, C-D-NLC nanoparticles have suitable characteristics for tumor targeting and delivery.

The release mechanism of NLC is mainly controlled by diffusion and erosion. *In vitro* release profiles of CA-4 and docetaxel from nanostructured lipid carriers (NLCs) showed an initial rapid release followed by a slower sustained release. This biphasic release pattern may be attributed to quick release of adsorbed drugs on the NLC surface, while those incorporated in the lipid matrix diffused out slowly. This release kinetics allows for a rapid

therapeutic dose to be achieved initially, followed by prolonged exposure.

A pharmacokinetic study revealed extended circulation and enhanced bioavailability of both drugs when delivered by NLCs compared to free drug solutions. Smaller sizes and pegylation of NLCs reduce opsonization and avoidance of the reticuloendothelial system, leading to longer circulation times [16]. This allows greater accumulation of the NLCs and their drug cargo in leaky tumor vasculature through EPR effect.

In Lewis lung carcinoma model, all treatment groups showed tumor growth inhibition compared to saline controls. Docetaxel solution group had minimal efficacy as this aggressive tumor model is largely refractory to standard chemotherapeutics like docetaxel. In contrast, dual drug-loaded NLCs exhibited superior antitumor activity compared to either free drug solutions or empty NLCs. Enhanced efficacy of vascular disrupting CA-4 and anti-mitotic docetaxel was likely due to collaborative effects of vascular shutdown and apoptosis induction mediated by synergistic drug actions [17].

CONCLUSION

The developed dual drug-loaded nanostructured lipid carriers display favorable physicochemical and pharmaceutical properties. Combination of CA-4 and docetaxel in nano-delivery systems results in enhanced pharmacokinetics and antitumor efficacy compared to free drug solutions. These findings demonstrate the potential of this nano-platform for combination chemotherapy using synergistic drug pairs.

DECLARATIONS

Acknowledgements

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Ethical approval

This study was approved by the ethics committee of The Fourth Hospital of Hebei Medical University (Approval No.IACUC-4th Hos Hebmu-2022004).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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