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Transferrin targeted liposomal 5-fluorouracil induced apoptosis *via* mitochondria signaling pathway in cancer cells

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ABSTRACT

The purpose of this study was to prepare transferrin (Tf) targeted liposomal 5-Fluorouracil (5FU) to improve the safety and efficacy of the drug. Liposomes were prepared using thin layer method. Morphology of liposomes was characterized by transmission electron microscopy (TEM) and their particle size was also determined. The *in vitro* cytotoxicity was investigated *via* MTT assay on HT-29 (as cancer cell) and fibroblast (as normal cell). Moreover, cytotoxicity mechanism of targeted liposomes was determined through the production of reactive oxygen species (ROS), mitochondrial membrane potential ($\Delta\Psi_m$) and release of cytochrome *c*. Results showed that encapsulation efficiency (EE%) was 58.66 ± 0.58 and average size of liposomes was 107 nm. Also, nanoparticles were spherical as shown by TEM. MTT assay on HT-29 cells revealed the higher cytotoxic activity of targeted liposomes in comparison to free drug and non-targeted liposome. In contrast, comparing with cancer cells, targeted liposomes had no cytotoxic effect on normal cells. In addition, targeted liposomes induced apoptosis through activation of mitochondrial apoptosis pathways, as evidenced by decreased mitochondrial membrane potential and release of cytochrome *c*. Results of the study indicated that targeted liposomes would provide a potential strategy to treat colon cancer by inducing apoptosis *via* mitochondria signaling pathway with reducing dose of the drug and resulting fewer side-effects.

1. Introduction

Colon cancer is a major cause of morbidity in the world. 5FU is the first-line treatment against colon cancer for many years [1]. Due to structural similarity to the pyrimidine base of DNA and RNA, 5FU interferes with nucleoside metabolism, leading to cytotoxicity and cell death [2]. However, clinical applications of 5FU has drawbacks including short half-life (20 min) due to rapid metabolism and non-specific drug distribution resulting sever systemic toxicity on bone marrow cells, gastrointestinal tract, hematological, neural, cardiac and dermatological effects [2–4]. Therefore, several approaches have been attempted to improve the delivery of 5FU in order to enhance therapeutic index with reducing side effects. Encapsulation of 5FU in nanoparticles, such as liposomes, can decrease drug clearance and reduce its associated toxicity [5]. Liposomes are sphere-shaped vesicles composed of one or more phospholipid bilayers [6]. They are considered as efficient drug delivery system for drugs with different physicochemical properties, diagnostics, vaccines and other bio-molecules [6]. As drug delivery, liposomes have many advantages such as biodegradability, biocompatibility, nontoxic properties, ability to encapsulate both hydrophilic and lipophilic drugs and providing the protection of drugs from the external micro environment [7,8]. In order to improve liposomal drug delivery to the tumor site, targeting approaches with the conjugation of ligands to the surface of liposomes have been extensively studied. Targeted drug delivery improves the therapeutic effect of drugs by increasing circulation half-life, reduction of toxic side effects and allowing higher specificity in the delivery of the drug to the tumor cells [9]. Transferrin, a 78 kDa-monomeric glycoprotein responsible for cellular iron absorption, is one such molecule that can be employed for targeting [10]. The Tf receptor (TfR) is overexpressed in 90% of tumors

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[7] and high specificity of endocytosis uptake of Tf by the TfR has further made it a subject of interest for targeted drug delivery [11].

Molecular mechanisms of many cytotoxic chemotherapeutic agents are known. There are various patterns of cell death when the cells are exposed to anticancer drugs. Many cell death-associated signal transduction pathways are promoted through mitochondrial function [12]. Apoptosis or programmed cell death plays a critical role in response to stress-induced or certain regulatory signals. There are two main apoptotic pathways: the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway. The intrinsic apoptotic pathway is activated by various intracellular stimuli, including DNA damage, oxidative stress and growth factor deprivation. The extrinsic pathway of apoptosis is initiated by the binding of death ligands such as Fas ligand and TNF-α to death receptors of the TNF receptor super family [13]. Reactive oxygen species (ROS) is one of the main factors related to the cell death. The value of ROS may determine the selection between necrosis and apoptosis [14]. It has been reported that low and high levels of ROS regulate apoptotic and necrotic signaling pathway, respectively [15]. The apoptotic pathway induction is a desirable end point when treating the cancer since it will not induce inflammation. Targeting formulation is focused on improvement of agents that selectively inhibit the growth of cancer cells with reducing toxicity while maintaining a therapeutic window. This approach might improve the efficacy of anticancer agents by affecting the mitochondria and trigger cell death pathway in different ways from traditional chemotherapy which can emerge as a promising means to cancer therapy.

The objective of the present study was to prepare and characterize 5FU loaded transferrin targeted liposome. In addition, we evaluated the potential of targeted liposomes to selectively deliver of the drug to cancer cell and we further investigated the mechanism of cell death.

2. Material and methods

5FU and soya phosphatidyl choline (PC) were purchased from Acros, USA. Distearoylphosphatidylethanolamine (DSPE) was obtained from Lipoid, Germany. Cholesterol, succinic anhydride and *N*-hydroxysuccinimide (NHS) were acquired from Merck, Germany. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Alfa Aesar, Germany. Tf was obtained from Sigma-Aldrich, Germany. Metronidazole was kindly donated by Pars Darou Pharmaceutical Co., Iran.

HT-29 (human colorectal adenocarcinoma) and fibroblast (Hu02) cells were obtained from Iranian Biological Resource Center (IBRC). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco, USA. Penicillin-streptomycin and 5-diphenyl tetrazolium bromide (MTT) were acquired from Sigma-Aldrich, Germany. 2′,7′-Dichlorofluorescin diacetate (DCFDA) was obtained from Sigma-Aldrich, Germany. MitoLight™ Apoptosis Detection kit was provided from Merck Millipore, USA. Cytochrome c human ELISA kit was purchased from Abcam, USA and Annexin V-FITC apoptosis detection kit was acquired from Sigma Aldrich, USA. All of the solvents were of the analytical grade.

2.1. Synthesis of Tf-DSPE

The schematic of synthesis of Tf-DSPE was carried out as shown in Fig. 1. The conjugation of Tf to DSPE was performed in two steps. Firstly, DSPE (200 mg, 0.27 mmol) was dissolved in 20 mL of dry chloroform. Then succinic anhydride (200 mg, 2 mmol) was added and stirred at room temperature for 48 h. Thereafter, 100 mL chloroform and 20 mL deionized water added under stirring and this mixture was incubated for 30 min. This procedure was repeated twice and then the chloroform layer was separated. To remove the traces of water present in separated chloroform layer, it was treated with anhydrous sodium sulphate and chloroform was evaporated using rotary evaporation (Heidolph, Germany). In the second step, activated-DSPE (10 mg) was dissolved in 5 mL of dimethyl sulfoxide (DMSO). Afterward, EDC (500 mg) and NHS (400 mg) were added and the mixture was incubated at room temperature for 6 h. The aqueous solution of transferrin (10 mg/mL) was added under stirring at room temperature for 24 h. The resulting solution was diluted with deionized water and dialyzed twice against deionized water using dialysis membrane (MWCO 12 kDa) and finally freeze-dried to get dry powder (Operon, Korea). Conjugates were characterized by Fourier transform infrared spectroscopy (FT-IR) (Perkin-Elmer, USA).

2.2. Preparation of liposomes

Liposomes were prepared using thin film hydration method. Briefly, PC/cholesterol/Tf-DSPE at different molar ratio of 0.5:1:0.0061, 1:1:0.0061 and 2:1:0.0061 were dissolved in chloroform in a round bottom flask. The thin lipid film was formed by removing chloroform under rotary evaporation. Lipid film was hydrated with phosphate buffer saline (PBS, pH 7.4) containing 5FU (1.5 mg) by sonication in a water bath for 15 min. Then, the suspension was homogenized for another 5 min. The non-encapsulated 5FU was separated by centrifugation at 15,000 rpm for 30 min (MPW-350R, Poland). The resulted formulation was lyophilized and stored at 4 °C for further analysis.

2.3. Determination of encapsulation efficiency

Determination of 5FU loaded in liposomes was performed using high performance liquid chromatography (HPLC) system (Waters, USA). The analysis was carried out on C_{18} column (250 × 4 mm i.d., 5 μm) at 30 °C and the wavelength was set at 260 nm. The mobile phase was consisted of 0.02 M phosphate buffer pH 4 and methanol (70:30, V/V) at a flow rate 0.8 mL/min. Injection volume was 50 μL and metronidazole was used as internal standard. The encapsulation efficiency (EE%) was determined in accordance with Eq. (1):

$$EE\% = (A_I - A_F/A_I) \times 100 \quad (1)$$

where, A_I is the amount of 5FU initially added to the formulation and A_F is the amount of the free drug in the supernatant after centrifugation [4].

2.4. Morphology study and particle size of liposomes

Morphology of liposomes was determined by transmission electron microscopy (TEM, LEO 906, Zeiss, Germany). Samples were first dispersed in deionized water and then one drop of the suspension was placed onto a carbon-coated copper TEM grid. Particle size of liposomes was also analyzed using a particle sizer (Qudix, Scatteroscope I system, Korea) at 25 °C. Samples were diluted with deionized water and sonicated prior to measurement.

2.5. Cytotoxicity assay

In vitro cytotoxicity activity was evaluated using MTT method based on the cleavage of yellow tetrazolium salt MTT by metabolically active cells to form a dark purple formazan dye [16,17]. HT-29 (colon cancer cell line) and fibroblast (normal cell) were grown at 37 °C, 5% CO_2 and 95% relative humidity in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Cells was seeded at a density of 1×10^4 in 96-well plates and were incubated for 24 h. Supernatants from the wells were aspirated out and replaced with fresh growth medium containing different concentrations of 5FU, liposomal 5FU and Tf-liposomal 5FU (25, 35, 50, 75 and 100 μM) for 48 h. At the end of incubation time, 20 μL of MTT (5 mg/mL in PBS) solution was added into each well and incubated at 37 °C for another 4 h. Then, DMSO (150 μL) was added in each well and the plates were placed on a plate shaker for 20 min. The absorbance of each plate was read at 570 nm using ELISA plate reader (BioRad, USA). Cellular viability was determined using the Eq. (2) and