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ARTICLE

Synthesis and biological application of BKT-140 peptide modified polymer micelles for treating tumor metastasis with an enhanced cell internalization efficiency

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Recent years, polymer micelles have been investigated as targeted drug delivery systems for treating tumors. However, passive targeted drug delivery systems failed in accumulation at dispersive tumor metastasis sites, which leads to a poor prognosis of tumor metastasis. Chemokine receptor CXCR4, which is often high expressed in metastatic tumor cells and regulates tumor cell trafficking during the metastasis process and related to invasive response of tumor cells, is a potential targeting site. In this study, BKT-140 peptide, a specific CXCR4 inhibitor, was conjugated on the surface of chitosan based polymer micelle (BKT-CSOSA). After peptide modification, in CXCR4 high expressing cells BKT-CSOSA represented a considerable increase of cell uptake by receptor-mediated cell internalization. However, in CXCR4 low expressing cells there was no obvious change in uptake efficiency but the main uptake pathway was altered from clathrin-mediated endocytosis into macropinocytosis. Doxorubicin loaded BKT-CSOSA micelles (BKT-CSOSA/DOX) showed an enhanced anti-metastasis effect in cell migration and cell invasion *in vitro*. It was further confirmed that BKT-CSOSA/DOX micelles decreased tumor metastasis sites' formation in lung. Both CSOSA and BKT-CSOSA micelles were mainly located in the liver, spleen and lung *in vivo*, but BKT-CSOSA represented an enhanced accumulation in lung. This study demonstrates that BKT-140 modification notably increased the cell internalization of polymer micelles in metastatic tumor cells and enhanced inhibition effect on lung metastasis sites formation.

1. Introduction

Recent years, polymer micelles have been investigated as targeted drug delivery systems for treating tumors. Passive targeted delivery systems such as Doxil (liposome doxorubicin) have improved the survival rate; chitosan based glycolipid-like polymer micelles showed remarkable effect in tumor therapy[1-3]. However, passive targeted drug delivery systems failed in accumulation at dispersive tumor metastasis sites, which leads to a poor prognosis of tumor metastasis.

Metastasis, the migration of tumor cells from primary tumor to secondary sites, is one of the main causes of cancer death. It frequentl occurs in liver, lung, bone marrow or brain, which brings vast fatal body functional injuries to patients [4]. The epithelial-mesenchymal transition (EMT) may be the trigger of early stage in tumor metastasis [5]. Despite the rapid deterioration of metastasis process, there are several other reasons make tumor metastasis refractory: On one hand, conventional ways like chemotherapy, radiotherapy or surgery treatments might increase the risk of tumor metastasis[6, 7]; on

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Electronic Supplementary Information (ESI) available: GPC data of polymer. Mass spectrum and HPLC data of the peptide. FTIR spectra of intermediated products. ^{1}H NMR spectra of unmodified polymer, peptide, PEG and the peptide modified polymer. See DOI: 10.1039/x0xx00000x

the other hand, drugs can hardly accumulate at dispersive metastasis sites. Even worse, exposed in low drug concentration, tumor cells are more likely to become invasive[8]. Thus, modifying drug delivery systems with appropriate ligands to increase specific targeted accumulation of drugs may be an effective solution for tumor metastasis therapy.

CXCR4 is one of the targets for metastatic tumor among abundant cell surface biomarkers. As a member of G-protein coupled receptor (GPRC) family, CXCR4 plays a critical role in tumor metastasis with its ligand CXCL12[9]. High-expressing CXCR4 tumor cells have higher probability to metastasize. Similar to the "seed and soil" hypothesis, circulating CXCR4-expressing tumor cells tend to adhere to CXCL12 high-secreting organs [9]. CXCR4/CXCL12 complex activates the downstream protein kinase B (AKT)/mitogen-activated protein kinases (MAPK) signaling pathways, which leads to gene alteration, actin polymerisation, cell skeleton reorganization and cell migration[10]. Crosstalk between the pathways can be interrupted by inhibitors of CXCR4, such as AMD3100 [11], CTCE9908 [12-14], ALX40-4C [15], peptide LFC131 [16], peptide T140 and its derivatives [17]. Recent studies reveal that the conjugation of these ligands and nanoparticles not only leads to receptor targeting but also induces receptor-mediated endocytosis.[16, 18]

Based on the above context, BKT-140 peptide, a highly affinitive ligand to CXCR4, was chosen as the targeting ligand to be modified on stearic grafted chitosan oligosaccharide (CSOSA) polymer micelles for tumor metastasis therapy. Doxorubicin (DOX) was utilized as the model drug. Alteration of cell uptake pathways after peptide modification was investigated. In order to evaluate the effectiveness of this novel drug delivery system, wound healing assay and cell invasive assay were applied to illustrate the *in vitro* anti-migration and anti-invasion effect. *In vivo* anti-metastasis study demonstrated the inhibiting effect on lung-metastasis formation.

2. Experimental

2.1 Materials

Chitosan Oligosacchraide (CSO) of low molecular weight (Mw=18.1kDa) was obtained by enzymatic degradation of Chitosan (95% deacetylated, Mw=450kDa, supplied by Yuhuan Marine Biochemistry Co., Ltd, Zhejiang, China). Stearic acid (SA) was purchased from Shanghai Chemical Reagent Co., Ltd, (Shanghai, China). NH_2-PEG_{2000}-NH_2, Rhodamine-B-Isothiocyanate (RITC), flourescein isothiocynate (FITC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Inc.,(St Louis, MO). BKT-140 peptide (4F-benzoyl-Arg-Arg-Nal-Cys-Tyr-Cit-Lys-D-Lys-Pro-Tyr-Arg-Cit-Cys-Arg-NH_2) was synthesized by Hangzhou Dgpeptides Co., Ltd, China. N,N'-Disuccinimidyl carbonate (DSC) was obtained from Bio Basic Inc.,(Toronto, Canada). Ditertbutyl dicarbonate ($(Boc)_2O$) and 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) were purchased from Shanghai Medpep Co, Ltd, (Shanghai, China). Pyrene was purchased from Aldrich Chemical Co, (Milwaukee, WI). Doxorubicin hydrochlorate (DOX·HCl) was purchased from Zhejiang Hisun Pharmaceutical Co, Ltd, (Taizhou, Zhejiang, China). Chitosanase was purchased from Dyadic International Inc., (Jupiter, Florida). Trypsin and Dulbecco's modified Eagle's medium were purchased from Gibco-BRL Life Technologies (Carlsbad, CA). Fetal bovine serum was purchased from Sijiqing Biology Engineering Materials Co, Ltd, (Zhejiang, China). Other chemicals used were of analytical or chromatographic grade.

2.2 Synthesis of BKT-140 modified CSOSA (BKT-CSOSA)

Glycolipid amphipathic polymer CSOSA was prepared as described before[2]. Briefly, SA and EDC were dissolved in acetone and then added into ethanol-acetone mixed solvent (ethanol: acetone=3:7, v/v). The mixture was stirred for 0.5h at 60 ^{0}C. CSO (Mw 18.1kDa) was dissolved in hot water when the SA and EDC mixture was added in drop wise. The reaction solution was stirred for another 4h and then was dialyzed against DI water using a dialysis membrane (MWCO: 7kDa, Spectrum Laboratories, Laguna Hills, CA) for 48 h. Then the production was collected by lyophilization following further wash with ethanol to remove by-products. The washed product was collected by lyophilization again.

To synthesize BKT-140 modified CSOSA, NH_2-PEG_{2000}-NH_2 was used to connect peptide and CSOSA. 60μL $(Boc)_2O$ was attenuated by 20mL anhydrous DMSO and 30mg BKT-140 peptide was added into DMSO under light protection. 26.7mg EDC was added into the reaction system after stirring for 10 h under light protection at room temperature. 2 h later, 35 mg NH_2-PEG_{2000}-NH_2 was added and stirred for 24 h. Then 7.5mg DSC was added and stirred for 24 h. So the Reaction Liquid 1 was obtained. Then 220 mg CSOSA dissolved in 50mL DI water was added into Reaction Liquid 1. After stirring for 24 h, the mixture was treated with HCl to remove the t-Boc (tert-butox-ycarbonyl) groups. The final product was collected by lyophilization after dialysis (MWCO 7kDa) against DI water for 48 h.

2.3 Preparation of DOX-loaded CSOSA and BKT-CSOSA micelles

Doxorubicin hydrochloride (DOX·HCl) was stirred with twice the mole ratio of triethylamine in DMSO for 24 h to obtain Doxorubicin base (DOX). The drug loaded micelles were prepared by dialysis method[2]. Briefly, DOX was solved in DMSO while CSOSA or BKT-CSOSA were solved in mixed solvent (H_2O: DMSO = 1:1, v/v). 5mg/mL DOX in DMSO was added into CSOSA or BKT-CSOSA solution (5mg/mL). After stirring for 2 h, the mixture solution was dialyzed against DI water with a dialysis membrane (MWCO 7kDa) for 12 h. Unloaded drug was removed by centrifugation at 3000 rpm for 10 min.

Then, drug encapsulation efficiency and drug loading of CSOSA/DOX and BKT-CSOSA/DOX micelles were

determined. 400μL of CSOSA/DOX micelles was centrifuged at 10,000 rpm for 25 min in a centrifugal-ultrafiltration tube (Microcon YM-10, MWCO 3000, Millipore Co., USA). Fluorescence spectrophotometer (F-2500 fluorescence spectrophotometer, HITACHI Co, Ltd, Japan) was utilized to determine the amount of DOX in ultra-filtrate. Excitation wavelength was at 505 nm, emission wavelength at 565 nm and slit openings at 5 nm. To determine the total amount of DOX (C_0), a mixed solution (H_2O: DMSO=1:9, v/v) was used to destroy the micelles.

Drug encapsulation efficiency (EE, %) and drug loading (DL, %) of DOX-loaded micelles was then calculated by the following equations.

$$EE\% = [(C_0 \times 100 - C_f) \times V]/M_D \times 100\% \quad (1)$$

$$DL\% = [(C_0 \times 100 - C_f) \times V]/[M + (C_0 \times 100 - C) \times V] \times 100\% \quad (2)$$

where M_D is the added amount of drug, mg; M is the amount of blank micelles used, mg; V is the final volume of CSOSA/DOX or BKT-CSOSA/DOX solution, mL; C_f is the DOX concentration in ultra-filtrate; C_0 is the DOX concentration in the final DOX-loaded micelle solution.

2.4 Characteristics of CSOSA and BKT-CSOSA

FTIR (FT/IR-4100, JASCO, Japan) was used to obtain Infrared Spectrum (IR) of intermediate products BKT-PEG_{2000}-NH_2. ^1H NMR spectrometer (AC-80, Bruker Biospin, Germany) was used to obtain ^1H NMR spectra of chemicals. BKT-140 peptide were dissolved in deuterated DMSO, while CSOSA polymer, NH_2-PEG_{2000}-NH_2 and BKT-CSOSA polymers were dissolved in D_2O at the concentration of 10 mg/mL.

A Zetasizer (3000HS, Malvern Instruments Ltd., UK) was used to exam the hydrodynamic diameters of blank and DOX-loaded micelles in DI water at the concentration of 0.5 mg/mL. Transmission electron microscopy (TEM) (JEOL JEM-1230 Japan) was used to observe the morphological characters of DOX-loaded micelles. The samples was placed on copper grids with films and stained with 2% (w/v) phosphotungstic acid for TEM viewing.

Pyrene was used as a probe to determine the critical micelle concentration (CMC) of CSOSA, PEG-CSOSA and BKT-CSOSA by fluorescence measurement [19].The intensity ratio of the first peak I_1 (374 nm) to the third peak I_3 (384 nm) and the polymer concentration was analyzed to calculate CMC.

2.5 *In vitro* release study

Phosphate-buffered saline (PBS, pH 7.4) was used as the dissolution medium for *in vitro* drug release determination. Each dialysis membrane bag (MWCO: 3.5kDa) containing 1 mL CSOSA/DOX or BKT-CSOSA/DOX solution was placed in a tube containing 20 mL PBS. The tubes were put in an incubator shaker (HZ-8812S-B, Hualida Laboratory Equipment Company, Tai Cang, China), which kept shaking horizontally at 75 rpm at 37 ^{0}C. At point-in-time, the dissolution medium out of the dialysis membrane was poured out and replaced by fresh medium. Drug concentration was then determined by fluorescence spectrophotometer. All the tests were repeated thrice.

2.6 Cell culture

Mouse metastatic melanoma cells (B16), human non-small cell lung cancer cells (A549) and human breast adenocarcinoma cells (MCF-7) were obtained from the Cell Resource Center (Chinese Academy of Medical Sciences, Beijing, China). B16 cells were cultured in DMEM medium (GIBCO) supplemented with 10% fetal calf serum (v/v), 100 U/mL streptomycin and 100 U/mL penicillin at 37 ^{0}C in a humidified incubator with 5% CO_2. A549 and MCF-7 cells were cultured in the same environment except for the medium 1640 (GIBCO) with 10% newborn calf serum (v/v).

2.7 Quantification of CXCR4 expression level and receptor affinity of BKT-140 peptide

B16, A549 and MCF-7 cells ($1x10^4$) were seeded in a 24-well cell culture cluster (COSTAR, corning, USA) separately with 1 mL culture medium overnight at 37 ^{0}C 5% CO_2. After medium was removed, cells were rinsed with PBS three times and fixed with 200 μL 4% formaldehyde in PBS at room temperature for 30 min, followed by washing with PBS for three times. Then the cell samples were blocked by 2% bovine serum albumin (BSA) in PBS for 40 min. Then cell samples were incubated with a rabbit polyclonal CXCR4 antibody (H-118, sc-9046, SANTA CRUZ BIOTECHNOLOGY, Inc.) diluted solution overnight. After rinsed with PBS, the samples were then stained with 200 μL secondary antibody solution (Dylight649 conjugated Goat anti-Rabbit IgG) for another 12 hours. Hochest33342 diluted solution was used to stain the cell nucleus. Immunofluorescent stained samples were observed by confocal laser scanning microscopy (Carl Zeiss LSM 510, Germany).

The expression level of CXCR4 on cell surface was evaluated by a BD FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA). Briefly, 10^6 cells were collected and be immunofluorescent stained as described before. Flow cytometry results were obtained on the same machine at the same settings on the same day.

The affinity of BKT-140 peptide to CXCR4 was verified by immunofluorescent staining. The cell samples were blocked by 100 μg BKT-140 2 h before incubation with primary CXCR4 antibody. Then the samples were stained as described before and observed by confocal laser scanning microscopy.

2.8 Cellular uptake

CSOSA and BKT-CSOSA polymer micelles were labeled by Rhodamine B Isothiocyanate (RITC) (RITC: micelles=2:1, mol/mol). B16 or MCF-7 cells were cultured in the 24-well plate and incubated for 24 h to attach. With or without pretreatment of cell uptake pathway inhibitors (Chlorpromazine 10 μg/mL or Cytochalasin D 5 μg/mL) or CXCR4 inhibitor (AMD3100 10 μg/mL) for 1 h, cells were

incubated with RITC-CSOSA micelles, RITC-BKT-CSOSA micelles (both 20 μg/mL) for 24 hours. Another group of cells were pretreated with BKT-140 for 1 h then incubated with RITC-CSOSA micelles (20 μg/mL). Hochest33342 was used to stain the cell nucleus. After washing the cells with PBS, the cell samples was observed by confocal laser scanning microscopy (Carl Zeiss LSM 510, Germany) or collected for detecting the cellular uptake ratio by a BD FACS Calibur Flow Cytometer (BD Biosciences, San Jose, CA).

2.9 *In vitro* cellular cytotoxicity

MTT assay was used to evaluate *in vitro* anti-tumor activity of the DOX-loaded micelles. Briefly, $1x10^4$ /well B16 cells or MCF-7 cells were placed in a 96-well microtiter plate (Nalge Nunc International, Naperville, IL, USA). After cultured at 37 ^{0}C for 24 h, the cells were exposed to a series of concentrations of blank or DOX-loaded CSOSA, BKT-CSOSA micelles for another 48 h. Then, cells were incubated with 20 μL MTT solution (5 mg/mL) each well for further 4 h at 37 ^{0}C. After the culture medium was removed, 100 μL DMSO was added in each well. Finally, the optical density of each well was determined at 570 nm using an automatic reader (Bio-Rad, Model 680, USA). Cell viability was calculated in reference to cells incubated with culture medium alone. All the experiments were repeated three times.

2.10 Wound-healing assay

B16 cells or MCF-7 cells were seeded in a 24-well cell culture plate. When cells formed a confluent monolayer, a wound was created by manually scraping with a standard 200 μL-pipette tip[20]. The wound width was observed and measured by a microscope 1 h after wounding. DOX ·HCL, CSOSA/DOX micelles and BKT-CSOSA/DOX micelles water solution containing 20 ng DOX were added into culture medium immediately. Filled wound was observed with microscope after 24 hours. All the experiments were repeated at least three times. The percentage of area was calculated as follows:

Filled wound area (%) = (original wound area – remaining wound area)/original wound area ×100%.

2.11 Cell Invasive assay

Cell invasive assays were conducted using 24-well Transwells® fitted with Matrigel-coated 8 μM pore size polycarbonate filters (Corning, Massachusetts, USA)[21]. Briefly, B16 cells or MCF-7 cells were seeded into upper inserts in 0.5 mL serum-free DMEM medium with 0.1% BSA, whereas 0.8 mL DMEM medium containing 10% FBS was added into the lower chamber. Certain concentration of DOX ·HCL, CSOSA/DOX and BKT-CSOSA/DOX was added into the upper inserts. After incubation with micelles for 24 hours at 37 ^{0}C with 5% CO_2, the cells that migrated into the lower chamber was stained by methanol with 5% Crystal Violet (w/w) and counted.

2.12 *In vivo* distribution

All animal procedures were performed according to national regulations and approved by Ethics Committee of the Institution on Laboratory Animal Care in Zhejiang University. *In vivo* distribution of micelles was performed on experimental lung metastasis model. Briefly, 100 μL of cell suspension containing $1.0x\ 10^5$ B16 cells were injected to each C57BL/6 mice intravenously through tail vein. 2 weeks later, when the lung metastasis sites had formed, Dir-loaded CSOSA and BKT-CSOSA micelles were intravenously injected into the tail vein of C57BL/6 mice (5 mg/kg of Dir), repectively. After 48h, the mice were sacrificed, followed by collection of heart, liver, spleen, lung, kidney and hind leg bone. The fluorescent images were taken by Maestro *in vivo* Imaging System (CRI Inc., Woburn, MA).

2.13 *In vivo* anti-lung metastasis

Experimental lung metastasis model was established as described above. Then all the C57BL/6 mice were randomly divided into 5 groups (6 animal each). This day was pointed as day 0. At day 1, 3, 5, saline, DOX ·HCl, CSOSA/DOX and BKT-CSOSA/DOX (total dosage: DOX 9 mg/kg body weight) were injected via tail vein. Body weight of mice was measured every 3 days for treatment safety evaluation. The mice were sacrificed at day 21 and the lungs were removed and weighted. The metastasis sites were counted directly to evaluate the anti-metastasis efficacy.

2.14 Statistical analysis

All the data were presented as means of at least three separate experiments. To compare differences between groups, two-tailed Student's t-test was used. Statistical comparisons were performed by one-way ANOVA for multiple groups and P value less than 0.05 was accepted as statistically significant.

3. Results and discussion

3.1 Synthesis of BKT-CSOSA

Briefly, the basic structure of micelle was obtained by coupling reaction between amino groups of CSO and carboxyl groups of SA in the presence of EDC. As is shown in Figure 1A, BKT-140 modified CSOSA was then prepared by the chemical reaction between BKT-140 and NH_2-PEG_{2000}-NH_2 in the presence of EDC. DSC was utilized as a versatile reagent for active ester synthesis. The t-Boc-BKT-PEG-CSOSA was obtained by conjugating the remaining amino groups of NH_2-PEG_{2000}-NH_2 and the remaining primary amino groups of CSOSA in the presence of DSC. Finally obtained solid BKT-CSOSA was white soluble power. The degrees of amino-substitution (SD %) of CSOSA and BKT-CSOSA were measured as 4.03% and 5.59%, respectively.

BKT-140 peptide, also known as 4F-benzoyl-TN14003, was chosen as the targeting ligand in this study because it is a highly affinitive ligand to CXCR4 [17]. It is reported that BKT-140 peptide exhibited improved stability in mouse serum compared

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to other T140 peptide derivatives[17, 22]. ^{111}In-labeled BKT-140 peptide analogue exhibits CXCR4 targeting capacity in

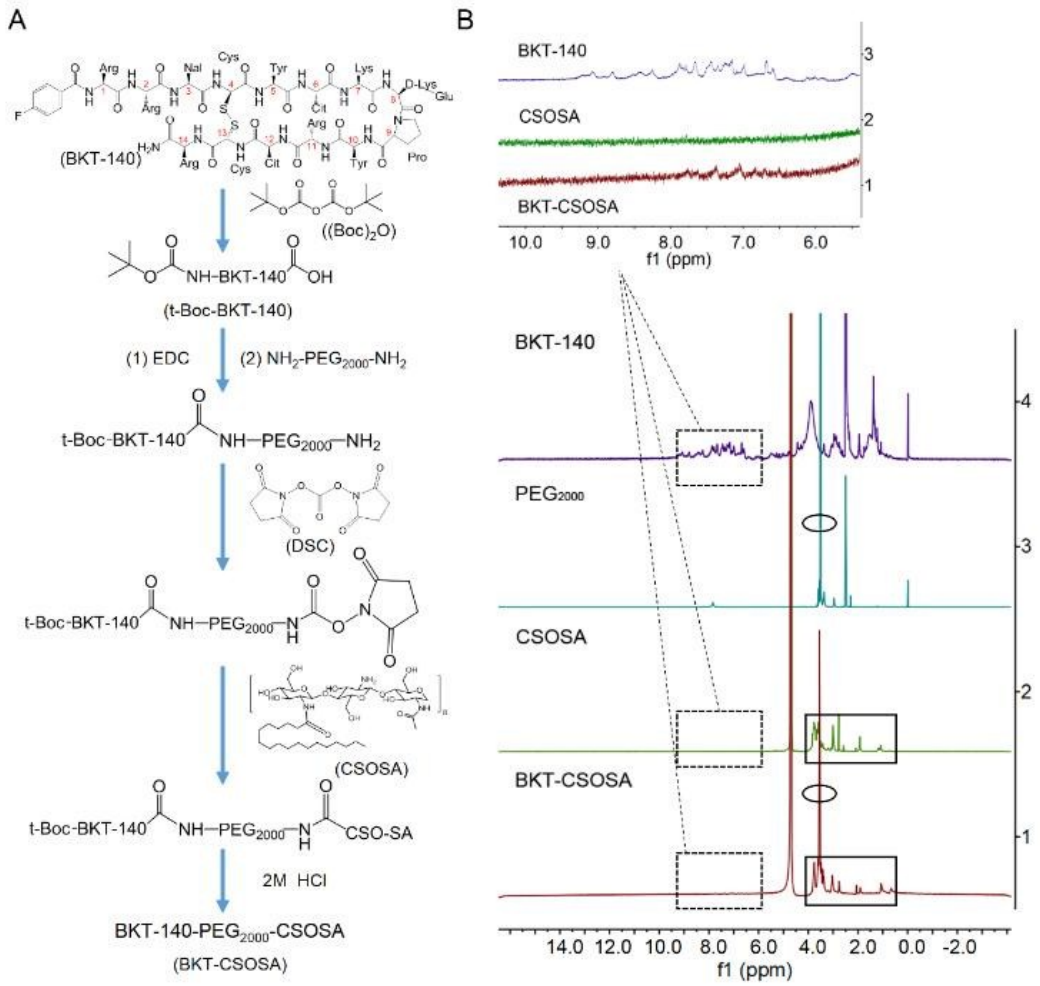

Figure 1. Synthesis and ^{1}H NMR spectra of BKT-CSOSA. (A) Synthetic route of BKT-CSOSA. (B) ^{1}H NMR spectra of BKT-140 peptide, NH_2-PEG_{2000}-NH_2, CSOSA and BKT-CSOSA. The important peaks were pointed out and magnified (upper)

molecular imaging[23]. As shown in Figure 1A, Arg^2, Nal^3, Tyr^5 and Arg^{14} amino acid residues constitute the intrinsic pharmacophore of BKT-140. [23] The single disulfide bridge between Cys^4 and Cys^{13} is also essential in interaction with CXCR4. In order not to interfere the function, we need to conjugate micelles and the peptide via a site distant from the pharmacophore. Therefore, CSOSA was conjugated with BKT-140 via the Glu added on D-Lys^8, a single amino group at one end of the hairpin structure.

The structure of intermediate products were confirmed by IR (Figure S3). The chemical structures of BKT-140 peptide, CSOSA, NH_2-PEG_{2000}-NH_2 and BKT-CSOSA were measured by ^{1}H NMR (Figure 1B & Figure S4). The peaks at about 1.94 ppm, 2.10 ppm and 3.76 ppm were attributed to -CH_3, -NH_2 and -COOH of CSOSA respectively. And the peaks at about 3.58 ppm were attributed to -CH_2CH_2O- of NH_2-PEG_{2000}-NH_2. The tiny but indelible waves near peaks at 6.68 ppm, 6.99 ppm and 7.78 ppm (upper half of Figure 1B) belong to BKT-140

peptide. These results indicated that BKT-140 peptide was conjugated to CSOSA.

3.2 Characteristics of CSOSA and BKT-CSOSA

Particle size and zeta potential of micelles were presented in Table 1. Average size of CSOSA/DOX and BKT-CSOSA/DOX micelles were 117.4 ± 6.5 nm and 166.6 ± 4.5 nm. TEM images (Figure 2A) showed spherical morphologies of drug loaded micelles. As a result, BKT-CSOSA was larger in size than CSOSA due to the modification of PEG_{2000} and BKT-140 peptide (shown in Table 1 and Figure 2). Since DOX brought stronger cohesive force inside the hydrophobic core of micelles, CSOSA/DOX and BKT-CSOSA/DOX had smaller sizes compared to CSOSA and BKT-CSOSA micelles. Zeta potential of CSOSA and BKT-CSOSA were positive in DI water. BKT-140 modification increased the positive charge of both blank and DOX loaded micelles. Doxorubicin was successfully encapsulated in CSOSA and BKT-CSOSA micelles with an efficiency of 92.32% and 79.53%, indicating that the modification had influenced the drug encapsulation.

In vitro DOX release from drug loaded micelles was shown in Figure 2B. There was no obvious difference between CSOSA/DOX and BKT-CSOSA/DOX, indicating that BKT-140 peptide and PEG_{2000} rarely influent drug release. Cumulative drug release curve showed a typical biphasic pattern and about 70% drug was released after 96 h. CMC of CSOSA and BKT-CSOSA were 50.94 μg/mL and 61.96 μg/mL respectively.

Table 1 Characteristics of CSOSA or BKT-CSOSA micelles

	Average Particle Size(nm)	Polydispersity index	Zeta Potential(mV)	Encapsulated efficiency (EE, %)
CSOSA	171.7 ±3.5	0.032 ±0.002	16.5 ±0.81	---
BKT-CSOSA	195.5 ±2.0	0.111 ±0.001	19.9 ±0.23	---
CSOSA/DOX	117.4 ±6.5	0.188 ±0.002	13.2 ±0.70	92.32 ±2.70
BKT-CSOSA/DOX	166.6 ±4.5	0.043 ±0.003	14.4 ±0.32	79.53 ±1.31

Note: Data represent the mean ±standard deviation (n = 3)
Abbreviations: CSOSA, stearic conjugated chitosan micelle; BKT-CSOSA, BKT140 peptide modified CSOSA micelles; DOX, doxorubicin; CSOSA/DOX, DOX loaded CSOSA micelles.

3.3 Quantification of CXCR4 expression level and verification of BKT-140 peptide's affinity

Expression levels of CXCR4 in B16, A549 and MCF-7 cells were measured by immunofluorescence (IF). As shown in Figure 3A, B16 cells presented the strongest fluorescent intensity while MCF-7 cells presented weak flourescence. CXCR4 expression level was further confirmed by flow cytometry (Figure 3B). Similar to the results of IF micrographs, B16 cells had apparently higher CXCR4 surface expression relative to MCF-7 cells. Nearly 50% A549 cells expressed CXCR4.

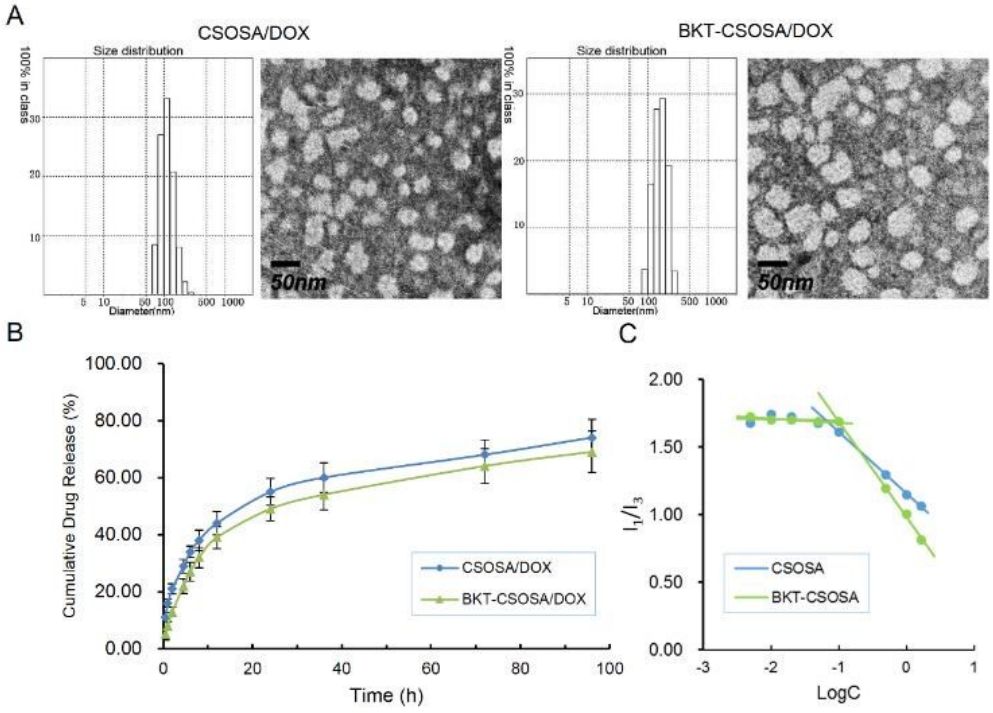

Figure 2. Characteristics of CSOSA, BKT-CSOSA and DOX-loaded micelles. (A) Size distribution and TEM images of CSOSA/DOX and BKT-CSOSA/DOX micelles. Scale bar: 50nm. (B) *In vitro* drug release curve of CSOSA/DOX and BKT-CSOSA/DOX. The error bar presents the standard deviation. (C) Critical micelle concentration (CMC) of CSOSA and BKT-CSOSA.

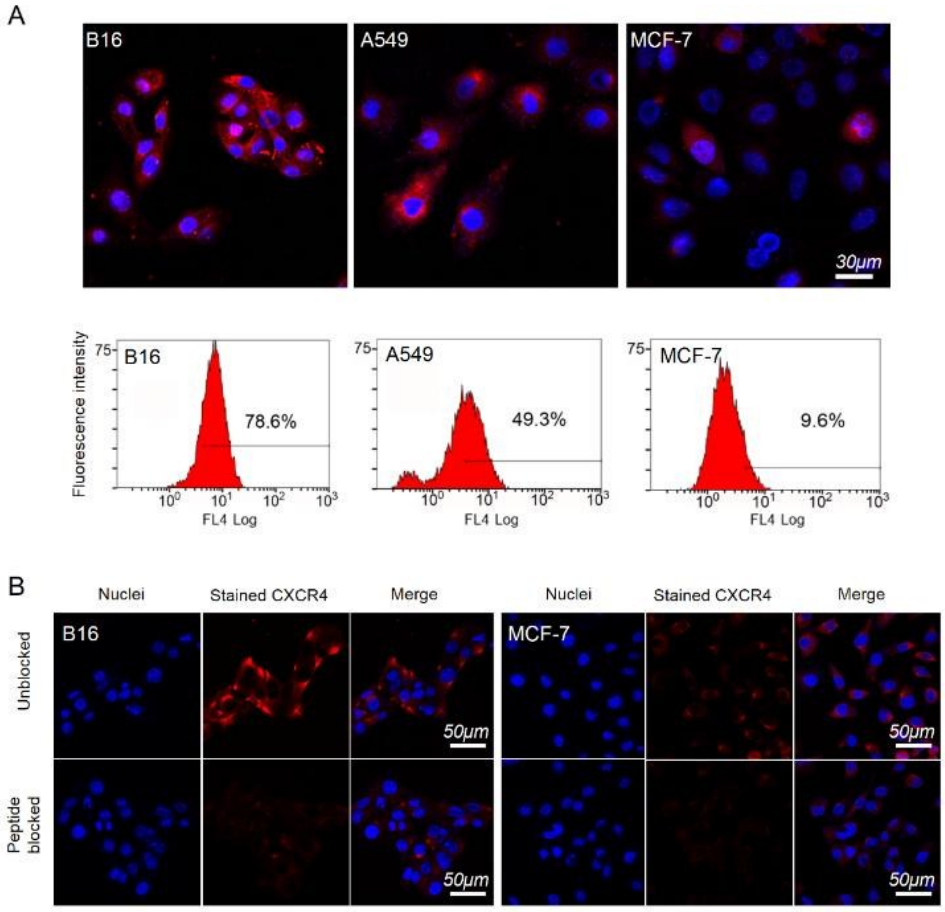

Figure 3. Qualification of CXCR4 expression level in B16, A549 and MCF-7 cells. (A) Confocal microscopy images of immunofluorescent stained CXCR4 on B16, A549 and MCF-7 cells. Scale bar: 30μm. (B) CXCR4 expressed percentage of B16, A549 and MCF-7 cells. Tested by a BD FACS Calibur Flow Cytometer (FCM). (C)BKT-140 peptide interrupted antibody binding to CXCR4, showing high affinity to its receptor. Scale bar: 50μm. In all the confocal images, IF stained CXCR4 was red and cell nucleus was blue.

To confirm the affinity of BKT-140 peptide to CXCR4, we utilized IF to exhibit the competitive ability of BKT-140 compared with specific CXCR4 antibody. Figure 3C showed that BKT-140 peptide obviously interfered IF staining in B16 cells and brought no remarkable change in IF stained MCF-7 cells.

3.4 Cell uptake mechanism study

To investigate the influence of BKT-140 peptide on CSOSA cell uptake, we compared the behavior of 3 groups: RITC-CSOSA, BKT-140+RITC-CSOSA and RITC-BKT-CSOSA, of which the cells were pretreat with BKT-140 peptide for 1 h in BKT-140+RITC-CSOSA group. Figure 4A shows that RITC-BKT-CSOSA had remarkably higher cell uptake relative to unmodified RITC-CSOSA on B16 cells. B16 cells pretreated with BKT-140 peptide also had a higher cell uptake of CSOSA compared with control group. However there was no remarkable difference between RITC-CSOSA and RITC-BKT-CSOSA observed on MCF-7 cell line. Figure 4B states that cell uptake ratio of RITC-BKT-CSOSA were increased with time in both B16 and MCF-7 cells. Faster internalization of BKT-CSOSA was observed in 8 h in both cell lines. After 24 h incubation, RITC-BKT-CSOSA showed nearly 12 fold increase in cell uptake compared with unmodified RITC-CSOSA in B16 cells.

To investigate the changes in cell uptake mechanisms caused by BKT-140 modification, Chlorpromazine, Cytochalasin D and AMD3100 were used as specific inhibitors of clathrin-mediated endocytosis, macropinocytosis and CXCR4-mediated cell uptake pathway, respectively. CSOSA and BKT-CSOSA were internalized through different pathways in both B16 and MCF-7 cells. As shown in Figure 4C, BKT-CSOSA was mainly internalized via CXCR4-mediated cell uptake pathway in CXCR4 high expressing cells (B16 cells). While BKT-CSOSA was primarily uptake through macropinocytosis

pathway in MCF-7 cells. Unmodified CSOSA was internalized through clathrin-mediated endocytosis without BKT-140's influence in both cell lines (Figure 4C & 4D), which confirmed what we reported before[24].

We hypothesize that the conjugated BKT-140 peptide was functioning like cell-penetrating peptides (CPPs) in this delivery system. As is shown in Figure 1A, BKT-140 peptide

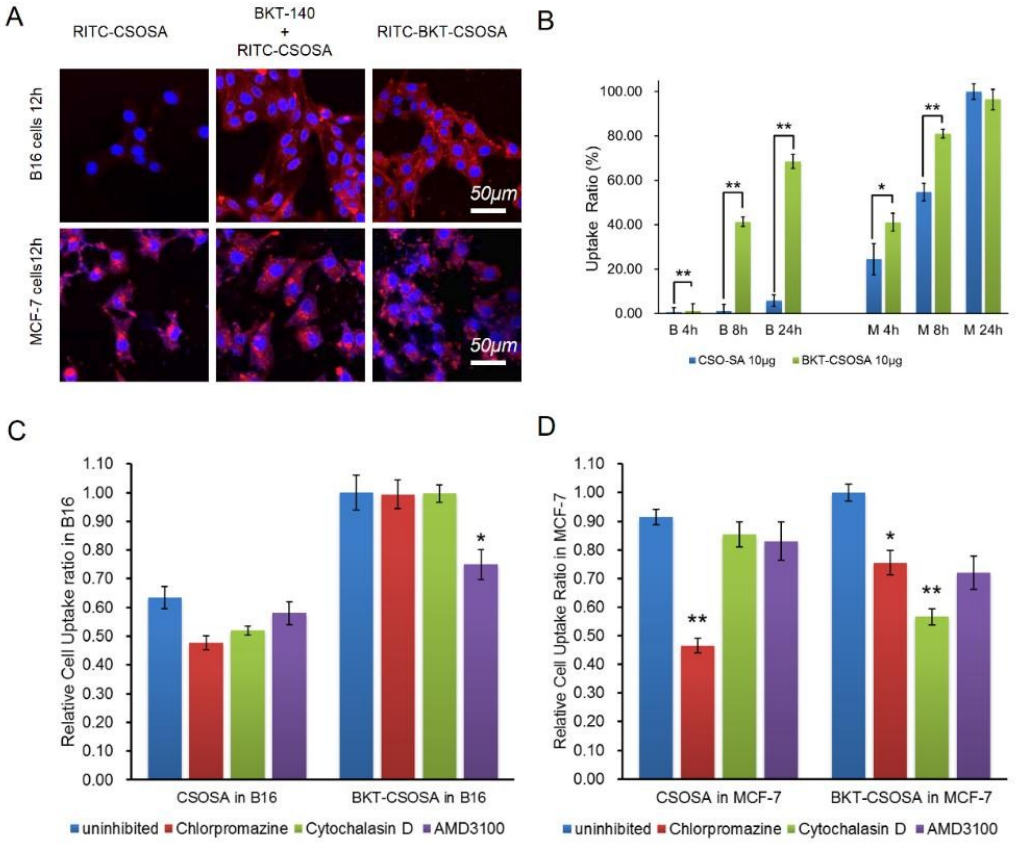

Figure 4. Cell internalization and uptake pathways study of RITC-CSOSA and RITC-BKT-CSOSA. (A) Confocal observation of cell uptake. RITC-CSOSA: cells were exposed to Rhodamine B Isothiocyanate (RITC) labeled CSOSA; BKT-140+RITC-CSOSA: the cells were pretreat with BKT-140 peptide for 1h then incubated with RITC-CSOSA; RITC-BKT-CSOSA: the cells were exposed to RITC labeled BKT-CSOSA. All the photographs were taken 12h after micelles added into the cell wells. Scale bar: 50μm (B) Uptake ratio of RITC-CSO-SA & RITC-BKT-CSOSA on B16 cells ("B") or MCF-7 cells ("M"). The uptake ratio was tested by FCM. (C) and (D) show Intracellular mechanism study of BKT-140 modified CSOSA micelle in B16 cells (C) and MCF-7 cells (D). Relative cell uptake ratio has been calculated compared to uninhibited RITC-BKT-CSOSA uptake, which has been set to 1. Each column presents average value of 5 measurements. The error bar presents the standard deviation. (n=3, mean ± SD). *, $p<0.05$ and **, $p<0.01$, respectively.

contains four Arg amino acid residues and two Lys residues, which were positively charged at pathological pH. Since Arg and Lys residues are primary components of CPPs and energy-dependent macropinocytosis is the primary endocytotic pathway responsible for CPPs-mediated intercellular delivery[25]. Arg and Lys residues induced electrostatic interaction between cell surface and BKT-CSOSA micelles in MCF-7 cells, leading a more efficient internalization of BKT-CSOSA (Figure 4B). While in the CXCR4 high expressing cells (B16 cells), receptor-ligand interaction between BKT-140 and high expressed CXCR4 increased adhesion of micelles on cell surface making more chances for micelles to be internalized.

3.5 Cytotoxicity of CSOSA and BKT-CSOSA

To examine the cytotoxicity of DOX·HCl, CSOSA/DOX and BKT-CSOSA/DOX on B16 and MCF-7 cells, MTT assay was performed. Figure 5A and 5B showed that DOX·HCl exhibited stronger inhibition effect compared to DOX loaded micelles with IC_{50} value at 0.38 μg/mL in B16 cells and 0.23 μg/mL in MCF-7 cells, which may due to the quick transport by passive diffusion of free drugs with high concentration gradient [26]. CSOSA/DOX showed weaker cytotoxicity in B16 cells due to

poor cell uptake efficiency (Figure 4A), while BKT-CSOSA/DOX exhibited an enhanced inhibition effect in B16 cells, showing the advantage of BKT-140 peptide modification. Besides, the increased percentage of other endocytosis pathways probably reduced the drug degradation[27] in

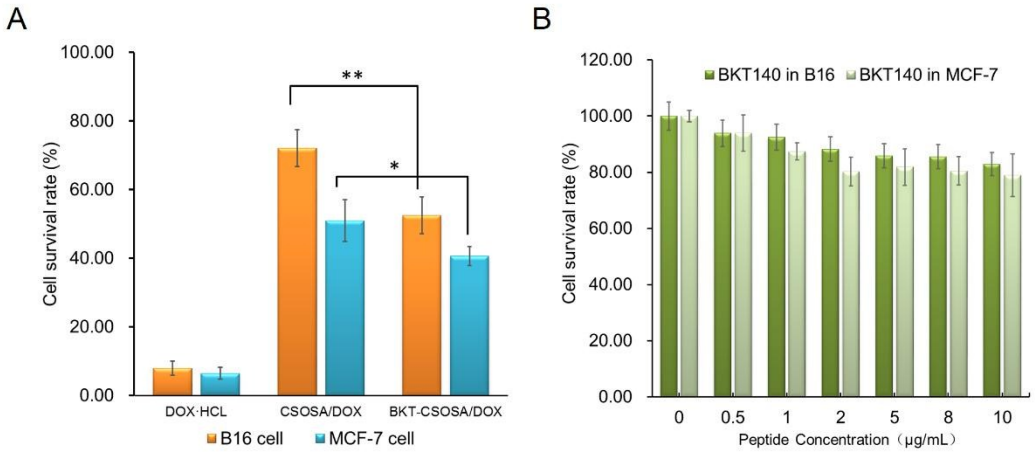

Figure 5. Cytotoxicity of DOX load micelles and BKT-140 peptide in B16 and MCF-7 cells. (A) Cell survival rate of B16 cells and MCF-7 cells incubated with DOX·HCL, CSOSA/DOX and BKT-CSOSA/DOX (DOX 5μg/mL) for 48 hours. (B) Cell survival rate of B16 cells and MCF-7 cells incubated with BKT-140 peptide at series concentrations for 48 h. Cytotoxicity was measured with MTT assay. (n = 3, mean ± SD). *, p<0.05 and **, p<0.01, respectively.

lysosomes related to clathrin-mediated pathway, thus BKT-CSOSA exhibited enhanced cell cytotoxicity in both B16 and MCF-7 cell lines (Figure 5).

As shown in Figure 5B, BKT-140 peptide did not inhibit cell viability even at concentration up to 10 μg/mL, which was similar to other antagonists of CXCR4 (AMD3100 [28] or peptide S[29]). This verified the fact that BKT-140 did not induce epithelial tumor cell death[30]

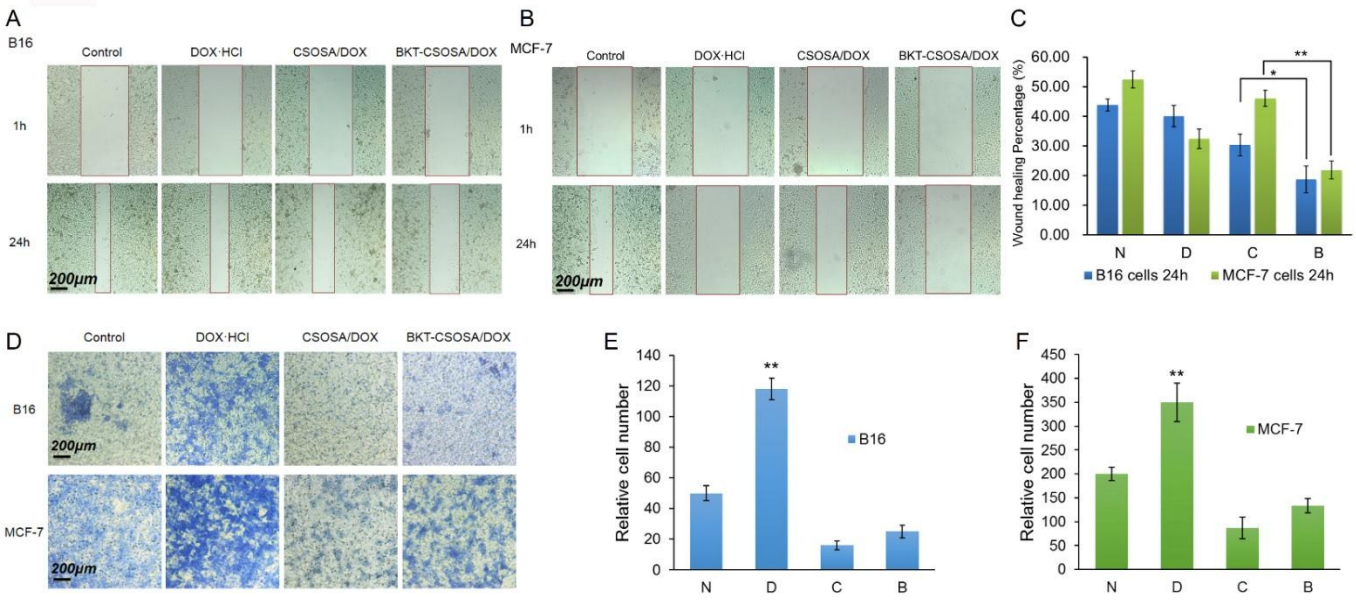

Figure 6. Wounding-healing assay and Cell invasion assay. (A) and (B) are images showing typical extent of healing of B16 cells (A) and MCF-7 cells (B) treated with saline, DOX·HCL, CSOSA/DOX or BKT-CSOSA/DOX. Scale bar: 200μm. (C) Wound healing percentage measured by microscope. Group "N""D""C""B" represents for "Control""DOX·HCl""CSOSA/DOX" and "BKT-CSOSA/DOX" (n=3, mean ± SD). (D) Photographs of B16 and MCF-7 cells invasion after treatment with saline, DOX·HCL, CSOSA/DOX or BKT-CSOSA/DOX. Scale bar: 200μm. (E) and (F) represent relative cell number of invasive cells in B16 cells(D) and MCF-7 cells(E). Data were given as mean ± SD (n = 3). * and ** represent p<0.05 and p<0.01, respectively

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3.6 *In vitro* anti-metastasis assay

To evaluate the effect of BKT-CSOSA/DOX micelles in inhibiting tumor invasion process, cell migration were assessed by wound-healing assay. The healing rate of the wound represented migration potential of tumor cells. It can be observed directly from Figure 6A and 6B that in B16 cells CSOSA/DOX and BKT-CSOSA/DOX had a slower wound healing rate, showing an improved anti-migration effect compared with DOX·HCl. BKT-CSOSA/DOX represented a better inhibition effect in comparison with CSOSA/DOX, indicating that BKT-140 peptide improved therapy efficiency of CSOSA/DOX (Quantitative results was shown in Figure 6C). To further confirm the anti-metastasis ability of drug loaded micelles, cell invasive was examined using Transwell invasive assay. Despite lower expression level of CXCR4, MCF-7 cells was more invasive than B16 cells. It was interesting to found that DOX·HCl induced cell invasion in both cell lines, while CSOSA/DOX and BKT-CSOSA/DOX represented anti-invasion effect in both cell lines (Figure D). As is shown in Figure 6D, 6E, DOX·HCl apparently induced cell migration in both B16 and MCF-7 cells, while CSOSA/DOX and BKT-CSOSA/DOX decreased cells transport to the bottom chamber. It was observed that 6-fold decrease in B16 cells and 5-fold decrease in MCF-7 cells in cell migration induced by CSOSA/DOX (DOX 200 ng/mL) while 4-fold and 3-fold decrease induced by BKT-CSOSA/DOX (DOX 200 ng/mL).

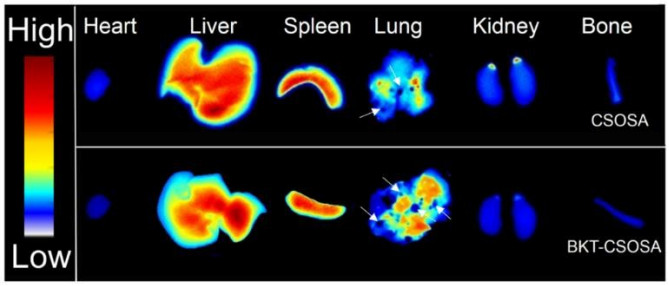

Figure 7. Comparison of tissue distribution between CSOSA and BKT-CSOSA micelles. White arrows point at the metastasis sites formed in lung.

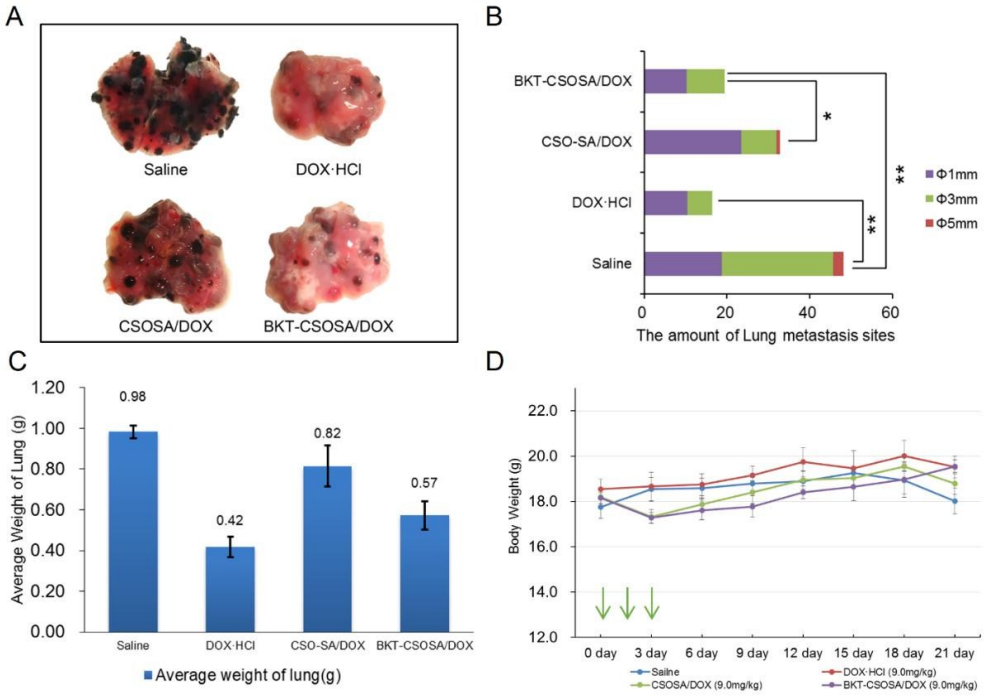

Figure 8. *In vivo* anti-metastasis experiment. (A) The photograph of metastasis sites in lungs. (B) The amount of melanoma lung metastasis sites in 21 days. (n=5, mean ± SD), * and ** represent $p<0.05$ and $p<0.01$, respectively. (C) Average weight of lung in each group (n=5, mean ± SD). (D) Body weight change curve of each group. Arrows show the days when the certain dose of saline, DOX·HCl, CSOSA/DOX or BKT-CSOSA/DOX were injected to mice. The error bar presents the standard deviation.

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3.7 *In vivo* distribution and anti-lung metastasis study

The 48 h *in vivo* distribution result showed that BKT-140 modification made micelles exhibit specifically enhanced targeting effect in lung and decreased accumulation in liver (Figure 7). The white arrows were pointed at metastasis sites in lung. It was shown that BKT-CSOSA micelles were stayed around the tumor metastasis sites.

We also investigated the *in vivo* inhibition effect on melanoma lung metastasis formation (Figure 8). Compared with saline group, CSOSA/DOX showed a certain extent anti-metastasis effect. While BKT-CSOSA/DOX represented significant inhibitory effect on lung-metastasis with decreased amount of lung metastasis sites, which was close to DOX•HCl group (Figure 8B). The diameters of metastasis sites were also limited by BKT-CSOSA/DOX. Due to pulmonary inflammation, the weight of lungs would increase along with tumor deterioration. As shown in Figure 8C, average lung weight value in BKT-CSOSA/DOX group was smaller than CSOSA/DOX group. The changing trend of lung weigh accorded with the results of lung metastasis sites counting.

DOX•HCl exhibited the most effective therapeutic action probably because of the fast and direct cytotoxicity. Because of the highly affinitive of BKT-140, BKT-CSOSA/DOX improved drug accumulation in lung (Figure 7). Thus, the anti-metastasis effect was enhanced, making the therapy results close to DOX•HCl group.

4. Conclusions

In summary, BKT-140 peptide, a CXCR4 specific ligand, was conjugated to glycolipid-like polymer micelle (CSOSA) forming a novel drug delivery system for treating tumor metastasis. Modification by BKT-140 significantly enhanced cell uptake of CSOSA polymer micelles in CXCR4 high expressing cells. The enhanced cell uptake increased cytotoxic and anti-metastasis efficiency of DOX loaded micelles both *in vitro* and *in vivo*, indicating CSOSA based micelle a potential appropriate drug delivery system for tumor metastasis therapy.

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Graphic abstract

A modification of CSOSA polymer micelles with BKT-140 peptide increased cell internalization by receptor-mediated cell internalization in CXCR4 high expressing cell line. Doxorubicin loaded BKT-CSOSA micelles (BKT-CSOSA/DOX) showed an enhanced anti-metastasis effect compared with CSOSA/DOX.