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High paclitaxel-loaded and tumor cell-targeting hyaluronan-coated nanoemulsions

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ABSTRACT

The purpose of this study was to develop hyaluronan-coated nanoemulsions (HNEs) with high solubilizing capacity and tumor cell targeting capability for the poorly soluble paclitaxel. The HNEs were composed of DL-a-tocopheryl acetate, soybean oil, polysorbate 80, and ferric chloride and were coated with hyaluronic acid (HA) as a targeting moiety. The nanoemulsions (NEs) and HNEs with or without paclitaxel (PTX) were prepared using high-pressure homogenization with a microfluidizer and were lyophilized with D-mannitol. The particle diameter and zeta potential of the HNEs were 65 ± 15 nm and -39.5 ± 0.33 mV, respectively. The concentration of PTX loaded in the NEs was 6 mg/mL, which was higher than that in any other nanocarrier. The HNEs were coated with HA on the outer surface of the sphere and the amount of HA was $0.82 \pm 0.10\%$ (w/w). The lyophilized formulation was stable at 4 °C for 12 months and the reconstituted HNE solution was stable for at least 96 h, even though Taxol® can be maintained for only 72 h. In the cell affinity studies with SK-OV-3 (cluster of differentiation 44 [CD44]$^+$) and OVCAR-3 (CD44$^-$) cells, the HNEs displayed a 10-fold higher targeting capability than the NEs did. Therefore, the HNEs displayed high drug loading capability, excellent stability, and targeting of tumor cells overexpressing CD44, which suggested they were a potentially effective nanocarrier for carrying poorly soluble paclitaxel and targeting tumors.

1. Introduction

In recent years, in an attempt to reduce the side effects of cancer chemotherapy, formulation scientists have investigated numerous nanocarrier systems, including nanoparticles [1], polymeric micelles [2], liposomes [3], emulsions [4], and nanogels. These studies were focused on attempts to find technologies that effectively targeted cancer cells, including passive and active targeting [5]. Although nanocarriers provide numerous advantages as drug carrier systems, considerable limitations exist, such as low bioavailability, precipitation after injection, inadequate tissue distribution, low drug loading doses, large injection volume, unpredictable toxicity, low solubilizing capacity for poorly soluble drugs, and instability during storage.

In particular, numerous studies have focused on developing nanoparticles with a high drug loading capacity for poorly soluble drugs, such as paclitaxel, to enhance patient compliance and reduce adverse effects [6]. However, these systems, including natural, poly(lactic-*co*-glycolic acid) (PLGA), solid lipid nanoparticles, liposomes, and inclusion complexes, were not satisfactory solutions to the existing problems of nanocarriers [7,8]. Recently, the maximum drug loading dose for nanocarriers in various research studies was up to approximately 3 mg/mL for paclitaxel (PTX), a poorly soluble and representative anticancer drug [6]. Therefore, we investigated nanoemulsions (NEs) as drug carrier systems with efficient drug loading capabilities for poorly soluble drugs.

NEs are composed of an oil and a surfactant, cosurfactant, or both with a particle size < 200 nm. Furthermore, NEs have been extensively used for the delivery of poorly soluble chemotherapeutic drugs because of their unique solubilizing properties that are associated with their composition and emulsion specificity [9]. Compared to other nanoparticle systems, NEs as parenteral drug carriers have several advantages, including a simple manufacturing process, high bioavailability, excellent solubilizing capacity in oil droplets for highly lipophilic drugs, high drug loading capacity, long shelf life, and no accumulation in the human body [10]. However, because NEs are only composed of oils and emulsifiers, but do not include target molecules, it is difficult to actively target them to specific cancer cells or other disease sites. There have been challenges associated with ensuring that the target molecule or moieties in NEs adhere to specific sites and there are some limitations to be addressed, such as unpredictable toxicity caused by the

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nonspecific distribution of the active drug to normal cells and weak binding to cancer cells. Therefore, it has been necessary to investigate methods for producing non-toxic and strong bonds between the target molecule and the oil phase of the NEs to achieve better tumor targeting.

Currently, various molecules, such as folic acid, RGD, biotin, lipoprotein, cholesterol, and hyaluronic acid (HA), have been used in drug carrier systems for anticancer agents to target active drug to tumor tissues [11]. HA, which is a negatively charged linear polysaccharide composed of repeating units of glucuronic acid (GlcUA) and *N*-acetyl-D-glucosamine (GlcNAc), is widely distributed throughout the epithelial and neural tissues. It has been investigated as a target molecule owing its unique properties [12]. HA specifically binds to cluster of differentiation 44 (CD44), which is a cell surface biomarker that is overexpressed in tumors and has low expression in normal tissues. The specific binding of HA to CD44 in cancer therapy provides a new strategy for active tumor targeting. Several nanocarrier formulations using HA, which can be connected to the nanocarrier particles by covalent bond modification, electrostatic attraction, or ionic bonding, have been developed, such as HA-drug conjugates, HA nanogels, and HA decorated nanoparticles [13]. These nanocarriers have good targeting capabilities for cancer cells, but their limited solubilization of poorly soluble drugs is a serious challenge.

Therefore, in this study, we investigated NEs as drug carrier systems with efficient drug loading rates for poorly soluble drugs and a chelating method for non-toxic and strong bonding between HA as a targeting molecule and the oil phase of the NEs for tumor targeting. The physicochemical properties and PTX-solubilizing capacity of NEs and HA-coated NEs (HNEs) were evaluated and the tumor cell targeting capability was studied in CD44-expressing cell lines.

2. Materials and methods

2.1. Materials

Paclitaxel was provided by Cipla Pharmaceutical Co. Ltd. (Uttar Pradesh, India). DL-a-tocopheryl acetate was purchased from DSM Pharmaceutical Tech. Co. Ltd. (Shanghai, China). Myvacet 9–45 K^{TM} was purchased from Kerry Inc. (Waunakee, WI, USA). Miglyol 812^{TM} was purchased from CREMER OLEO GmBH & Co. KG (Hamburg, Germany). Soybean oil was purchased from Croda Pharmaceutical Co. Ltd. (Yorkshire, UK) and injectable polysorbate 80 was purchased from Seppic Pharmaceutical Co. Ltd. (Fairfield, NJ, USA). Triacetin, tributylin, mannitol, ferric chloride ($FeCl_3$), chloromethylbenzoyl chloride, and tetra-n-butyl ammonium hydroxide (TBA) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). HA was purchased from Bioiberica Co. Ltd. (Barcelona, Spain). Anti-human/mouse CD44 fluorescein isothiocyanate (FITC) was purchased from eBioscience (San Diego, CA, USA) and sodium salicylate was purchased from Duksan Co. Ltd. (Gyeonggi, Korea).

The SK-OV-3 ($CD44^+$) human ovarian cancer cell line was acquired from the American Type Culture Collection (ATCC, HTB-77; Rockville, MD, USA) and the OVCAR-3 ($CD44^-$) human ovarian cancer cell line was acquired from the Korean Cell Library Bank (KCLB)-00000287 (Seoul, Korea).

The Roswell Park Memorial Institute (RPMI) 1640 cell culture medium (Waymouth), penicillin, streptomycin, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer solution, and heat-inactivated fetal bovine serum (FBS) were purchased from Gibco Life Technologies, Inc. (Big Cabin, OK, USA). All other chemicals were of reagent grade and were obtained commercially.

2.2. Preparation of NEs and HNEs

2.2.1. Solubility

To determine the appropriate components for the oil phase of the NEs to ensure that the poorly soluble drug was solubilized, the solubility of PTX in various oils (Myvacet 9–45 K^{TM}, DL-a-tocopheryl acetate, triacetin, trybutylin, and Myglyol 812^{TM}) was determined after adding an excess amount of the drug to 3 mL of each of the selected oils. The mixtures were kept at 25 °C in 10-mL beakers with magnetic stirring at 200 rpm for 24 h to achieve an equilibrium state. The equilibrated samples were removed from the beaker and centrifuged at 12,500 rpm for 30 min. The supernatant was collected and filtered through a 0.45-μm membrane filter to remove the free PTX. The PTX content of the oils was then assayed using a high-performance liquid chromatography (HPLC) system based on the method described in section 2.4. The solubility of PTX in the mixtures of oils and surfactants (polysorbate 80, polysorbate 20, poloxamer 407, sorbitan laurate, and soy lecithin; oil:surfactant, 1:1, v/v) was determined using a method similar to that described above. To evaluate the compatibility of the oils and surfactants, the stability of PTX in the various oils was determined after storage at 25 °C for 7 days.

2.2.2. Preparation of NEs

The NEs were comprised of an oil phase, surfactant, and water phase. First, the oil phase, which was composed of DL-a-tocopheryl acetate and soybean oil, was selected based on its compatibility and solubilizing capability with the hydrophobic drug PTX. Polysorbate 80 was chosen as the surfactant based on its emulsification ability and emulsion stability with the oil phase. Moreover, these materials are known to possess good biocompatibility, good biodegradability, and low toxicity and have been used for the preparation of parenteral NEs in other research studies [14]. The quantities of DL-a-tocopheryl acetate, soybean oil, and polysorbate 80 in our NE formulation were 720, 80, and 800 mg, respectively. The NEs were prepared using a high-pressure homogenization method. Briefly, the oil and surfactant were gently mixed in a 50-mL round-bottomed flask. Thereafter, 8.4 mL of water for injection was added to the mixture of oil and surfactant with gentle magnetic stirring for 1 h. The mixture of the two phases was slowly added to a chamber containing microfluidizers and the emulsion was further homogenized using a high-pressure homogenizer (M-110S, Microfluidizer®, New York, NY, USA) at 15000 psi seven times, then cooled to 20 °C. Finally, the prepared NEs were added to 500 mg D-mannitol as a cryo-protectant and lyophilized using a freeze dryer (Genesis 25XL, Virtis freeze dryer^{TM}, Warminster, PA, USA).

2.2.3. Preparation of HNEs

The HNEs were comprised of an oil phase, surfactant, and water phase. HA (molecular weight (MW) 500 kDa) was used as a targeting molecule and the HNEs were prepared with a high-pressure homogenization method using a microfluidizer. The HA was coated onto the surface of the NEs using chelating covalent bonding attraction [15]. First, 2 mg of $FeCl_3$ was dissolved in 20 mL of ethanol, then the 20 mL of ethanol containing $FeCl_3$ was dissolved in a mixture of 7.2 g of DL-a-tocopheryl acetate, 0.8 g of soybean oil, and 8.0 g of polysorbate 80 at 25 °C. Thereafter, the ethanol was evaporated using a vacuum rotary evaporator and the mixture with the $FeCl_3$ in the oil phase was formed in a 200-mL round-bottomed flask. Second, 252, 126, and 25.2 mg samples of HA were dissolved in 84 mL of water for injection and were then added to the previously prepared oil phase with gentle magnetic stirring for 1 h. The two phases were mixed using high-pressure homogenization and lyophilization using the method described in Section 2.2.2.

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