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Dual chemotherapy and photodynamic therapy in an HT-29 human colon cancer xenograft model using SN-38-loaded chlorin-core star block copolymer micelles

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ABSTRACT

Chlorin-core star-shaped block copolymer (CSBC) may self-assemble to form micelles, which act as nanosized photosensitizing agents for photodynamic therapy (PDT) and further encapsulate hydrophobic drugs. This functionalized micellar delivery system is a potential dual carrier for the synergistic combination of photodynamic therapy and chemotherapy for the treatment of cancer. In this study, SN-38 encapsulated CSBC micelles were successfully prepared using a lyophilization–hydration method. Our results show that the prolonged plasma residence time of SN-38/CSBC micelles as compared with free CPT-11 permit increased tumor accumulation and consequently, improved antitumor activity. The combined effects of SN-38/CSBC micelles with PDT were evaluated in an HT-29 human colon cancer xenograft model. Interesting, SN-38/CSBC-mediated PDT synergistically inhibited tumor growth, resulting in up to 60% complete regression of well-established tumors after 3 treatments. These treatments also decreased the microvessel density (MVD) and cell proliferation within the subcutaneous tumors. Therefore, this SN-38/CBSC delivery system has the potential to offer dual therapies for the synergistic combination of PDT and chemotherapy for the treatment of cancer.

1. Introduction

Photodynamic therapy (PDT) is a treatment that utilizes the combined action of photosensitizers (PSs) and specific light sources for the treatment of various cancers. Following the activation of PSs by a photochemical reaction, reactive oxygen species (ROS) are generated to destroy cancer cells [1,2]. The advantages of PDT are that it is a precisely targeted treatment that utilizes selective illumination that can be repeated in the same site if necessary, and it is less invasive than surgery. Despite significant advantages, biodistribution of PSs is limited. In addition phototoxicity to the skin, due to mainly the hydrophobicity and non-selectivity of PSs, is another considerable limitation [3]. In recent years, nanostructured materials such as polymer–drug conjugates [4], liposomes [5], nanoparticles [6] and polymeric micelles [7,8] have been considered as potential carriers for hydrophobic drug delivery that may resolve the aforementioned problems.

Polymeric nanoparticles including micelles composed of amphiphilic block copolymers have shown great promise in drug delivery [9–12]. Generally, amphiphilic block copolymers can self-assemble to form nanosized spherical structures consisting of a hydrophilic outer shell and hydrophobic inner core in aqueous medium. Polymeric micelles composed of amphiphilic block copolymers have demonstrated not only good biocompatibility and but also high stability both in vitro and in vivo, and they have been successfully used to encapsulate various poorly soluble agents [13,14]. In our previous study, chlorin-core star-shaped block copolymer (CSBC) could self-assemble to form the versatile micelles, which can act as nanosized photosensitizing agents for PDT and further encapsulate hydrophobic drugs such as paclitaxel [15]. This functionalized micellar delivery system is a potential dual carrier for the synergistic combination of PDT and chemotherapy for cancer treatments.

Camptothecin (CPT), a cytotoxic alkaloid first isolated from *Camptotheca acuminata* [16], is a promising antitumor agent that targets the nuclear enzyme topoisomerase I (Top I) and inhibits the religation of the cleaved DNA strand, resulting in tumor cell death [17,18]. However, poor solubility in water as well as physiologically acceptable organic solvents limits the clinical use of CPT. 7-ethyl-10-hydroxy-CPT (SN-38), a biologically active form, is metabolized from irinotecan hydrochloride (CPT-11) by carboxylesterases in the

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liver and in tumors; SN-38 is approximately 1000-fold more cytotoxic against various cancer cells than CPT-11. Although CPT-11, a water-soluble, semi-synthetic derivative of CPT, is active against various cancers [19–23], its metabolic conversion rate is approximately 2–8% of CPT-11 [24,25]. Thus, SN-38 might be of great use for cancer treatment; however, it is virtually insoluble in all pharmaceutically acceptable solvents. To overcome the solubility problem, its delivery by micelles was explored to determine if it was possible to use SN-38 for in vivo experiments and further clinical use.

In many cases, tumors are supplied by a leaky neovasculature characterized by an incomplete endothelial barrier and possess poor lymphatic drainage. This phenomenon, which is known as an enhanced permeability and retention (EPR) [26], provides an opportunity for nanosized carriers to reach their target site. Thus, amphiphilic block copolymer-based nanoparticles would be an ideal candidate carrier for antitumor drugs, utilizing the so-called 'passive' targeting, which may in turn improve drug efficacy and reduce the high toxicity to normal cells that accompanies most chemotherapy treatments.

In this study, SN-38-loaded CSBC micelles were prepared using a lyophilization–hydration method. The SN-38/CBSC micelles were characterized by dynamic light scattering (DLS) and atomic force microscopy (AFM), and their encapsulation efficiency, drug content, and in vitro release were also analyzed. Cellular cytotoxicity by the SN-38/CBSC micelles was evaluated in human colon cancer HT-29 cells, and their pharmacokinetics, biodistribution, and antitumor efficacy were compared with CPT-11 in an HT-29 xenograft model. The antitumor efficacy of the SN-38/CBSC micelles combined with PDT was compared with CPT-11; SN-38/CBSC micelles combined with PDT significantly improved bioavailability and antitumor efficacy.

2. Materials and methods

2.1. Materials

5,10,15,20-Tetrakis(4-aminophenyl)-21H,23H-porphine (TAPP) was purchased from Tokyo Chemical Industry (TCI, Tokyo, Japan) and monomethoxy polyethylene glycol (mPEG) (M_W 5000) was supplied by Fluka (Milwaukee, WI, USA). Before polymerization, mPEG was vacuum-dried at room temperature for 24 h. All other HPLC grade solvents, including methanol, ethanol, *n*-hexane, dichloromethane (DCM), acetone, dimethyl sulfoxide (DMSO), acetonitrile, pyridine, and tetrahydrofuran (THF) were obtained from Tedia Inc. (Fairfield, OH, USA). DCM and THF were dried over calcium hydride (CaH_2) and distilled before use. Stannous (II) octoate (SnOct), 3-caprolactone (CL), maleic anhydride dicyclohexyl carbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), pyrene thionyl chloride, triethylamine (TEA), potassium carbonate, *p*-toluenesulfonylhydrazide, tetrachloro-*p*-benzoquinone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and other chemicals were obtained from Sigma Aldrich (Milwaukee, WI, USA). SN-38 (7-ethyl-10-hydroxy-camptothecin) was purchased from ScinoPharm Taiwan Ltd.

2.2. Preparation of SN-38-loaded CSBC micelles

The CSBC micelles were prepared by conjugation of the activated mPEG-*b*-PCL–COCl with the amino group of 5,10,15,20-tetrakis (4-aminophenyl)-21H,23H-chlorin (TAPC) as described previously [15,27]. Briefly, the activated polymer (0.2 mmol) was dissolved in 5 ml THF in the presence of TEA; chlorin (0.015 mmol) dissolved in 1 ml THF was then added, and the mixture refluxed for 48 h. The product was collected by precipitation in diethyl ester and then filtrated and re-dissolved in THF. Finally, the mixture was transferred into dialysis tubes (Spectrapore, MWCO 25 000 or 50 000) and immersed in 500 ml of acetone to remove any free polymer and chlorin. The purified copolymer was recovered by rotary evaporation and dried under vacuum at 40 °C (yield: 57%). The molecular weights of the synthesized polymers were characterized by ^{1}H NMR (Bruker Avance-500 MHz FT-NMR) using deuterated chloroform ($CDCl_3$) as the solvent and gel permeation chromatography (GPC; Waters 510 pump/410 differential refractometer) with a THF eluent at a flow rate of 1 ml/min. Calibration was accomplished using monodispersed polystyrene standards.

The micelles were prepared using a lyophilization–hydration method. Briefly, SN-38 and CSBC were dissolved in 1 ml dimethyl sulfoxide (DMSO) accompanied by gentle agitation until the formation of a clear solution. Different drug to polymer weight ratios (D/P ratio = weight of SN-38/weight of polymer), ranging from 1:5 to 1:20, were tested. The resulting homogeneous solution was then freeze-dried overnight at a condenser temperature of −60 °C and under a vacuum of 10 Pa. The lyophilized cake was hydrated in 0.9% NaCl at 70 °C and then sonicated for 5 min using an ultrasonic cell crasher at 70 °C for further dispersion of the micelles. The micellar solution was filtered through a 0.45 μm cellulose acetate filter membrane to remove non-incorporated drug crystals and copolymer aggregates.

2.3. Characterization of SN-38-loaded CSBC micelles

The micelle solutions were freeze-dried and re-dissolved in DMSO. The SN-38 content incorporated into CSBC micelles was determined by fluorescence with excitation and emission wavelengths of 326 and 426 nm, respectively. The drug encapsulation efficiency (E.E.) and SN-38 content of the micelles were calculated using the following equations: E.E. (%) = (weight of SN-38 in the micelles/total weight of SN-38 in the loading solution) × 100% and drug content (%) = [weight of SN-38/(weight of SN-38 + weight of polymer)] × 100%.

The mean diameter and polydispersity of micelles were measured by DLS (Coulter N4 Plus, Coulter Electronics Ltd., Luton, UK) using the Size Distribution Processor (SDP) mode. The morphology of the micelles was observed by AFM (MFP-3D, Asylum Research, Santa Barbara, CA, USA).

2.4. Release profiles of SN-38 from the CSBC micelles

In vitro release of SN-38 from the CSBC micelles was analyzed using a dialysis-bag diffusion technique at 37 °C [28]. The SN-38/CSBC micelles were dispersed in 5 ml of PBS (Phosphate Buffered Saline) buffers at pH 8.0, pH 7.4, or pH 5.0, and the dialysis bag was immersed in 200 ml of release medium with continuous gentle stirring at 37 °C. At selected time intervals, 200 μl aliquots of the aqueous solution were withdrawn from the release medium. The amount of SN-38 was estimated by fluorescence as described above. All experiments were carried out in triplicate.

2.5. In vitro cytotoxicity

The human colon cancer cell line, HT-29 cells, was maintained in a humidified 5% CO_2 incubator at 37 °C in DMEM (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% heat-activated fetal bovine serum (FBS) and 1% antibiotics (Antibiotic–Antimycotic; GIBCO BRL, Gaithersburg, MD, USA). HT-29 cells were first seeded onto 96-well plates at a density of 10,000 cells per well and cultured. After 24 h, cells were incubated in media containing different concentrations of SN-38, CBSC micelles, SN-38/CBSC micelles, or CPT-11 for 3 days and washed with PBS. Cell viability was determined using an MTT assay and a scanning multi-well ELISA reader (Microplate Autoreader EL311, Bio-Tek Instruments Inc., Winooski, VT, USA). The fraction of live cells was calculated by dividing the mean optical density obtained from treated cells by the mean optical density from untreated control cells.

To determine the cytotoxicity of photosensitizing CBSC micelle-mediated PDT with or without SN-38, the cells were incubated with CSBC micelles or SN-38/CSBC micelles in 10-fold serial dilutions from 10,000 to 0.01 μg/ml (equivalent to 1000–0.001 μg/ml of SN-38) for 24 h at 37 °C. The cells were immediately exposed to different doses of light (7 J/cm^2), and cell toxicity was determined 48 h later. After addition of photosensitizing micelles, all procedures were carried out in subdued light. The light source used to activate the CSBC micelles, which consisted of an array of light emitting diodes (LEDs) emitting in the spectral region of 650–670 nm, with the highest intensity at about 660 nm, was supplied by the Industrial Technology Research Institute (ITRI, Hsin Chu, Taiwan). The total fluence rate was 19.5 mW/cm^2.

2.6. Antitumor efficacy of the SN-38-loaded CSBC micelles

The in vivo experimental protocols were approved by the National Taiwan University College of Medicine and College of Public Health Institutional Animal Care and Use Committee (IACUC). Female BALB/c athymic (nu+/nu+) mice (5–6 weeks old) were purchased from the National Laboratory Animal Center, Taiwan. HT-29 tumors were initially established by a subcutaneous injection of 10^6 cells in PBS. Tumor sizes and body weight were measured every 3 days for the duration of the experiment. Tumor volume was calculated as $1/2(4\pi/3)(L/2)(W/2)H$ where L is the length, W is the width, and H is the height of the tumor. Treatments were started when the tumors reached a volume of 100 mm^3, which was designated day 0. Mice were divided into different groups for the treatments (PBS-control, CPT-11, or SN-38/CBSC micelles), consisting of five mice in each group. Distinct formulations were administered via tail vein injections at multiple doses of SN-38/CSBC micelles (equivalent to 10 mg/kg of SN-38) or CPT-11 (10 mg/kg) in PBS on days 0, 4, and 8. The tumor size and change in body weight of each mouse were recorded. Percentage of tumor growth inhibition (TGI%) was calculated from the relative tumor volume at day 30.

For the combined treatment, SN-38/CSBC micelles were also administered via tail vein injections at one dose of SN-38/CBSC micelles (SN-38: 10 mg/kg and 2.03 mg/kg chlorin equivalent) on day 4 and multiple doses on days 0, 4, and 7. After 24 h, tumors were exposed to light from the diode laser (λ, 652 ± 2 nm; fluence rate,