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RESEARCH PAPER

Tumor-Specific Delivery and Therapy by Double-Targeted DTX-CMCS-PEG-NGR Conjugates

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ABSTRACT

Purpose To synthesize and evaluate the antitumor efficacy of double-targeted docetaxel (DTX)-carboxymethyl chitosan (CMCS)-PEG-NGR (DTX-CPN) conjugates that could target to CD13 over-expressed tumor neovascular endothelium cells and tumor cells.

Methods DTX was conjugated to CMCS via biodegradable linker and cNGR was applied to endow the conjugates with double targeting ability. The physiochemical properties and stability of this DTX-CPN conjugates were characterized. Cellular uptake study was carried out to evaluate the targeting ability of DTX-CPN conjugates. Cytotoxicity and apoptosis analysis were conducted to evaluate *in vitro* antitumor effects. *In vivo* antitumor efficacy was investigated in B16 murine melanoma model.

Results DTX-CPN conjugates could self-assemble into nanoparticles in water and were stable in plasma. cNGR modification could promote the cellular uptake of DTX-CPN conjugates in CD13 positive HUVEC and B16 cells, leading to more significant cytotoxicity and apoptosis effect than non-targeted conjugates. DTX-CPN conjugates also exhibited better antitumor effect than non-targeted conjugates and Duopafei® in a B16 murine melanoma model.

Conclusions Double-targeted DTX-CPN conjugates could efficiently target to tumor neovascular cells and tumor cells, and achieve good antitumor effects. DTX-CPN conjugates may be promising candidate for one-double targeting cancer therapy.

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KEY WORDS CARBOXYMETHYL CHITOSAN · cNGR · Docetaxel · Double-targeted · Polymer-drug conjugates

ABBREVIATIONS

CMCS	Carboxymethyl chitosan
cNGR	Cylic NGR
CP	CMCS-PEG
CPN	CMCS-PEG-NGR
DTX	Docetaxel
DTX-CP	DTX-CMCS-PEG
DTX-CPN	DTX-CMCS-PEG-NGR

INTRODUCTION

Angiogenesis plays important role in cancer progression and metastasis. It is believed that that blocking the formation of new blood vessels could inhibit tumor progression and even starve existing tumors of oxygen and nutrients, eventually leading to the recession of the tumors (1,2). It is reported that there is approximately one endothelial cell per 100 tumor cells and tumor vessels are poorly formed with thin walls, often comprising just a basement membrane and endothelial cells (3), therefore, the endothelial cells of tumor vasculature are more readily accessible to drugs. Since tumor vasculature cells are genetically more stable, they have a much lower tendency to develop drug resistance than tumor cells (4). Furthermore, anti-angiogenic therapy could weaken the tumor vessels and decrease interstitial fluid pressure within the tumor, making better penetration of chemotherapeutic agents into tumor tissue (5). Therefore, destroying tumor endothelial cells turns out to be more potential than killing the tumor cells directly. The unique characteristics of the tumor neovascular endothelium make it a popular target for the design of innovative chemotherapy.

A number of proteins, such as vascular endothelial growth factor receptor (VEGFR) (5), $\alpha_v\beta_3$ (6), $\alpha_v\beta_5$ integrins (7) and APN/CD13 (4) are barely expressed in normal blood vessel cells but are over-expressed in neovascular endothelial cells and some kind of tumor cells. Development of double-targeted drug delivery system that target to these up-regulated proteins both in endothelial cells of angiogenic vessels and tumor cells offers opportunities for novel therapeutic targeting approaches, since these double-targeted drug delivery system enable maximizing the utility of anticancer drugs by combination blood vessel destruction with the conventional antitumor actions together (8).

NGR peptides show high affinity to tumor neovascular endothelial cells by recognizing the up-regulated tumor-specific isoforms of APN/CD13 that are over-expressed on these cells and it was reported that the specificity of NGR to APN/CD13 was three-fold higher than that of RGD peptides to $\alpha_v\beta_3$ integrins (4). In addition, many tumor cell lines are proved to express high levels of CD13, such as A549, SKOV-3, Hela, HT1080 and B16 cells, making them becoming target cells for NGR peptides (9,10). Compared to linear ones, cyclic NGR (cNGR) displays stronger affinity and higher specificity towards the APN/CD13 (11). Thus, utilization of cNGR peptide as tumor targeting moiety has been considered as a very promising strategy for cancer therapy and cNGR modified drug delivery system exhibited good targeting to CD13 positive tumor neovascular endothelial cells and tumor cells (12,13).

Docetaxel (DTX), a semisynthetic taxoid derived from the european yew tree (Taxus baccata), is an inhibitor of microtubule depolymerization and displays confirmed therapeutic activity against a broad spectrum of different kinds of tumors, including breast, ovarian, head and neck, and non small cell lung cancer etc. (14). DTX also exhibits anti-angiogenic effect at low doses due to its potent cytotoxic effect (15). However the clinical application of DTX is limited by severe side effects due to its insolubility and widespread distribution among the body (16). In order to increase the solubility of DTX, the present marketed formulation of DTX contain high concentration of polysorbate 80 (Tween-80) and ethanol, which are known to be harmful to liver and kidneys, causing dose-dependent hemolysis, hypersensitivity reactions, fluid retention, musculoskeletal toxicity and neurotoxicity (16). These adverse effects have hindered medical utility of DTX for intravenous administration to a great extent. Besides, due to the low molecular property, DTX shows short half time and non-selective distribution among the body, leading to only a small amount of DTX localizing in tumor (17). Therefore, there is an urgent need to develop efficient drug delivery system free of surfactants for DTX.

In recent years, polymer-drug conjugates have been emerging as an novel and potential platform for the delivery of low molecular anticancer drugs due to many advantages of them, which include increased drug loading capacity, enhanced stability, prolonged *in vivo* circulation time, enhanced intracellular uptake, better controlled release, tumor targeting by EPR effect and improved therapeutic efficacy (18). It was encouraging that some potential polymer-drug conjugates such as poly(L-glutamic acid)-paclitaxel (PGA-PTX) (19), HPMA-DOX (20), PEG-docetaxel (PEG-DTX) (21) and Cyclodextrin-CPT (22) have already advanced to clinical trials and PGA-PTX (also known as OpaxioTM, Xyotax® or CT-2103) developed by Cell Therapeutics Inc. was closet to market (23). Polymer-drug conjugates show bright prospects of clinical application and it is believed that this landmark will soon achieved as the success of PEGylated proteins (24).

Selection of a suitable polymer is crucial for the development of a successful polymer-drug conjugates. Firstly, Biocompatibility, biodegradability and safety for repeated administration are the fundamental requirements for polymers applied in polymer-drug conjugates. Secondly, It is believed that polymers with high molecular weight is beneficial for more drug accumulation in tumor tissue via EPR effect and prolonging the half time of drugs (25). Furthermore, polymers with multiple functional groups (unlike PEG which is only with two terminal functional groups) are more preferable due to the higher ability of carrying the drug/targeting residue payload (26). In recent decades, polysaccharides based drug delivery systems have been becoming more and more popular due to their outstanding merits, such as good biocompatibility, biodegradability, safety, solubility, abundant of resources in nature and low cost in of purification for polysaccharides (27). Carboxymethl chitosan (CMCS) is a kind of polysaccharides, which is proved to be non-toxic, non-immunogenic and biocompatible, and its pharmacokinetics and biodegradation mechanism are well characterized (28). Most importantly, there are abundant functional groups in CMCS, e.g. carboxyl groups, amino groups and hydroxyl groups, which are necessary for conjugation with low molecular drugs and targeting ligands. All these advantages mentioned above make CMCS a promising candidate as the polymer in the polymer-conjugates development and a few examples about drugs conjugation to CMCS via amine bonds have been reported (29,30).

These excellent biomedical and physiochemical properties of CMCS could be advantageously exploited to enhance the therapeutic effect of DTX via formation of polymer-drug conjugates. In order to utilizing the advantage of anti-angiogenic tumor therapy, cNGR was employed as the targeting ligand to modify CMCS and the developed CMCS-PEG-NGR (CPN) was used to afford DTX-CPN conjugates. The resulting nanosized DTX-CPN conjugates were expected to target tumors by passive targeting effect due to the structure of leaky capillaries in the tumor tissue. Besides,

cNGR modification could endow DTX-CPN conjugates with double targeting ability to CD13 positive tumor neovascular endothelial cells and tumor cells, achieving "one ligand modification for double targeting" (one-double targeting) drug delivery (tumor vascular- and tumor- targeted drug delivery) (Fig. 1). Furthermore, introduction of PEG as a hydrophilic shell layer could extend blood-circulation time by reducing mononuclear phagocyte system uptake of nanocarriers. Therefore, conjugation of cNGR peptide via a PEG spacer could provide high extracellular stability as well as high accessibility to tumor for DTX-CPN conjugates (31,32).

In this study, DTX-CPN conjugates were synthesized by reproducible multi-step chemical processes and the final products were characterized to confirm their structure and composition. Stability test was carried out to evaluate the stability of DTX-CPN conjugates in the plasma. The cell uptake study was carried out to assess the targeting effect of cNGR modification. *In vitro* cytotoxicity and Hochest staining studies were performed to evaluate the antitumor effect of DTX-CPN conjugates *in vitro*. *In vivo* antitumor effect of DTX-CPN conjugates was evaluated on B16 melanoma bearing mice.

MATERIALS AND METHODS

Materials

CMCS (average Mw = 50000; degree of carboxymethyl substitution = 60%; degree of deacetylation = 85%) was obtained from Jinan Haidebei Biological Engineering Co. (China). NHS-PEG-NHS (Mw = 2000) was from Biomatrik Technology Co., Ltd (JiaXing, China). cNGR peptide ($GGCNGRCONH_2$, disulfide bridge: 3–7) was synthesized by Shanghai Apeptide Co., LTD. (Shanghai, China). DTX and Duopafei® were provided by Qilu Pharmaceutical Co., Ltd. (Jinan, China). 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC · HCl), N-Hydroxysulfosuccinimide sodium salt (sulfo-NHS), succinic anhydride and 4-dimethylamiopryidine (DMAP) were purchased from Aladdin® (Shanghai, China). Fluorescein isothiocyanate, (FITC), Hochest 33342 and 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT) were from Sigma-Aldrich (USA). Human hepatocellular liver carcinoma (HepG2), murine malignant melanoma (B16) and human umbilical vein endothelial cells (HUVEC) cell line were from Shandong Institute of Immunopharmacology and Immunotherapy (Jinan, China). Super neonatal bovine serum

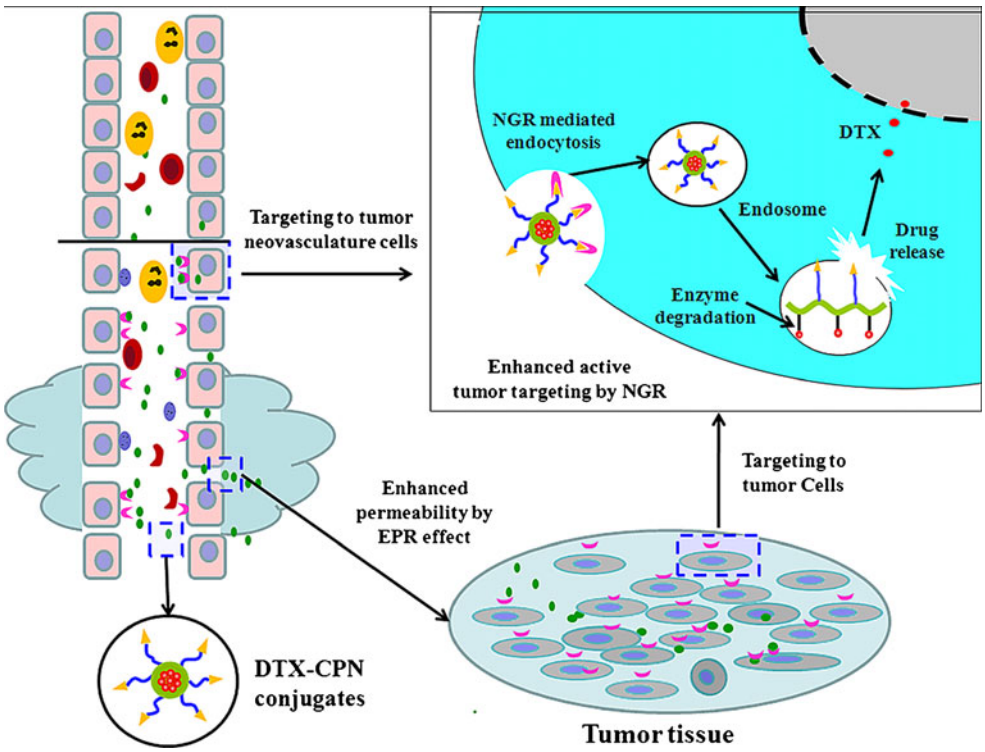

Fig. 1 Diagram of the mechanism of action of "one-double targeting" tumor therapy by nanosized DTX-CMCS-PEG-NGR (DTX-CPN) conjugates. After intravenous administration, on one hand, DTX-CPN conjugates are too large to be excluded from normal endothelium, but are small enough to extravasate from tumor neovasculature and localize in the interstitial space of the tumor issue, making more drug accumulating within the tumor (passive targeting by EPR effect). On the other hand, DTX-CPN conjugates could attach to and be internalized into tumor neovascular endothelial cells and tumor cells through NGR-mediated endocytosis, fuse with the endosomes, and subsequently degraded by enzymes to release DTX intracellularlly, which could further increase the therapeutic efficacy by specially and efficiently causing damage to CD 13 positive tumor neovascular endothelial cells and tumor cells (active targeting).

was obtained from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (China). Female Kunming mice weighing 18–22 g were supplied by the Medical Animal Test Center of the New Drugs Evaluation Center, Shandong University. All animal experiments complied with the requirements of the National Act on the Use of Experimental Animals (People's Republic of China).

Synthesis and Characterizations of DTX-CPN Conjugates

The synthesis scheme of DTX-CPN conjugates was shown in Fig. 2a.

Synthesis of CPN

NHS-PEG-NHS (38 mg), cNGR (12 mg) and 10 μL of triethylamine (TEA) was dissolved in 3 mL phosphate buffered saline (PBS, pH 7.4, 100 mM) and reacted for 2 h in ice bath under stirring. Next, the resultant solution was added to CMCS (50 mg) dissolved in (PBS, pH 8.0) and maintained stirring for 24 h. The final reaction solution was dialyzed with a dialysis bag (molecular weight cutoff: 8000–14000 Da) (Sigma-Aldrich) against ultrapure water for 48 h and lyophilized. The synthesis of CMCS-PEG (CP) was identical to CPN in the absence of cNGR.

Synthesis of 2′-Succinyl DTX

The synthesis of 2′-succinyl-DTX was carried out according to the method described by Esmaeili (33). Briefly, DTX (100 mg), succinic anhydride (22 mg) and DMAP (0.5 mg) were dissolved in 5 mL of Dichloromethane (DCM). The reaction was maintained for 24 h at room temperature under stirring. Then DCM was evaporated and ethyl acetate was added to dissolve the product. The organic phase was collected and washed with 10 mL of HCl (1%, w/v) and 10 mL of ultrapure water twice, respectively, to remove DMAP and unreacted succinic anhydride. Magnesium sulfate was added to the organic phase and incubated overnight to remove remained water. The final solution was subjected to SiO_2 column chromatography for purification.

Synthesis of DTX-CPN Conjugates

Succinyl-DTX (30 mg), EDC · HCl (13 mg) and sulfo-NHS (14 mg) were dissolved in 2 mL mixture of THF: H_2O (50:50) and reacted for 4 h at room temperature to afford the sulfo-

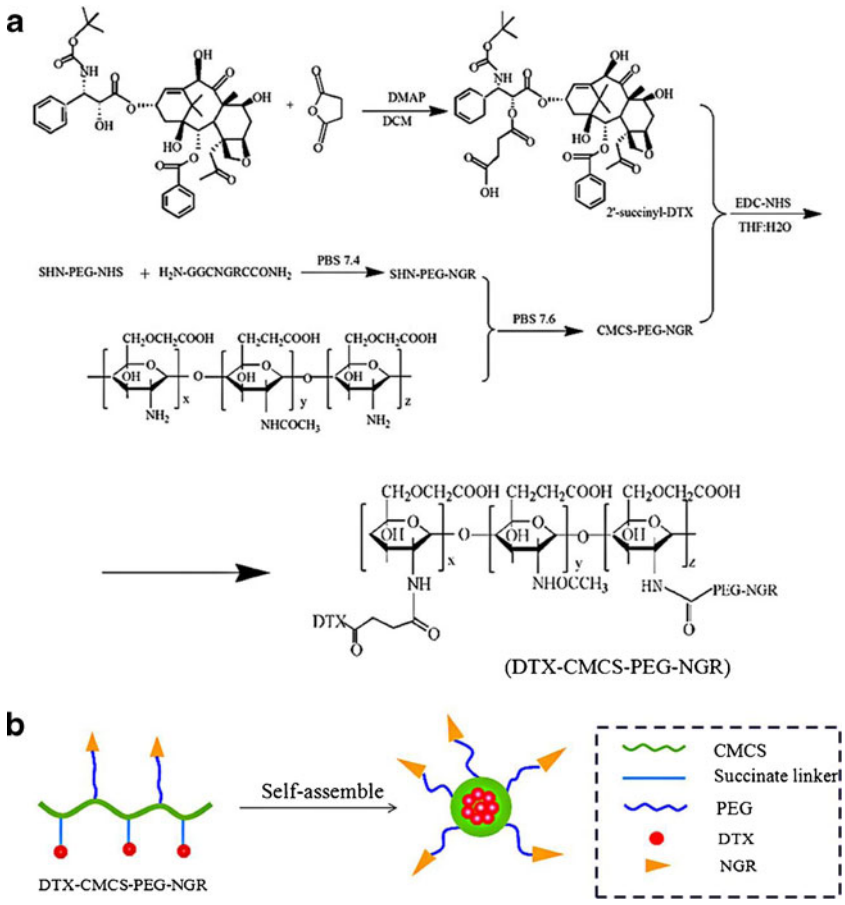

Fig. 2 (**a**) Synthesis scheme of DTX-CPN conjugates and (**b**) schematic diagram of DTX-CPN conjugates self-assembling into nanoparticle in water.

NHS-DTX-Suc. Subsequently, CPN (50 mg) was dissolved in 20 mL mixture of THF: H_2O (50:50) and the solution of sulfo-NHS-DTX-Suc was added to CPN. After stirring for 24 h, the reaction solution was dialyzed with a dialysis bag, whose molecular weight cutoff was 8000–14000 Da, against PBS (pH 7.4, 5 mM) for 48 h. As the dialysis process went on, unreacted reagents and unconjugated DTX, whose molecular weight were much smaller than 8000 Da, could be removed and DTX-CPN conjugates could self-assemble into nanoparticles. For the synthesis of the non-targeted DTX-CMCS-PEG (DTX-CP) conjugates, instead of CPN, CP was applied in the conjugation process. The structure of DTX-CPN was confirmed by ^{1}H-NMR (Avance™ DPX-300, Bruker BioSpin GmbH, Rheinstetten, Germany).

Determination of DTX Content in DTX-CPN Conjugates

The DTX content in DTX-CPN conjugates was determined by UV spectrophotometer (UV-2102PCS; UNICO [SHANG-HAI] Instruments Co., Ltd, Shanghai, China) at an absorption wavelength of 230 nm. Appropriate controls of CPN solution were run to subtract background absorbance.

Preparation of FITC-Labeled Conjugates

Briefly, 5 mg of FITC pre-dissolved in 100 μL of dimethyl sulfoxide (DMSO) was mixed with 10 mL of DTX-CP or DTX-CPN conjugates suspended in PBS (pH 7.4, 100 mM) and stirred for 24 h. The reaction mixture was dialyzed with a dialysis bag (molecular weight cutoff: 8000–14000 Da) against PBS (pH 7.4, 5 mM) to remove free FITC.

Morphology, Particle Size and Zeta Potential of DTX-CPN Conjugates

Transmission electron microscopy (TEM) was used to visualize the morphology of DTX-CPN conjugates after negative staining with phosphotungstic acid solution (2%, w/v). Size and zeta potential of DTX-CPN conjugates were measured with the Delsa™Nano Submicron Particle Size and Zeta Potential Particle Analyzer photon correlation spectroscopy (PCS) (Beckman Coulter, USA). All measurements were performed at 25°C. Calculation of the size and zeta potential was achieved by using the software supplied by the manufacturer. Experimental values were calculated from the measurements performed at least in triplicate.

Stability of DTX-CPN Conjugates

The chemical stability of DTX-CPN conjugates were examined by determining DTX release after being incubated in PBS/Tween 80 mixed medium (pH 7.4, containing 0.5% Tween 80 (w/v) to enhance the solubility of DTX), cell culture medium and fresh plasma from Wistar rats. DTX released from conjugates was then extracted by ethyl acetate. The ethyl acetate in the samples was evaporated under vacuum and the samples were redissolved in the mobile phase for quantification by HPLC (SPD-10Avp Shimadzu pump, LC-10Avp Shimadzu UV–vis detector, Shimadzu, Japan, $\lambda = 230$ nm) on a C18 column (4.6×250 mm reverse phase stainless steel column packed with 5 μm particles Inertdil ® ODS-3, GL Sciences Inc., Japan) with acetonitrile/water (55: 45) as eluting solution with a flow rate of 1.0 mL/min.

Physical stability of DTX-CPN conjugates was also evaluated. For long term storage, mannitol was added to DTX-CPN nanoparticles as the cryoprotectant (5%, w/v) before lyophilization. The changes of particle size before and after lyophilization were studied to verify the availability of this method. Besides, for short term storage, stability of redissolved DTX-CPN conjugates kept at 4°C was also studied by monitoring changes of particle size within 15 days.

In Vitro Cellular Uptake Study

In order to test the targeting property of DTX-CPN conjugates, cellular uptake of FITC labeled DTX-CPN conjugates was investigated after pre-incubation with free cNGR (34). HUVEC and B16 cells were chosen as the CD13 positive cells lines and HepG2 cells was chosen as CD13 negative cell line, respectively, which was based on the expression levels of CD13 on these cells surface according to the result of fluorescence-activated cell sorting (FACS). Cells were seeded in a 12-well plate cells at 1.0×10^5 cells/well and incubated overnight. Then, cells were incubated with different concentration (0 μg/mL, 1 μg/mL and 1 mg/mL) of cNGR for 1 h at 37°C to attach CD13 receptors, after that, the cells were washed 3 times with cold PBS (pH 7.4). Nextly, Cells were incubated with DTX-CP or DTX-CPN conjugates (containing 1 μg/mL of equivalent DTX) for 4 h. After incubation, conjugates solutions were removed and the cells were washed 3 times with cold PBS (pH 7.4). Cellular uptake of FITC labeled conjugates was observed using an inverted fluorescence microscope ($\lambda_{ex} = 540$ nm, $\lambda_{em} = 580$ nm; BX40, Olympus, Japan). Thereafter, cells were harvested by trypsinization and washed with cold PBS 3 times. The cell-associated fluorescence was quantitatively determined by FACSCalibur flow cytometry (BD Biosciences, USA). Data collection involved 10,000 counts per sample and only viable cells were gated for fluorescence analysis. All experiments were performed in triplicate.

In Vitro Cytotoxicity Study

The *in vitro* cytotoxicity against HepG2, B16 and HUVEC cells were applied to reflect the *in vitro* antitumor effect of DTX-CPN conjugates with free DTX dissolved in DMSO

as control. Cells were seeded in 96-well plates at an initial density of 1×10^4 cells/well and allowed to attach for 24 h. Then, the cells were treated with DTX or tested conjugates and incubated for 48 h. 20 μL of MTT (5 mg/mL in PBS) was added to each well. After further incubation for 4 h, the cell plate was centrifugated at 3000 rpm and the culture medium containing MTT was discarded. 200 μL of DMSO was added to each well to dissolve the MTT formazan crystals and the optical density was measured by a microplate reader (Model 680, BIO-RAD, USA) with a test wavelength of 570 nm and a reference wavelength of 630 nm. Untreated cells were taken as control with 100% viability and appropriate controls only with DMEM and MTT were run to subtract background absorbance. For cytotoxicity test, DMSO, instead of Tween 80/ethanol, was used as the solvent for DTX due to cytotoxic effect of Tween 80 (35). All experiments were repeated thrice.

Apoptosis Analysis

Cell apoptosis induced by DTX-CPN conjugates in HepG2, B16 and HUVEC cells were identified morphologically by Hochest 33342 staining. Briefly, cells were seeded on 24 well cell plate (1×10^5 cells per well) and cultured at 37°C for 24 h. Cells were then treated with DTX, DTX-CP or DTX-CPN conjugates, respectively, and incubated for 24 h at the concentration of 1 μg/mL of DTX equivalent. Subsequently, cells were washed with cold PBS twice and stained with Hoechst 33342 (10 μg/mL) at 37°C for 30 min in the dark. After staining, cells were washed with cold PBS and then photographed with inverted fluorescence microscope (Olympus IX71, Japan).

In Vivo Antitumor Efficacy

The antitumor effect of DTX-CPN conjugates were evaluated in Kunming mice bearing B16 malenoma. When the tumor volume was measured up to 100 mm^3, the mice were administered Duopafei® (20 mg Duopafei® is supplied in a blister carton containing one single-dose vial of 20 mg DTX preparation in 0.5 mL sterile pyrogen-free anhydrous Tween 80, and a single dose specific solvent vial containing 1.5 mL 13% ethanol in saline), DTX-CP or DTX-CPN conjugates (at the dose of 5 mg/kg of DTX equivalent for all DTX formulations), respectively, by intravenous administration once every 3 days for 30 days (36,37). Normal saline (NS) were used as control. Solutions of CP and CPN were administrated to assess the safety of blank drug carriers. Tumor volumes were measured by using calipers and calculated by using an equation of $(1/2)\ (L\times W^2)$, where W is the tumor measurement at the widest point and L stands for the tumor dimension at the longest point. Mice were monitored till the tumor volume reaching about 4000 mm^3. Mice with tumor volume beyond the upper limit size were euthanized. A Kaplan Meier plot of survival probability was generated from the survival data.

RESULTS

Synthesis and Characterization of DTX-CPN Conjugates

The chemical structure of DTX-CPN conjugates and the synthesis steps were shown in Fig. 2. For the characterization of CPN, as shown in Fig. 3e, ^{1}H-NMR spectrum of the synthesized CPN in D_2O showed corresponding peaks of PEG (3.4–3.6 ppm), cNGR (1.0–1.8 ppm) and CMCS (3.0–4.5 ppm of the glycol backbone). The structure of DTX-CPN conjugates was confirmed by ^{1}H-NMR. As shown in Fig. 3g, the ^{1}H-NMR spectrum of DTX-CPN conjugates showed both characteristic peaks of DTX (7–8 ppm) and CMCS-PEG-NGR (3–4 ppm), respectively. The weight percentage (wt %) of DTX determined by UV spectrophotogaphy was $20.11\pm0.83\%$ and $20.32\pm0.79\%$ for the resultant DTX-CP and DTX-CPN conjugates, respectively.

Morphology, Particle Size and Zeta Potential of DTX-CPN Conjugates

The morphology of DTX-CPN conjugates was visualized by TEM. As shown in Fig. 4a, b, DTX-CP conjugates and DTX-CPN conjugates were spherical or ellipsoidal in shape with good dispersity, which confirmed that these conjugates could self-aggregate into nanoparticles in water due to their amphipathic property. As shown in Fig. 4c, d, the mean particle size of DTX-CP conjugates and DTX-CPN conjugates determined by PCS were 130.5 ± 8.2 nm and 142.9 ± 5.6 nm, respectively. The average zeta potential for conjugates DTX-CP and DTX-CPN conjugates was -20.86 ± 1.8 mv and -15.57 ± 2.2 mv, respectively.

Stability of DTX-CPN Conjugates

The stability of DTX-CPN conjugates was studied by determining of the amount of DTX released from conjugates in a series of release medium. As shown in Fig. 5, the amount of DTX released from its conjugated form after 48 h incubation in PBS/Tween 80 (0.5%) mixed medium is very minimum ($3.23\pm0.41\%$ for DTX-CP and 4.54 ± 0.52 for DTX-CPN conjugates), which indicated that linkages were stable in this medium. DTX release in cell culture medium was faster than that in PBS/Tween 80 (0.5%), which was $8.49\pm0.51\%$ and $7.61\pm0.35\%$ for DTX-CP and DTX-CPN conjugates, respectively, after 48 h incubation. Although DTX release in plasma was the fastest, which was still only $10.75\pm0.41\%$ and $10.34\pm0.39\%$ for DTX-CP and DTX-CPN conjugates,

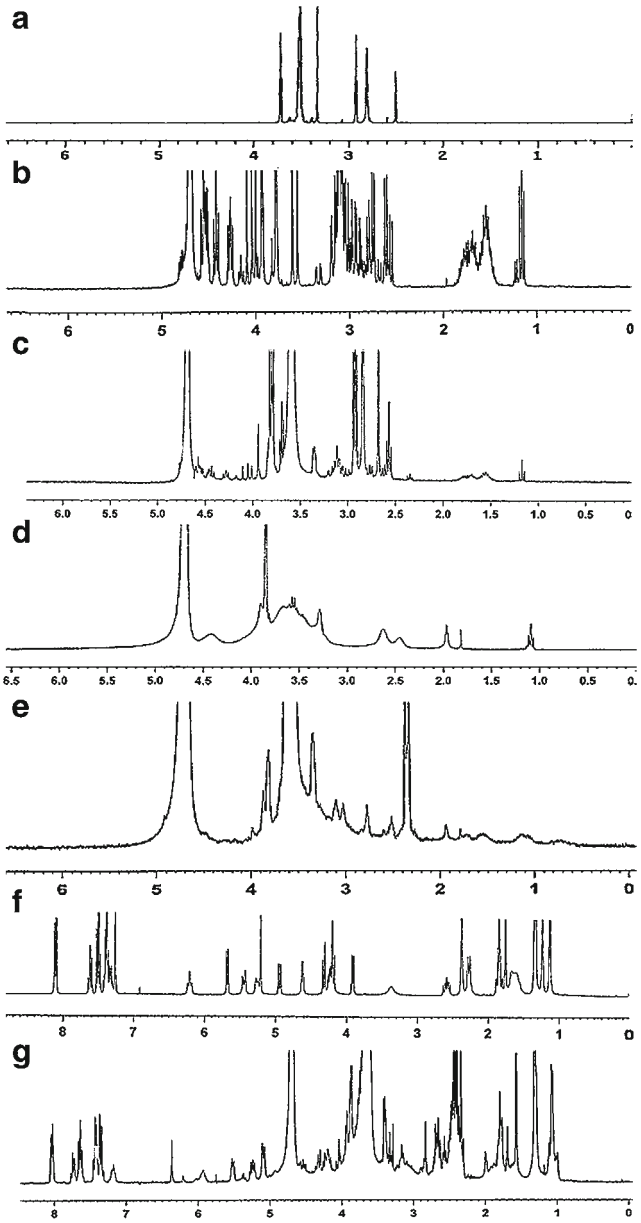

Fig. 3 ^{1}H-NMR spectra of (**a**) NHS-PEG-NHS in DMSO, (**b**) cNGR in D_2O, (**c**) PEG-NGR in D_2O, (**d**) CMCS in D_2O, (**e**) CMCS-PEG-NGR in D_2O, (**f**) DTX in DMSO, (**g**) DTX-CPN conjugates in D_2O.

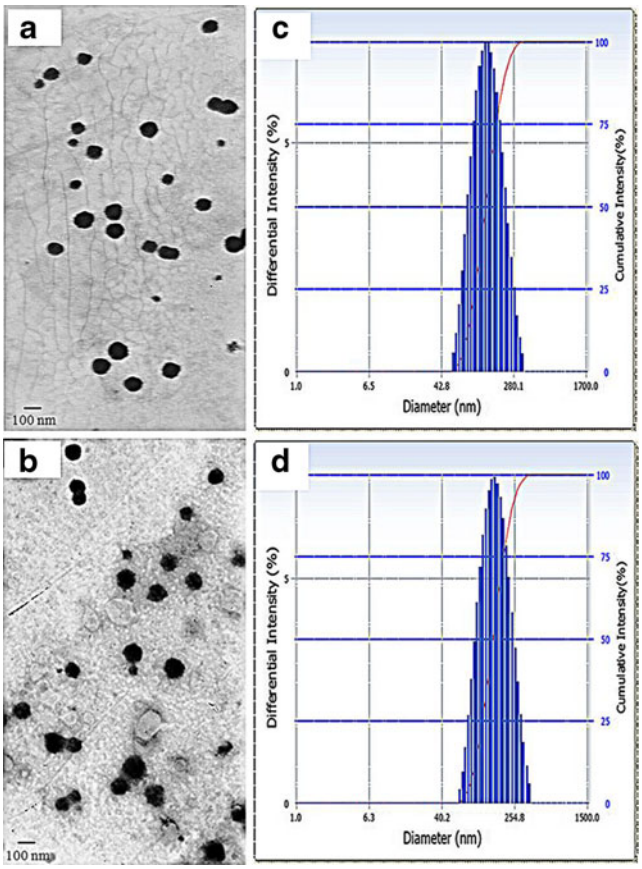

Fig. 4 TEM images of (**a**) DTX-CP conjugates and (**b**) DTX-CPN conjugates; Particle size distribution of (**c**) DTX-CP conjugates and (**d**) DTX-CPN conjugates.

respectively, after 48 h incubation. The good stability of DTX conjugates in the plasma ensured that non-significant DTX release happened in the circulation process. The reason for the relative faster DTX release in the cell culture medium and plasma than PBS/Tween 80 ($p > 0.05$) might be that enzymes existed in these medium were necessary for the cleavage of the linkages between drugs and polymers.

For the physical stability study, It was more technically feasible to store DTX-CPN conjugates in the lyophilized form for long term storage (38). Besides, DTX-CPN conjugates should also be stable enough to avoid aggregation or precipitation after being redissolved and before injection to ensure the stability for short term storage. The results of preliminary study indicated that particle size of DTX-CPN nanoparticles showed little increase than that before lyophilization (shown in Table I), indicating good feasibility of this technique. For short term storage, stability of DTX-CPN conjugates kept at 4°C was also studied and the result indicated that no aggregation or precipitation phenomenon could be observed during 15 days storage. Although paricle size of DTX-CPN conjugates nanoparticles increased a little compared with previous ones, but it was still permeable for intravenous injection (shown in Table II).

In Vitro Cellular Uptake Study

To test the targeting property of DTX-CPN conjugates mediated by the interaction between cNGR ligands and CD13 receptors, cellular uptake of the FITC labeled conjugates were investigated. As shown in Fig. 6, the cellular uptake of DTX-CP conjugates was much lower than that of DTX-CPN conjugates in HUVEC cells without cNGR pretreatment ($p < 0.01$). Besides, the cellular uptake of DTX-CPN conjugates in HUVEC could be significantly inhibited by free cNGR ($p < 0.01$), since the cellular uptake of DTX-CPN conjugates was decreased from 65.32% to 38.87% and 9.44% by pretreatment with 1 μg/mL

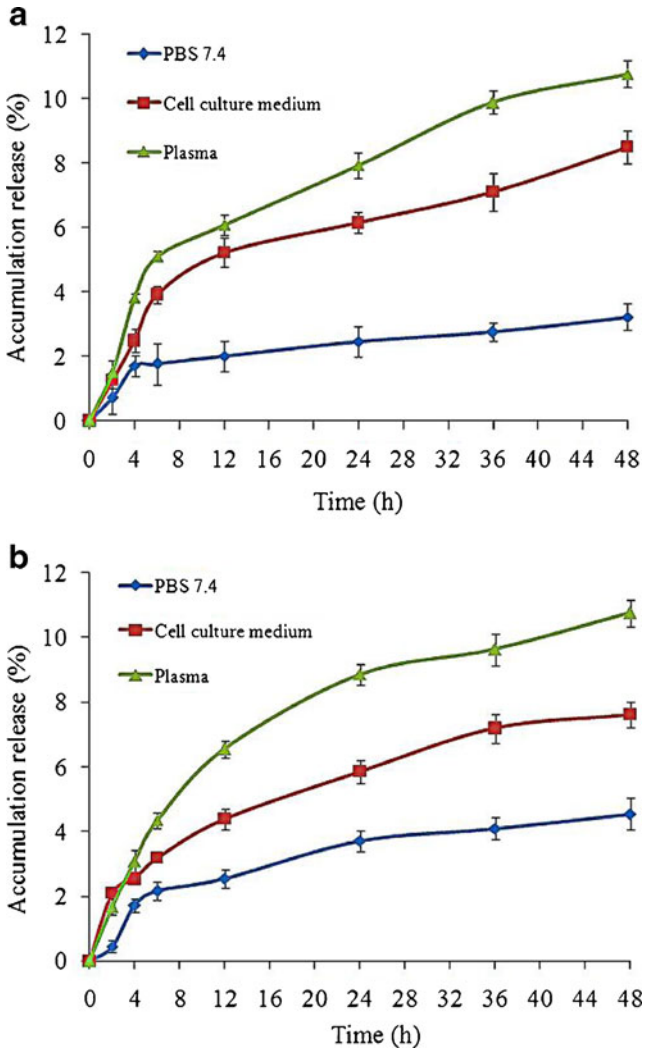

Fig. 5 Accumulative DTX release from DTX-CP and DTX-CPN conjugates in PBS/Tween 80, cell culture medium and rat plasma at 37 ± 0.5°C, respectively. Each data point represent mean ± SD. ($n = 3$).

and 1 mg/mL cNGR at 37°C, respectively ($p < 0.01$). However, cNGR pretreatment had no significant effect on the uptake of DTX-CP conjugates in HUVEC cells. For cellular uptake of DTX conjugates in B16 cells, similar uptake behavior was found as that in HUVEC. Cellular uptake of DTX-CPN conjugates in B16 cells was much higher than that of DTX-CP conjugates ($p < 0.01$) without cNGR pretreatment. While, uptake of DTX-CPN conjugates in B16 decreased from 80.95% to 54.09% and 30.52% by pretreatment with 1 μg/mL and 1 mg/mL cNGR at 37°C, respectively ($p < 0.05$). For cellular uptake of DTX conjugates in HepG2 cells (CD13 negative cell line), there was no significant difference between the cellular uptake of DTX-CPN and DTX-CP conjugates without cNGR treatment and pretreatment with cNGR peptide did not cause significant change of the cellular uptake for both of the conjugates. Based on these results, the higher cellular uptake of DTX-CPN conjugates in HUVEC and B16 cells than the non-targeted conjugates was indeed attributed to specific recognition and interaction between cNGR ligands and their receptors.

Table I Particle size of DTX-CP and DTX-CPN conjugates nanoparticles after lyophilization

Batches	1	2	3	Particle size (nm)
DTX-CP	155.6	149.9	158.3	154.6 ± 4.3
DTX-CPN	163.2	168.6	171.3	167.7 ± 4.1

Table II Particle size of DTX-CP and DTX-CPN conjugates nanoparticles after being stored at 4°C for 15 days

Batches	1	2	3	Particle size (nm)
DTX-CP	171.0	178.4	183.2	177.5 ± 6.1
DTX-CPN	192.9	197.3	206.0	198.7 ± 6.7

In Vitro Cytotoxicity Study

In vitro cytotoxicity of DTX conjugates were evaluated by MTT method on B16 and HepG2 cells. As shown in Fig. 7, Both CP and CPN exhibited no significant toxicity even at the highest concentration, indicating good safety of them. However, like free DTX, both of DTX conjugates showed a clear dose-dependent cytotoxicity against these two cell lines. There was no significant difference between the cytotoxicity induced by the two kinds of conjugates against HepG2 cells ($p > 0.05$). However, DTX-CPN conjugates exhibited higher cytotoxicity than DTX-CP conjugates on B16 cells above the concentration of 0.01 μg/mL of DTX equivalent ($p < 0.05$), which indicated that the cNGR modification could help DTX-CPN conjugates target to and cause more damage to CD13 positive B16 cells than non-targeted ones at the same concentration of DTX equivalent.

Considering the important role of the angiogenesis in cancer progression, the cytotoxic effect of DTX-CPN conjugates on HUVEC was also investigated. As shown in Fig. 7c, DTX, DTX-CP and DTX-CPN conjugates showed clear dose-dependent cytotoxic effect against HUVEC cells at doses from 0.001 to 100 μg/mL of DTX equivalent. The cytotoxic effect of DTX-CPN conjugates on HUVEC was significantly higher than that of DTX-CP conjugates ($p < 0.05$) at all dose level tested, suggesting that DTX-CPN conjugates displayed greater toxicity against HUVEC cells and could inhibit the proliferation of tumor neovascular endothelial cells more effectively than DTX-CP conjugates due to cNGR modification.

Apoptosis Analysis

Hoechst 33342 staining of nuclei was observed to elucidate the cell apoptosis induced by DTX-CPN conjugates. As shown in

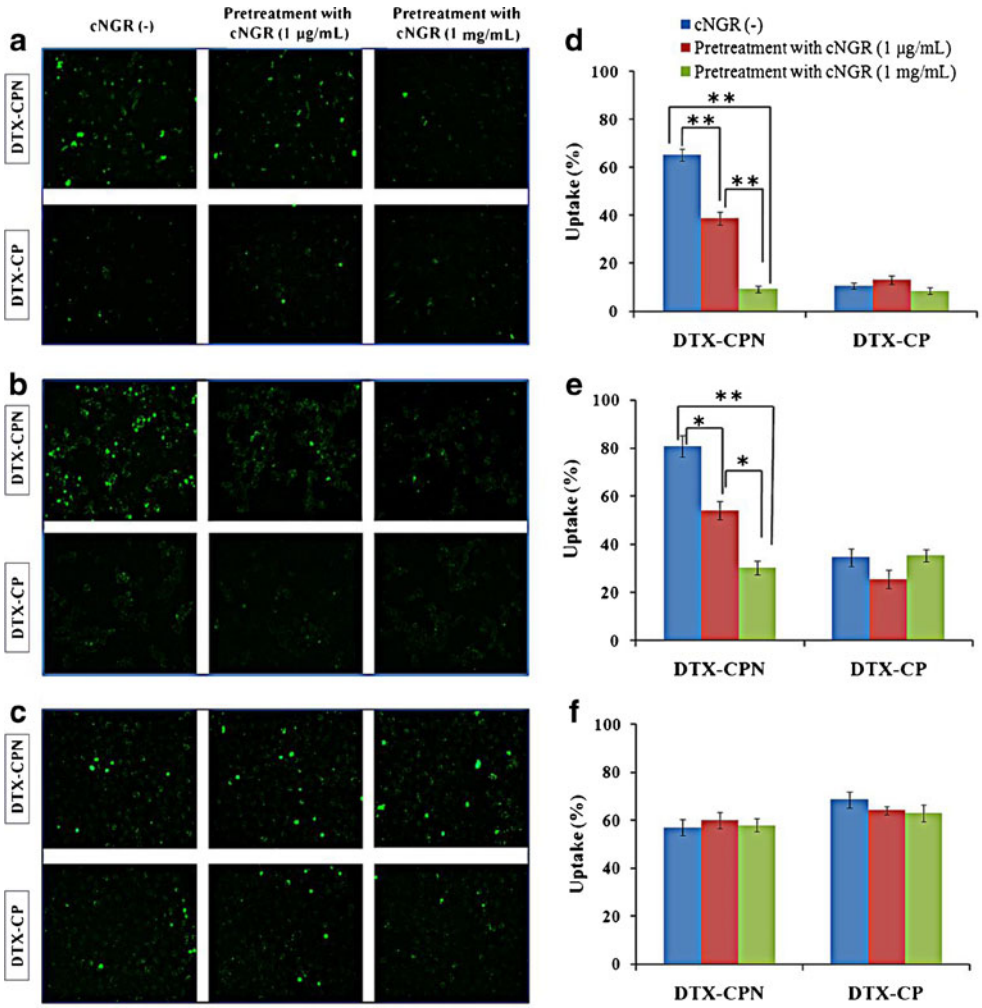

Fig. 6 Fluorescent microscopy images of FITC labeled DTX-CPN and DTX-CP conjugates incubated with (**a**) HUVEC, (**b**) B16 and (**c**) HepG2 for 4 h, respectively. Flow cytometry analysis of FITC labeled DTX-CPN and DTX-CP conjugates incubated with (**d**) HUVEC, (**e**) B16 and (**f**) HepG2 for 4 h, respectively. *$P < 0.05$, **$P < 0.01$.

Fig. 8, the nucleuses of the control cells were homogeneously stained, while nucleuses of the cells treated with drugs exhibited morphological evidence of apoptosis such as, nuclear shrinkage, chromatin condensation, nuclear fragmentation and formation of final apoptotic bodies. DTX-CPN conjugates were proved to be more effective than DTX-CP conjugates in inducing apoptosis of CD13 positive cells (HUVEC and B16) by causing more severe nuclear fragmentation and formation of final apoptotic bodies, which could be attributed to NGR mediated efficient cellular uptake of DTX-CPN conjugates. The result of apoptosis assay also confirmed the better *in vitro* antitumor effect of DTX-CPN conjugates in CD 13 positive cell lines.

In Vivo Antitumor Efficacy

In vivo antitumor activity of DTX-CPN conjugates was evaluated in B16 melanoma bearing mice. Survival curve of mice after different treatments were shown in Fig. 9a. Like control group administrated with NS, CMCS-PEG and CMCS-PEG-NGR group exhibited rapid animal death with 100% dying within 18 days, indicating no therapeutic effect for the blank polymer materials. Fifty percent of the mice died within 15 days and 21 days for the mice in Duopafei® and DTX-CP conjugates group, respectively. However, only 37.5% of the mice administrated with DTX-CPN conjugates died at the end of the 30 days treatment. Tumor volume change was shown in Fig. 9b. DTX-CP and DTX-CPN conjugates were more potent in inhibiting tumor growth than Duopafei® ($p < 0.05$). The reason accounting for this phenomenon might be that nanosized DTX-CP or DTX-CPN conjugates could accumulate in tumor tissue more effectively by EPR effect than Duopafei®. Besides, DTX-CPN was found to be more effective in inhibiting tumor growth than DTX-CP conjugates ($p < 0.01$), which was consistent with the results of *in vitro* antitumor studies.

DISCUSSION

Double-targeted DTX-CPN conjugates that could target to CD13 over-expressed tumor neovascular endothelium cells and tumor cells were designed and evaluated in this study. Conjugation of DTX to CMCS and the application of cNGR

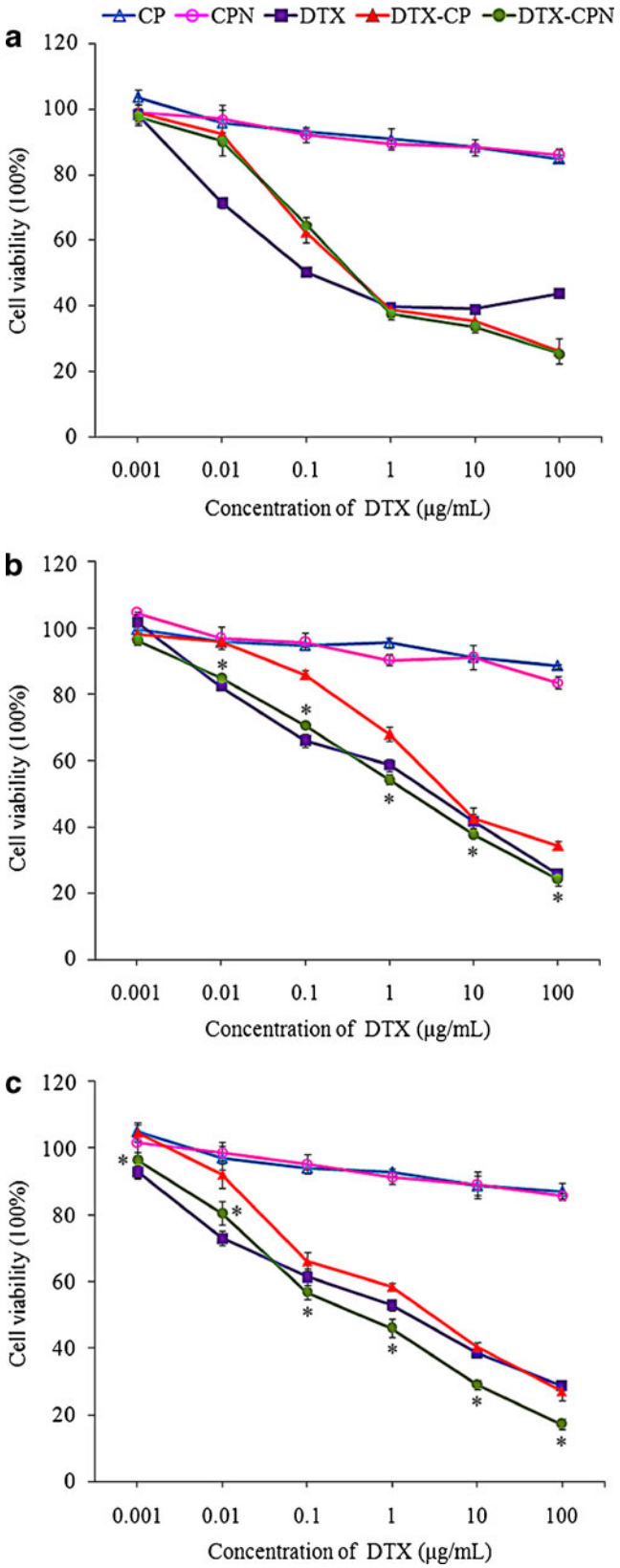

Fig. 7 *In vitro* cytotoxicity study of DTX-CPN conjugates against (**a**) HepG2, (**b**) B16 and (**c**) HUVEC cell lines. Each data point represents the value of mean ± SD. ($n = 5$). *$P < 0.05$.

peptide as targeting moiety were designed to improve DTX therapeutic efficacy and decrease its side effects. For DTX-CPN conjugates, synergistic therapy could be achieved by exerting therapeutic efficacy both in the noevascular endothelial microenvironment and the tumor cells (12).

Research in our lab focuses on the development of double-targeted nanosized drug delivery systems with "one ligand modification for double targeting" (one-double targeting) tumor therapy strategy (tumor- and vascular-targeting) (39). Advantages of this "one-double targeting" strategy are obvious, which could not only kill the tumor by more than one target (or pharmacological action site), but also simplify the manufacturing process. Previous work in our lab employed the Anti-VEGFR-2 antibody as the "one-double targeting" ligand to afford double-targeted nanostructured lipid carriers loaded with docetaxel (tNLC). Cytotoxicity of tNLC against VEGFRs over-expressed cell lines was superior to that of Duopafei® and nontargeted NLC (nNLC). The tNLC also displayed better tolerance and antitumor efficacy in a murine model bearing B16 melanoma compared with Duopafei® or nNLC (39). Compared with high molecular anti-VEGFR-2 antibody, cNGR is more preferable as "one-double targeting" ligand due to easiness of synthesis, structural simplicity, low probability of undesirable immunogenicity and high physicochemical stability (34,40).

CMCS is a kind biocompatible polysaccharide with good solubility. However, the affinity between hydrophilic CMCS and hydrophobic anticancer drug is very low, so traditional encapsulation method could not meet the requirement of the drug loading efficacy for DTX. Fortunately, there are many active chemical groups, such as amino groups and carboxyl groups, in CMCS molecular, which makes it particularly suitable for preparation of polysaccharide-drug conjugates. In this study, CPN was successfully synthesized by linking cNGR to CMCS via PEG linker and DTX was conjugated to CPN via succinate linker to afford DTX-CPN conjugates (Fig.2). Peak assignment for ^{1}H-NMR spectrum of DTX-CPN (Fig. 3g) confirmed distinctive peaks attributed to CPN segments (Fig. 3e) and DTX (Fig. 3f), which indicated successful synthesis of DTX-CPN conjugates . The drug loading for DTX-CPN conjugates was up to ~20%, which demonstrated the superiority of conjugates in loading hydrophobic drugs.

DTX-CPN conjugates could self-assemble into nanoparticles in aqueous medium, in which DTX and CPN were served as the hydrophobic core and hydrophilic out-layer, respectively. As can been seen in Fig. 4a, b, both DTX-CP conjugates and DTX-CPN conjugates were well dispersed as spherical or ellipsoidal particles. The particle size and zeta potential of DTX-CPN conjugates were similar to that of DTX-CP conjugates, which indicated that targeting modification with cNGR peptide had little effects on the physicochemical property of conjugates. Unlike the microvessel pore size in normal tissues (less than 2 nm

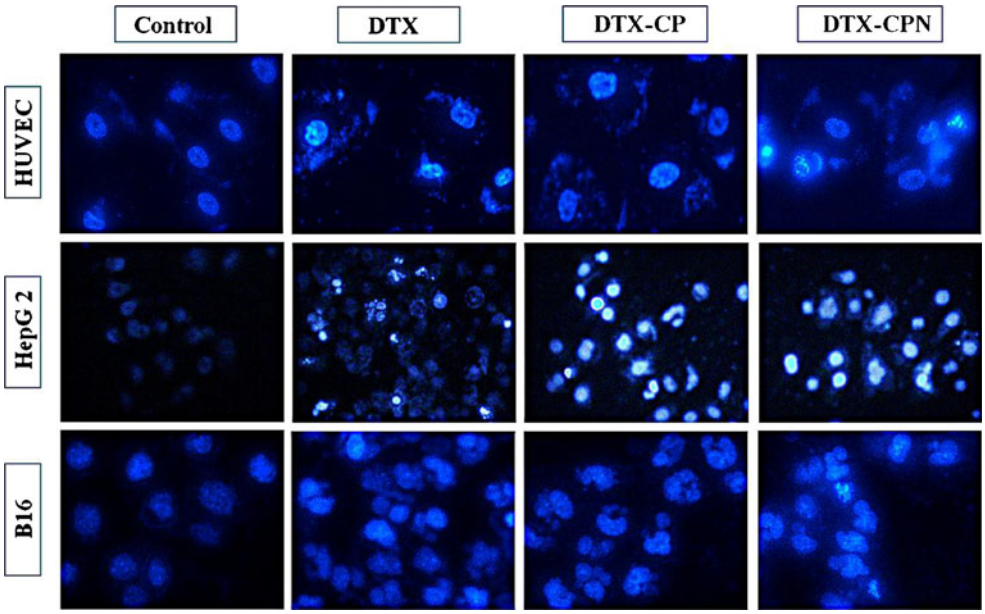

Fig. 8 Induction of apoptosis by DTX-CPN conjugates on HUVEC, HepG2 and B16 cells. Fluorescence microscopy images nucleuses following 24 h incubation with free DTX or DTX conjugates at equivalent concentration of DTX 1 μg/mL.

between endothelial cells), gaps between adjacent angiogenic blood vessels cells in the majority of experimental tumors ranges from 380 nm to 780 nm (41) and it is believed that limiting the size of nannoparticles to less than 200 nm can promote extravasation from tumor microvessels and accumulation of nanoparticles inside the interstitial space due to EPR effect. (42,43) Therefore, both DTX-CP and DTX-CPN conjugates had the potential of extravasating from the tumor blood vessels and selectively accumulating in solid tumors. Besides, human plasma proteins, cell membrane of the blood cells and the endothelial membrane are found to be negatively charged, so positive charged nanoparticles are toxic and could be quickly removed from circulation by reticulo-endothelial system (RES), which reduces the amount of drugs located in tumor tissue (44). The negative charged property was beneficial for prolonging the circulation time and improving pharmacokinetics of DTX-CP and DTX-CPN conjugates by suppressing plasma protein adsorption and minimizing nonspecific cellular uptake (45).

The stability of polymer-drug conjugates during transport process is of great significance for alleviation the side effects to normal tissues (46). For a successful polymer-drug conjugate, the polymer–drug linker should be stable during transportation process to reduce the toxicity to nonspecific sites and achieve better tumor targeting. In fact, clinical failures with HPMA copolymer-CPT conjugates and HPMA copolymer-PTX conjugates were ascribed to premature drug release in the blood (26). The result of stability test (Fig. 5) showed that DTX release in these three tested release medium was very slow. The fastest drug release was in plasma, which was only about ~10% after 48 h incubation. The good stability of DTX-CP and DTX-CPN conjugates in the plasma limited rapid drug access to the normal tissues during circulation, which was beneficial for reducing side effects and promoting drug accumulation in tumor sites by passive and active tumor targeting for these nanosized conjugates (25,47).

Ligand-targeted drug delivery systems have been investigated intensively to further improve the specificity and decrease side effects of therapeutic drugs by receptor-ligand interaction and subsequent endocytosis. The results of cellular uptake study (Fig. 6) demonstrated that intracellular uptake of DTX-CPN conjugates in CD13 positive HUVEC and B16 cells were higher than that of DTX-CP conjugates and free cNGR could decrease the uptake of DTX-CPN conjugates, which indicated cNGR peptide endowed DTX-CPN conjugates with active targeting ability and inclusion of cNGR could promote CD13-mediated endocytosis of DTX-CPN conjugates in CD13 positive cell lines. Besides, free cNGR peptide could compete with DTX-CPN conjugates for the CD13 receptors on the surface of HUVEC and B16 cells and thus block the receptor mediated endocytosis. While for the conjugates internalized by HepG2 cells, there were no receptor mediated endocytosis and competing phenomenon due to the lack of CD13 receptors on the surface of HepG2 cells (34).

The cytotoxic effect of DTX-CPN conjugates were examined on B16 and HepG2 cells. The result of cytotoxic study (Fig. 7) demonstrated that both of the blank polymer materials for DTX conjugates, which were CP and CPN, showed good safety and biocompatibility. Besides, DTX-CPN conjugates exhibited more severe cytotoxic effect in CD13 positive B16 cells than DTX-CP conjugates. A possible mechanism underlying this phenomenon might include the promoted cellular internalization of DTX-CPN conjugates through NGR–CD13 recognition, which suggested that NGR-mediated endocytosis was crucial for the enhanced cytotoxicity of DTX-CPN conjugates in CD13 positive tumor cells (39).

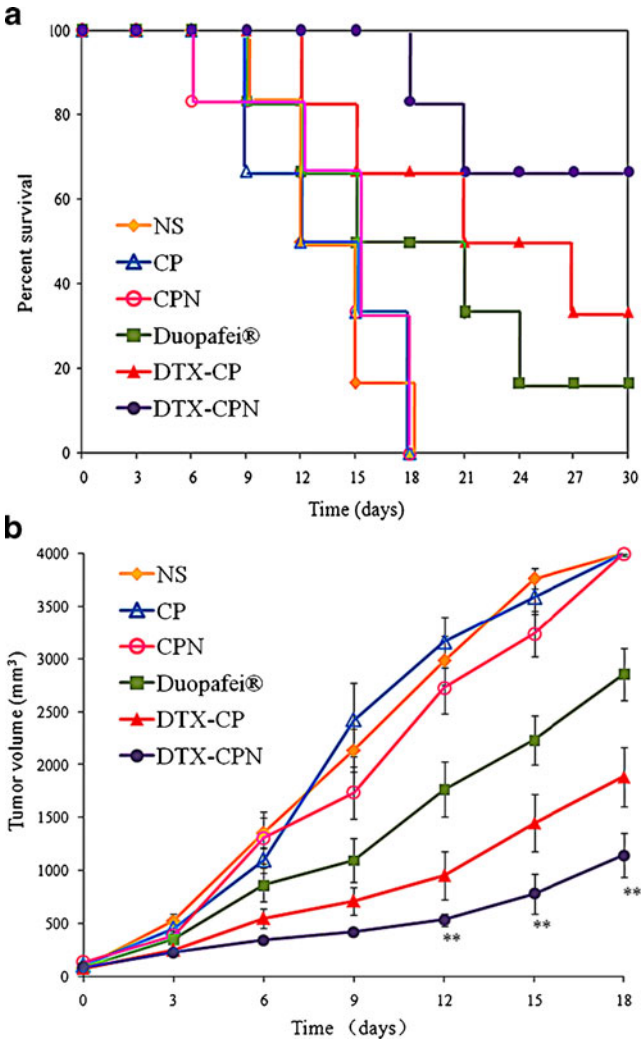

Fig. 9 Antitumor efficacy of Duopafei®, DTX-CP and DTX-CPN conjugates in KunMing mice bearing B16 tumor. (**a**) Kaplan–Meier survival plots. (**b**) Mean tumor growth curves. Data were shown as means ± SD. ($n = 6$); $^{**}P < 0.01$.

It is worth noting that the result of the stability test showed that DTX-CPN conjugates were stable in cell culture medium (only 8.49 ± 0.51% and 7.61 ± 0.35% for DTX-CP and DTX-CPN conjugates, respectively, after 48 h incubation). However, the result of *in vitro* cytotoxicity study showed that DTX conjugates exhibited significant and dose dependent cytotoxic effect as free DTX after being incubated with the live cells, which suggested that considerable amount of free DTX was released from the conjugated form under the procession of cells. The reason might be that enzymes existed in the lysosome could degrade the linkers between DTX and CPN efficiently, a phenomenon known as the "lysosomotropic drug delivery", which was the one of the initial targets for the design of polymer-drug conjugates. Besides, free DTX could penetrate cells readily, while cellular uptakes of DTX-CP and DTX-CPN conjugates were restricted to endocytosis (48) and there was time lag for the same amount of free drug releasing from the conjugated form when incubated with live cells. The altered cellular pharmacokinetics made the comparison between the cytotoxicity effect of free DTX and its conjugated form impossibly any meaningful.

Considering the tumor growth and progression is angiogenesis dependent, HUVEC was adopted as the blood cell model to elucidate the cytotoxic effect of DTX conjugates. It was clear from the data in Fig. 7c, cytotoxic effect on HUVEC caused by DTX-CPN conjugates was significantly higher than that of DTX-CP conjugates with the same concentration of DTX equivalent, which could be explained by that DTX-CPN conjugates could bind to HUVEC cells more effectively and subsequently be internalized by the cells through CD13 receptor mediated endocytosis. The increased cytotoxic effect of DTX-CPN conjugates on HUVEC reflected the effectiveness of cNGR modification.

DTX can induce cell apoptosis by acting on tubulin through stabilization of microtubules (49), so HUVEC and B16 cells, both expressing high levels of CD13, were used as the models of tumor neovascular endothelial cells and tumor cells, respectively, to evaluate the apoptosis effect of DTX conjugates. As shown in Fig. 8, DTX-CPN conjugates were more effective than DTX-CP conjugates in inducing apoptosis of HUVEC and B16 cells, which was in agreement with the result of cytotoxicity study. Since tumor progression is angiogenesis-dependent, the stronger ability of inducing apoptosis in neovascular endothelial cells for DTX-CPN conjugates could also contribute to its stronger antitumor activity than DTX-CP conjugates. Based on the results of the cytotoxicity and apoptosis tests, DTX-CPN conjugates could target to and cause more damage to CD13 positive tumor neovascular endothelial cells and tumor cells via NGR mediated endocytosis and it is reasonable to predict that DTX-CPN conjugates could cause more damage to tumors than non-targeted conjugates by the mechanism of "one-double targeting" tumor therapy for *in vivo* application.

In vivo antitumor efficacy of DTX-CPN conjugates was evaluated on B16 melanoma bearing mice. As shown in Fig. 9, the result indicated that both DTX-CPN and DTX-CP conjugates exhibited better antitumor effect than Duopafei®, the possible reason might be that the ratio of free DTX entering into tumor tissue was little and free DTX could be eliminated rapidly for Duopafei® systematically administrated, while both of DTX conjugates could be passively targeted to tumor tissue by EPR effects and maintain the effective therapeutic drug concentration for long time by sustained cleavage of the conjugates (50). The mechanism of enhanced antitumor activity of DTX-CPN conjugates than DTX-CP conjugates might be that DTX-CPN conjugates could achieve "one-double targeting" tumor therapy by binding to and being internalized into tumor neovascular endothelial cells and tumor cells via specific interactions between cNGR and CD13 receptors, which could lead to more potent antitumor activity by causing vascular damage, cutting off the

supply of nutrients and oxygen and killing tumor cells directly through the cytotoxic and apoptosis effect of DTX-CPN conjugates. In contrast, after extravasating through the leaky capillaries in the tumor tissue, DTX-CP conjugates could only localize in the interstitial space and were subjected to decomposition, degradation or phagocytosis due to lack of efficient receptor mediated endocytosis (39).

CONCLUSIONS

In this study, cNGR modified DTX-CPN conjugates were successfully synthesized and evaluated for its efficacy in "one-double targeting" tumor therapy. DTX-CPN conjugates increased the solubility of DTX significantly, which could eliminate the toxicity associated with Tween80/ethanol formulation. The result of stability test indicated that DTX-CPN conjugates were stable in the plasma, which ensured less drug release during transport and more drug accumulation in tumor by EPR effect and active targeting effect of cNGR modification. Double-targeted DTX-CPN conjugates exhibited superior antitumor activity both *in vitro* and *in vivo* due to property of targeting to both tumor neovascular endothelial cells and tumor cells. The polymer applied in DTX-CPN conjugates was of good biocompatibility. The inclusion of cNGR ligand achieved selective and enhanced anti-angiogenic and cytotoxic activity *in vitro*, and comprehensive antitumor activity *in vivo*. In summary, DTX-CPN conjugates for "one-double targeting" tumor therapy showed great potential for clinical application and worthy of further research.

ACKNOWLEDGMENTS AND DISCLOSURES

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