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Folate receptor-mediated celastrol and irinotecan combination delivery using liposomes for effective chemotherapy

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ABSTRACT

Drug targeting using functionalized nanoparticles provides a new standard in anticancer therapy. Liposomes, safe and effective drug delivery carriers, can incorporate both hydrophilic and hydrophobic drugs for combination chemotherapy treatment of cancers. The objectives of the current study were to synthesize and test the effectiveness of a nanotechnology-based strategy utilizing folic acid (FA)-conjugated liposomes that incorporate both celastrol (Cs) and irinotecan (Ir) for targeted breast cancer therapy. Our results revealed the successful preparation of Cs and Ir-loaded folate-targeted liposomes (Lipo/Cs/Ir-FA) with a small particle size (~190 nm) and polydispersity index (~0.10). The formulation exhibited higher drug release profiles for both Ir and Cs at pH 5.0 compared to those at physiological pH, favoring cancer cell-targeted release. Furthermore, *in vitro* cell studies showed high uptake and enhanced apoptosis in folate receptor-positive breast cancer cells (MCF-7 and MDA-MB-231), but not in folate receptor-negative lung cancer cells (A549). Moreover, an *in vivo* study in a mouse tumor model using MDA-MB-231 xenografts supported effective drug delivery behavior of the folate-conjugated liposomes by selective targeting of tumor tissue and minimizing systemic adverse effects. Therefore, our formulation could provide an effective therapy for targeted cancer treatment.

1. Introduction

Cancer is a leading cause of death worldwide, accounting for 8.8 million deaths in 2015, with breast cancer being one of the most common (approximately 571,000 deaths) [1]. Chemotherapy has been widely applied for the treatment of breast cancer. However, most anticancer agents cannot differentiate between cancerous and normal cells, resulting in severe systemic toxicity. In addition, acquired drug resistance during chemotherapy treatment further reduces treatment efficacy. Drug loaded nanocarriers improve drug delivery systems such as enhance accumulation of drugs at targeted area, increase cellular uptake, prolonged circulation time and improve systemic stability, moreover, it can reduce of toxic effects of the encapsulated compounds on healthy tissues [2]. With the proper treatment strategy, nanocarriers such as liposomes improve drug delivery to the target cancer cells [3,4].

Liposomal nanocarriers are biocompatible; the phospholipid bilayer protects the encapsulated active form of the drug until it reaches the tumor tissue, and minimizes exposure of the encapsulated drug to healthy tissue while in circulation [5]. They have been studied and used widely as nanoscale drug carriers due to their versatility in carrying both hydrophobic and hydrophilic drugs [6] and long blood circulation times due to pegylation of their surfaces [7,8]. In addition, for specific delivery of drugs to pathologic tissues, folic acid (FA) has been widely used for conjugation to the drug delivery system [9]. For the purpose of folate-based targeting, a variety of folate-linked molecules and complexes have been designed for selective delivery of drugs to folate receptor (FR) positive cancer cells [10]. Dual drug-loaded liposome with folate-grafted pegylation significantly enhanced cytotoxicity on prostate cancer cell line [11]. By incorporating a phospholipid-anchored folate into a liposome bilayer, the drug-containing liposomes can be targeted to FR expressing cells [10].

Celastrol (Cs), a quinone methide triterpenoid isolated from the

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Chinese Thunder of God vine, has been used to treat chronic inflammatory and autoimmune diseases [12]. It induces apoptosis in various cancer cell lines *via* inhibition of inhibitor of kappa B kinase (IKK) [13], the proteasome [14], topoisomerase activity [15], and vascular endothelial growth factor (VEGF) receptor expression [12], as well as *via* induction of heat shock proteins [13]. Irinotecan (Ir), a water-soluble camptothecin, is commonly used in clinical practice for cancer treatment. It exerts anti-tumor activity by inhibiting the intranuclear enzyme topoisomerase I [16]. In tumor cells, the level of topoisomerase I enzyme is higher than in normal cells. Irinotecan was shown to exert its cytotoxic activity through inhibition of DNA replication by acting upon the DNA topoisomerase I enzyme [17,18]. DNA topoisomerase relieves the torsional stress that develops during DNA replication by inducing single strand breaks. Irinotecan binds to the topoisomerase I-DNA complex, thereby leading to replication arrest and the formation of double-strand DNA breaks, which can lead to cancer cell death if not repaired.

Therefore, we used celastrol and irinotecan as combined drug because (i) they have different mechanisms to reach targeted tissue and can induce the synergistic anticancer effect; (ii) they are encapsulated in both a lipophilic and aqueous environments of bilayer corresponding to the properties of solubility and both drugs can be protected from sensitive area and liposomal nanoparticle can deliver these drugs by sustained release mechanism (iii) to reduce the main adverse effects of irinotecan in humans are gastrointestinal toxicity and myelosuppression, which limits its usage and administration [19]. The objective of our present study was to prepare and evaluate Cs/Ir combination-loaded liposomes with folate-targeting activity for the effective treatment of breast cancer with minimal adverse effects. The overall scheme is outlined in Fig. 1.

2. Materials and methods

2.1. Materials

Cs was obtained from Aktin Chemical, Inc., (Chengdu, China). Ir was a kind gift from Hanmi Pharmaceuticals, Co. Ltd. (Hwaseong, South Korea). FA, cholesterol, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). MCF-7, MDA-MB-231, and A549 cells were obtained from the Korean Cell Line Bank (Seoul, South Korea). Dipalmitoylphosphatidylcholine (DPPC) was obtained from NOF America Corporation (White Plains, NY, USA). 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG-NH_2) was purchased from Avanti Polar Lipid (Alabaster, AL, USA). Coumarin-6 and LysoTracker® Red were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All other chemicals were of reagent grade and were used without further purification.

2.2. Synthesis of DSPE-PEG-FA

DSPE-PEG-FA was synthesized by a carbodiimide-mediated coupling of FA to DSPE-PEG-NH_2 [20]. Briefly, 10.0 mg FA was dissolved in 1 mL dimethyl sulfoxide (DMSO). DSPE-PEG-NH_2 (35 mg) and pyridine (250 μL) were added to the reaction solution, followed by 20 mg dicyclohexylcarbodiimide. After completion of the reaction (approximately 24 h at room temperature), the pyridine was removed by rotary evaporation and 5 mL water was added to the solution. The solution was dialyzed with a 3500 Da molecular weight cutoff (MWCO) dialysis bag (Sigma-Aldrich) against 1000 mL 50 mM saline for 1 day and against 1000 mL distilled water for 2 days to remove excess unreacted substrates. The product was lyophilized. All processes were conducted in the dark. Fig. S1A depicts the synthesis of DSPE-PEG-FA. The product was a yellow dry powder. The composition of the prepared DSPE-PEG-FA conjugate was analyzed by Fourier-transform infrared spectroscopy (FTIR) and proton nuclear magnetic resonance (^{1}H NMR) (Avace300, Bruker, Germany).

The amount of FA in DSPE-PEG-FA was quantified by a previously published ultraviolet (UV) method (PerkinElmer U-2800, Hitachi, Japan) [20]. Briefly, FA (10 mg) was dissolved in 10 mL DMSO, and the solution was diluted to different concentrations (5–80 μg/mL). These dilutions were measured with a UV–vis spectrophotometer (UV-2102PCS; UNICO Instruments Co., Ltd, Shanghai, China) at a wavelength of 365 nm, and a standard curve in the range of 5–80 μg/mL was obtained. The content of conjugated FA was estimated from the standard curve of free FA under the same UV–vis conditions, assuming an identical molar absorbance for free FA and polymer-conjugated FA.

In addition, the characteristic peak of prepared DSPE-PEG-FA conjugate was analyzed by using FTIR and ^{1}H NMR. The FTIR spectra of FA, DSPE-PEG-NH_2, and DSPE-PEG-FA conjugate were compared. Moreover, the specific chemical shifts of DSPE-PEG-FA were recorded using an Agilent Technologies VNMRS 600 MHz NMR spectrometer (Varian Inc., USA); DMSO-d_6 was used as the solvent for this conjugate [21].

2.3. Preparation of folate-targeted liposomes

Liposomal nanoparticles were prepared by the thin-film hydration technique [22]. Briefly, the organic phase was prepared by dissolving 16.5 mg DPPC, 6 mg cholesterol, and 2.5 mg DSPE-PEG-FA, along with 3 mg Cs in a mixture of chloroform and methanol (4 mL, 4:1 v/v). A thin lipid film was formed by evaporating the organic solvent in a rotary evaporator (EYELA, Tokyo, Japan) maintained at 40 °C. The resulting thin film was hydrated with phosphate-buffered saline (PBS) containing dissolved 3 mg Ir. The large vesicles were then extruded through a 100-nm pore filter using a mini-extruder (Avanti Polar Lipids, Inc., Alabaster, AL, USA) for 11 cycles to yield monodispersed nanosized liposomes. The exact amount of polymer required to cover the liposomal surface was determined by mixing various ratios of the two solutions until nanoparticles with a uniform size distribution were produced.

2.4. Particle size and zeta-potential analysis of Lipo/Cs/Ir-FA

The Z-averaged hydrodynamic particle sizes, polydispersity indexes (PDIs), and zeta-potentials of nanoparticles were determined using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) with the dynamic light scattering (DLS) method. Software (Nano DTS software, version 6.34) that employs cumulative analysis was used to determine particle size, PDI, and zeta-potential. The mean value was calculated from three individual measurements [23].

2.5. Drug loading

To calculate drug-loading efficiency, drug-loaded nanoparticles were separated from unbound drugs using an Amicon centrifugal filter device (MWCO of 10,000 Da, Millipore). The formulations were centrifuged through the filter at 5000 rpm for 10 min, and unbound drugs in the filtrate were analyzed using high performance liquid chromatography (HPLC). An Inertsil® C18 column (150 mm × 4.6 mm, 5 μm particle size, Cosmosil, Nacalai Tesque Inc., USA) was used to perform isocratic elution with a mobile phase consisting of acetonitrile: ethanol: distilled water: trifluoroacetic acid (TFA) (3:5:2:0.01, v/v/v/v) pH 2.5 for Cs, and water: acetonitrile: methanol (50:25:25,v/v/v) pH 3.5 for Ir. A flow rate of 1.0 mL·min^{-1} was used for both drugs, and the column temperature was maintained at 25 °C A 20 μL sample was injected for each analysis and UV absorbance was measured at a wavelength of 430 nm for Cs and 255 nm for Ir. The drug encapsulation efficiency (EE (%)) was calculated as follows

$$\mathrm{EE}\,(\%) = \frac{W_{lipo}}{W_T} \times 100$$

where W_{lipo} is the weight of Cs/Ir entrapped in the liposome, and W_T is

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