Preparation nanoparticles of β -cyclodextrin loaded Scutellarein anti-tumor activity research by targeting integrin $\alpha v \beta 3$

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Abstract

Background

The problems associated with poor water solubility of anticancer drugs are one of the most important challenges in achieving effective cancer therapy. The present study was designed to evaluate the effect of Scutellarein on human colon cancer cells in vitro by using a target $\alpha\beta$ -3 novel Scutellarein (Scu)-loaded niosome nanoparticle (β -CD-CL-Scu-cRGD).

Results

β -CD-CL-Scu-cRGD has a diameter of 140.2nm and a zeta potential of -11.3 mV with a constant physicochemical stability. The MTT assay showed both Scu and β -CD-CL-Scu-cRGD caused a decrease in cell proliferation and viability of HT29, but β -CD-CL-Scu-cRGD showed better activity in vitro. Colony formation assay and flow cytometry assay showed that β -CD-CL-Scu-cRGD has a better effect on cell proliferation and apoptosis.

Conclusions

Although further in vivo studies are necessary, our results suggested that β -CD-CL-Scu-cRGD could be an outstanding carrier to deliver Scu for potential therapeutic approaches into colon cancer.

1. Introduction

Colon cancer is one of the most common malignant tumors of the digestive system in clinical practice, and its incidence ranks the third in the world, which seriously endangers people's life and health ^[1, 2]. At present, the clinical treatment of colon cancer is usually based on comprehensive treatment, including radiotherapy, surgery, and Chinese medicine adjuvant chemotherapy ^[3]. Scutellarein (5, 6, 7, 4'-tetrahydroxy-flavone) is an active monomer component isolated from traditional Chinese medicines with anti-inflammatory and analgesic properties such as *Erigeron breviscapus* and *Oroxylum indicum* ^[4]. Scutellarein has neuroprotective and anti-inflammatory effects ^[4-6]. It also has good activity in anti-lung cancer, fibrosarcoma, and other cancers, but research on colon cancer is rarely reported ^[7]. However, the therapeutic potential of Scutellarein is limited due to its low bioavailability as well as indigent water solubility and absorption.

Drug-carrying nanoparticles increase the permeability of biofilms and can be swallowed by macrophages as foreign bodies to target sites where ligands, antibodies and enzyme substrates are attached ^[8]. Nanoparticles are highly dispersed and have a large surface area, which is conducive to increasing the contact area between the drug and the biofilm at the absorption site and improving the absorption and bioavailability of drugs ^[9]. Increasing the local concentration of the drug reduces the concentration of the rest of the body, thus greatly reducing the systemic toxicity of the drug ^[10]. Cyclodextrin is a kind of non-toxic, water-soluble, and can be chemically modified cyclic oligosaccharides, which are connected by 6-8

D-glucopyranose units^[11]. β -Cyclodextrin (β -CD) can increase the solubility and stability of fat-soluble drugs, promote the absorption and bioavailability of drugs, cover up the bad smell of drugs, and reduce or eliminate the toxicity of drugs^[11, 12]. Cyclodextrin derivatives and their polymers can combine with drugs in physical or chemical ways, so it had been widely studied in the field of nano-drug loading.

Integrin $\alpha\beta 3$ is highly expressed on the surface of neovascular endothelial cells and a variety of tumor cells, which plays an important role in the process of cell adhesion, proliferation, differentiation, metastasis, and apoptosis^[13, 14]. It also plays an important role in angiogenesis and tumor migration and invasion. Artificial cyclic RGD peptides (arginine-glycine-aspartate) have a very high affinity with $\alpha\beta 3$ integrin, and the use of these cyclic RGD peptides can improve tumor targeted therapy^[15, 16]. RGD targeting integrins Ligand research has become a hot spot in tumor pathology and pharmacology.

The purpose of this study is to introduce a nanoparticle that was composed of β -cyclodextrin and Scu and decorated with cRGD peptide as a targeted drug delivery system for anti-tumor therapeutics. The physical and chemical properties and anti-tumor activity in vitro and in vivo of β -CD-CL-Scu-cRGD were evaluated.

2. Materials And Methods

2.1 Materials

β -Cyclodextrin was purchased from (www.best-reagent.com) (ChengDu, China). ϵ -caprolactone and Stannous iso-octanoate were purchased from aladdin (ShangHai, China). (EDC/NHS) was purchased from Sigma (San Francisco, American). cRGD peptide was synthesized by China Peptides Co. Ltd (ShangHai, China). Indole green fluorescent probe IR780 was provided by Xian ruixi Biological Technology Co. Ltd (Xi An, China). Scutellarein was provided by MUST BIO-TECHNOLOGY CO. LTD (ChengDu, China).

2.2 Synthesis of nanoparticles coated with β -cyclodextrin wrapped Scutellarein

2.2.1 Synthesis of β -CD-CL

β -CD (227.3 mg, 0.2 mmol) and ϵ -caprolactone (4.65 mL, 42 mmol) were added into a 50 mL flask with three necks, slowly drop 10 mL of p-xylene under magnetic stirring to make the reaction substance was completely dissolved. After dissolution, under the protection of N₂, add (Sn(Oct)₂ (0.1% w/w)) as the catalyst and react for 36 hours at 120°C. When the reaction was completed, add 15mL dichloromethane to the reaction product, and then drop it to 300 ml ice ether at a constant speed. There will be a lot of precipitation, and filter to obtain filter cake. Put it in a vacuum drying oven at 40 °C for 24h until constant weight, and the product β -CD-CL was 3g.

2.2.2 β -CD-CL nanoparticles was prepared by ultrasonic emulsification

10 mg Scu was added to 1 mL of chloroform and methanol solution (3:1) and fully dissolve. Then, 100mg of carrier material β -CD-CL was precisely weighed and fully dissolved in 3 mL chloroform until it was clear and transparent. Scu solution was mixed with β -CD-CL trichloromethane solution as oil phase, and then under vortex oscillation condition 10 mL (polyvinyl alcohol) PVA aqueous solutions (3%) was added into it. At last, ultrasonic emulsification was performed by ultrasonic breaker (100 W, 5 min, ice bath). Put the ultrasonic solution in the fume hood and stir overnight to remove the organic solvent. After the volatilization of organic solvent, β -CD-CL nanoparticles were ultrafiltrated with a 100 kD ultrafiltration tube to remove the residual PVA. Finally, the nanoparticles were resuspended with MES buffer at pH 6.5.

2.2.3 Surface modification of β -CD-CL nanoparticles with cRGD by carbodiimide method

19.2 mg EDC and 11.5 mg NHS were added into 10 mL of the above Scu-IR780-NPs solution, incubate at room temperature for 2 5min, then remove the by-products of the EDC/NHS reaction through a 100 KD ultrafiltration tube, and add a PB solution containing 10mg cRGD (pH7.4). React at room temperature for 4h. Finally, the unreacted cRGD was removed by ultrafiltration several times, then resuspended and dispersed in ultrapure water.

2.3 Characterization techniques

FT-IR spectra were obtained by Thermo Fisher Scientific, NICOLET IS50 (America). ^1H NMR spectra were obtained by Bruker avence 250 (Germany) instruments. Transmission electron microscopy TEM (JEOL JEM-2100 microscope operating at 120 kV and linear resolution of 2.5 Å) was used to observe the morphology of the nanoparticles. The morphology of β -CD-CL-Scu-cRGD under 40,000 $\times$ magnification was observed with a transmission electron microscope (TEM).

The molecular weight of the polymer β -CD-CL was determined by gel permeation chromatography (Waters1515, Waters) (Massachusetts, USA). Particle size and zeta potential were detected by DLS (Brookhaven Instruments) (USA).

2.4 Entrapment efficiency (EE%) and drug loading (DL%)

Scu was detected by HPLC at 0.02, 0.04, 0.06, 0.08, and 0.1 mg/ml to make a calibration curve. The drug contained in β -CD-CL-Scu-cRGD dissolved by methanol demulsification. The content of the drug was determined by HPLC, and drug encapsulation efficiency (EE%) and drug loading (DL%) content calculated according to the standard curve. The equations used were as follows:

$$\text{EE\%} = (\text{Weight of drug in nanoparticles} / \text{Weight of feed drug}) * 100$$

$$\text{DL\%} = (\text{Weight of drug in nanoparticles} / \text{Weight of nanoparticles}) * 100$$

2.5 In vitro stability of β -CD-CL-Scu-cRGD

The β -CD-CL-Scu-cRGD (100 mg) was dissolved in 14 mL tetrahydrofuran. Then, 2 mL of the β -CD-CL-Scu-cRGD solution was diluted four-fold using 5A medium and incubated at 37°C. The tetrahydrofuran with β -CD-CL-Scu-cRGD was divided into seven equal parts according to the above method. At every given time point (1, 2, 3, 4, 5, 6, 7, day), 1 mL of the sample solution was collected from the 5A medium, excess acetonitrile was added to remove the ingredients from 5A medium, followed by centrifugation, then the size and zeta potential of β -CD-CL-Scu-cRGD were measured by using TEM and DLS as scribed above.

2.6 In vitro anticancer activity assay

2.6.1 Cell culture

HT29 cell from National Collection of Authenticated Cell Cultuures was maintained in MCCOY'5A containing 10% FBS (Gibco, USA), penicillin (100 μ g/mL), Glutamine (100 μ g/mL), and incubated in a humid atmosphere at 37°C with 5% CO₂.

2.6.2 MTT assay

HT29 cells of the logarithmic growth phase were seeded into a 96-well culture plate (5000 cells/well) and medicine administration was conducted after 24 h of cell culture. 24 and 48 h after medicine administration, add 20 μ L MTT (5mg/mL) to each well, and incubated for 4 h at 37°C with 5% CO₂. Then, aspirate and discard the culture medium. Add 150 μ L of DMSO to each well, shake to dissolve totally, measure the absorbance value of each well on an enzyme-linked immunoassay with a wavelength of 490 nm to calculate the cell proliferation rate.

2.6.3 Colony formation assay

The cells at logarithmic growth stage were pretreated to prepare cell suspension. Inoculate 200 cells per well in a 6-well plate containing 4 mL of complete medium. Then, gently rotate to disperse the cells evenly. Place the 6-well plate in an incubator at 37°C and 5% CO₂. Change the medium every 3 days, and the drug concentration (40 μ g/mL) remains unchanged. After 2 weeks, discard the culture medium, and carefully immerse the plate with PBS. Add 5 mL of pure methanol to fix the cells for 15 min. Aspirate and discard methanol before adding the appropriate amount of Giemsa staining solution. Finally, slowly wash away the dyeing solution with running water, and air dry.

2.6.4 Flow cytometry assay

The cells are seeded in a 6-well plate at 300,000 per well. The concentrations of the drugs Scu and β -CD-CL-Scu-cRGD were both (40 μ g/mL), and incubated for 24 h. All cells were collected and stained with Annexin V-FITC (MultiSciences) and PI. Cell apoptosis was determined by flow cytometry using annexin V and propidium iodide (PI) double-staining technique for phosphatidylserine membrane redistribution. The apoptotic cells were measured by BD Caliber flow cytometry (Becton Dickinson, USA).

2.7 Statistical analysis

Statistical data was presented as mean $\pm$ standard deviation (SD), and analyses were carried using SPSS software (19.0 revision, IBM, Chicago, IL, USA). Graphs were obtained by GraphPad Prism software (6.0 revision, La Jolla, CA, USA). One-way ANOVA was used to compare the group differences. $P < 0.05$ was considered statistically significant.

3. Results

3.1 β -CD-CL polymer characterization of $^1\text{H-NMR}$

To develop an effective scutellarein delivery system, we constructed a Scutellarein-loaded niosome nanoparticle using the β -CD-CL as package material (Fig 1A). The polymer of β -CD-CL was characterized by $^1\text{H-NMR}$ analyses. $^1\text{H-NMR}$ information revealed that there are obvious proton peaks at 5.74, 5.69, 4.83, 4.46, 4.34, 3.98, 3.63, 3.36, 2.27, 1.53, 1.29. However, the signals at 3.98, 2.27, 1.53, and 1.29 ppm are significantly stronger than other signals. The target compound is mainly composed of β -cyclodextrin and polycaprolactone (PCL) fragments. Among these, the signals at 5.74 and 5.69 ppm correspond to the protons on the two hydroxyl groups facing inward on the cyclodextrin structure, and the signals at 4.83, 3.63, and 3.36 ppm correspond to the cyclodextrin structural formula contains protons on the methylene and methine groups, and the stronger signals at 3.98, 2.27, 1.53, and 1.29 ppm correspond to the protons on the methylene group in PCL (Fig 1B). The peak corresponding to the target compound can be found in the NMR spectrum, indicating the formation of the product.

3.2 β -CD-CL polymer characterization of FT-IR

FT-IR was used to further verify the synthesis of β -CD-CL. The terminal hydroxyl ($-\text{O-H}$) of β -CD is 3392.5 cm^{-1} , the asymmetric stretching vibration peak (C-H) of the methylene group on β -CD is 2928.9 cm^{-1} , and the in-plane bending vibration peak (C-H) of the methylene group is 1417.4 cm^{-1} . The asymmetric stretching vibration peak of the (C-O-C) bond on β -CD is 1028.6 cm^{-1} . The infrared spectrum of β -CD-CL shows the ester group stretching vibration peak on β -CD-CL C=O is 1726.7 cm^{-1} , and the stretching vibration peaks of the methylene group in the structural unit are 2946.8 cm^{-1} and 2867.5 cm^{-1} . In addition, the hydroxyl stretching vibration peak (O-H) on β -CD-CL is 3439.9 cm^{-1} , and the intensity of the hydroxyl characteristic peak is greatly weakened due to the substitution of the primary hydroxyl groups on β -CD. The FT-IR spectrum of the copolymer β -CD-CL has both characteristic peaks of β -CD and ϵ -CL. According to the results, it can be preliminarily judged that β -CD and ϵ -CL are successfully connected.

3.3 Gel permeation chromatography detection of β -CD-CL

The polydispersity of molecular weight is one of the basic characteristics of polymers, and the properties of polymers are closely related to their molecular weight and molecular weight distribution. The molecular weight of β -CD-CL copolymer is 10396 detected by Gel permeation chromatography, which is consistent with the molecular weight of the polymer.

3.4 Physicochemical characteristics of β -CD-CL-Scu-cRGD

Transmission electron microscopy (TEM) was used to investigate the particle size of the β -CD-CL-Scu-cRGD. The Pdi of β -CD-CL-Scu was 0.254, and Zeta Potential was -19.3mV. While, the particle size of the β -CD-CL-Scu was 127.1nm (Table 1). As showing in (Fig2 A), the images revealed that the particles present a uniformly dispersed spherical shape with an obvious core-shell structure. According to the image of TEM the mean diameter of β -CD-CL-Scu-cRGD was obtained to be 140.2nm. β -CD-CL-Scu-cRGD drug-loaded targeting microspheres Pdi was 0.21, and Zeta Potential was -11.3mV (Table 1). High performance liquid chromatography was used to detect encapsulation efficiency and drug loading of β -CD-CL-Scu-cRGD. HPLC detects Scu to draw the standard curve as $Y=2E^{-0.8X} \times 0.00661$ ($R^2=0.995$). The Cumulative release efficiency of Scu was showed in (Fig2 B). The encapsulation efficiency (EE %) was 33.50% and the drug loading (DL%) was 20.22% (Table 1). To preliminarily evaluate the validity of nanoparticles, the stability of β -CD-CL-Scu-cRGD in neutral conditions was studied. β -CD-CL-Scu-cRGD displayed excellent biostability in neutral conditions solution for 7 days. (Fig4 C).

Table1: Physicochemical characteristics of nanoparticle

Sample	Particle size (nm)	PDI	Zeta potentials (mV)	EE%	DL%
β -CD-CL-Scu-cRGD	140.2	0.21	-11.3	33.50%	20.22%
β -CD-CL-Scu	127.1	0.254	-19.3	——	——

3.5 β -CD-CL-Scu-cRGD inhibited proliferation and promoted apoptosis in colon cancer cells

In order to investigate the effect of β -CD-CL-Scu-cRGD on malignant biological behavior of colon cancer cells, MTT, colony formation and flow cytometry were applied to detect proliferation and apoptosis. As shown in fig 5A, the β -CD-CL-Scu-cRGD can significantly inhibit the proliferation of colon cancer cells. Clonal formation assay is regarded as the gold standard for detecting tumor cell proliferation. The present study also revealed that β -CD-CL-Scu-cRGD can significantly decreased the clone efficiency of colon cancer cells (Fig 5B and C). The flow cytometry results revealed that the apoptotic rate of colon cancer cells was significantly increased following β -CD-CL-Scu-cRGD treatment (Fig 5D and E). These data indicated that the β -CD-CL-Scu-cRGD has a good anti-tumor effect in vitro.

4. Discussion

Natural products have always been the most productive source for drug development, especially as anticancer agents. Previous studies have demonstrated that scutellarein has anticancer properties ^[17–19]. However, the watersolubility and bioavailability of scutellarein are low ^[20, 21]. Based on these observations, the present study was designed to investigate the ability of scutellarein loaded in nanoparticles to attenuate the development of human colon cancer by using in vitro model. Our results

revealed that β -CD-CL-Scu-cRGD has a better potential to inhibit tumor formation of coloncancer cells than scutellarein.

In the past two decades, nanoparticle-based drug delivery systems have been developed to treat various diseases, including colon cancer [22, 23]. Many types of nanoparticles, including polymer-based, lipid-based, viral, inorganic, and drug -conjugated, have been designed to target and destroy cancer cells [24, 25]. β -CD-CL was appropriately designed for the delivery materials, and anti-tumor properties of Scutellarein significantly promoted when loaded in nano particle. In this study, the average diameter of β -CD-CL-Scu-cRGD was 140.2nm, the zeta potential was - 11.3mv, the drug encapsulation rate was 33.5%, and the drug load was 20.22%. It indicated that encapsulating Scutellarein effective. The physical and chemical properties of β -CD-CL-Scu-cRGD is a permeable, stable, and inexpensive component of Scutellarein targeted delivery to cancer cells. Compared with free Scutellarein, β -CD-CL-Scu-cRGD has a stronger cytotoxic effect on HT29 cells.

5. Conclusion

The present study is the first to investigate the effect of a novel nanoparticle-loaded Scutellarein on HT29. Our findings revealed that β -CD-CL -Scu-cRGD more efficiently control the viability, proliferation, and migration of HT29 than Scutellarein through targeting integrin receptors. Our data suggested that nano-niosome could be used as an ideal carrier to deliver Scutellarein for possible therapeutic approaches to HT29. However, these findings should be interpreted with caution. Further in vitro and in vivo investigations are warranted to confirm the anti-tumor effect of β -CD-CL-Scu-cRGD through targeting integrin receptors. Meanwhile, the lower encapsulation efficiency is mainly due to the poor solubility of scutellarein (chloroform, acetone, methanol, acetonitrile have been tried), compared to curcumin, paclitaxel, etc.

Declarations

Acknowledgments

Not applicable.

Authors' contributions

JUNDONG WANG and TIANHAO LI finished the experiment. , CHAOCHI YUE, SEN ZHONG, XIANGDONG YANG and JUN LI contributed to the acquisition of reagents and materials. YUANZHI LI supervised this project and assisted in the peer review response and the writing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Synthesis and ^1H -NMR of β -CD-CL. A: Synthetic schematic diagram of β -CD-CL. B: ^1H -NMR assignment and analysis of β -CD-CL.

Figure 2

Infrared spectrum analysis results of β -CD and β -CD-CL.

Broad Unknown Relative Peak Table

	Distribution Name	Mh (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	Mz/Mw	Mz+1/Mw
1		10396	14846	11285	20745	27084	1.428081	1.397314	1.824269

Figure 3

β -CD-CL gel permeation chromatography test results.

Figure 4

Physicochemical characteristics and of nanoparticle. A: The image of β -CD-CL-Scu-cRGD detected by TEM. B: The Cumulative release curve of Scu. C: 7-day stability test results of the complex under neutral conditions.

Figure 5

Effects of β -CD-CL-Scu-cRGD on proliferation and apoptosis of colon cancer cells (HT29). HT29 cells were treated with Scu (40 μ g/mL) or β -CD-CL-Scu-cRGD (40 μ g/mL). A: Cell proliferation was determined by MTT assay. B: Images of the cell colony assay showing stained colonies resulting from different treatments. C: Statistics of cell colony assay. D: Images of the cell apoptosis by flow cytometry. E: Statistics of cell apoptosis. * $p \leq 0.05$, ** $p \leq 0.001$, *** p and **** $p \leq 0.0001$.