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Development and evaluation of antisense shRNA-encoding plasmid loaded solid lipid nanoparticles against 5-α reductase activity

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ABSTRACT

In this study, we aimed to develop and evaluate an effective system for nucleic acid delivery. For this purpose, shRNA-encoding plasmid against 5-α reductase (p5α-Red) activity was selected as a model. In order to achieve the delivery of p5α-Red and to obtain lower cytotoxicity, higher transfection and silencing efficiency, we developed solid lipid nanoparticles (SLNs) as a delivery vector by the melt-emulsification process. As a first step, microemulsions were prepared by using Compritol ATO 888 as internal oil phase; Tween 80 as a surfactant (S); ethanol as co-surfactant (CoS) and ultra-pure water as the continuous water phase. Then, obtained o/w microemulsion was dispersed in cold ultra-pure distilled water (0-4 °C) to form SLNs. The formulated nanoparticles were electrostatically bound to p5α-Red to form SLN:p5α-Red vectors. The SLN:p5α-Red vectors have particle sizes of 62.65 nm, and zeta potential values of 12.9 mV. DNase I protection analysis showed that developed formulation is able to protect the p5α-Red from degradation. The permeation study revealed that SLN:p5α-Red vectors are able to pass the cellulose membrane and 80.2% of the SLN:p5α-Red vectors were dialyzed after 6 h. According to cytotoxicity test results, no significant cytotoxicity was observed on DU-145 cells in the concentration range of 0.3–0.6 μg/well. Furthermore, in vitro gene silencing experiment demonstrated that SLN:p5α-Red vector effectively reduced 5α-Red enzyme level 48 h after administration in DU-145 cell line. Considering the role of 5-α reductase in related diseases such as benign prostatic hyperplasia, androgenic alopecia and prostate cancer, the developed SLN:p5α-Red vector system may have promises in future therapy.

1. Introduction

RNA interference is a specific post-transcriptional gene silencing mechanism within a cell via the transfection of microRNAs (miRNA), exogenous small interfering RNAs (siRNA) or small hairpin RNAs (shRNA) [1–3]. Transfection of shRNA can be done by shRNA-encoding expression plasmids. These vectors have several advantages. They ensure sustained silencing of the targeted genes via prolonged expression of shRNAs. Furthermore, shRNA-encoding sequence integration into the genome is also possible with specially designed plasmids. On the contrary, shRNA-encoding expression plasmids are larger than shRNA because of including elements for plasmid formation like promoters and antibiotic resistance sequences. Consequently, shRNA-encoding expression plasmids are comparatively larger molecules and it is more challenging to transfect cells. High transfection efficiencies are required for gene silencing. For this purpose, a convenient plasmid delivery system is required [4–6].

Solid lipid nanoparticles (SLNs) have gained increased interest over the past decade due to the various functions that help to incorporate genes into cells as a nonviral gene delivery system [7,8]. SLNs are nanosized particulate systems prepared by lipids which are solid at room temperature. SLNs can be prepared by various techniques such as solvent emulsification-evaporation, microemulsion dilution, high pressure homogenization, solvent evaporation and ultrasonication methods [9,10]. They usually consist of physiologically well-tolerated ingredients already approved for pharmaceutical applications in humans, can readily be produced in large scale, have good storage capabilities including freeze-drying, can be sterilized and have low cytotoxicity [11,12]. SLNs could be produced in nanosize and have been shown to condense pDNA into nanometric colloidal particles. SLN:pDNA vectors are capable of transfecting mammalian cells in vitro and better results were obtained when compared with standard DNA carriers such as cationic lipid, cationic polymers, cationic liposomes or some other commercially available transfection kits [13–15]. The total surface charge and the size of the SLN:pDNA vectors play important roles in cellular uptake of cells. The size of SLNs and electrostatic interactions between vector and cell surface can be modified due to various production strategies to obtain higher transfection efficiency [16].

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5α-reductase were chosen as a target protein in this study for gene silencing due to its important role in steroid metabolism [17]. The conversion of testosterone to dihydrotesterone (DHT) is catalyzed by 5α-reductase and 5α-reductase inhibitor drugs such as finasteride, dutasteride, and turosteride are mainly used in benign prostatic hyperplasia (BPH) and androgenic alopecia (AA) [18,19]. Although, 5-α reductase inhibitors are primarily used to treat BPH and AA, these inhibitors are under investigation to be used against prostate cancer, as well [20–22]. Their efficacy against prostate cancer has been evaluated in recent years and there are some 5α-reductase inhibitors that have ongoing phase II and phase III trials for the markets [23]. Beside their promising uses in different disease, gynecomastia, erectile dysfunction, fatigue, hypoglycemia, and depression, are only a few of the possible side-effects of conventional 5α-reductase inhibitors. These effects reduce their systemic usage especially for AA indication [24,25]. Due to all these side effects, topical administration of nucleic acid-based therapeutics against 5α-reductase activity may be an alternative and effective delivery route without systemic side effects [26–29]. In this study, we aimed to develop SLN:pDNA vector system for delivery of shRNA-encoding plasmid against 5-α reductase in order to silence the expression of 5α-reductase enzyme transcripts. DU-145 cell line was chosen for its well-known 5-α reductase activity and to test the silencing effects of developed vector system [30–32]. Permeation ability of vector system was also evaluated for its possible use in topical therapy in the future for some diseases such as AA. Obtained data in this study may help to develop drugs or vectors with less side effects against BPH, prostate cancer or AA for further studies.

2. Material and methods

2.1. Materials

Plasmid DNA which encodes green fluorescent protein (pEGFP-C1) was purchased from Invitrogen, California, USA. 5-α reductase-shRNA-encoding plasmid (p5α-Red), 5-α reductase-control plasmid (p5α-Red-C) and 5-α reductase antibody was obtained from Santa Cruz Biotechnology, USA. Amplification and isolation of the plasmid were performed according to standard molecular biology protocols. Compritol ATO 888 was kindly donated by Gattefosse, France. XTT cell proliferation assay kit was provided from Biological Industries, Israel. Human prostate cancer cell line (DU-145) was purchased from ATCC, USA. Ethanol, Tween 80 and Sodium dodecyl sulfate (SDS) were provided from Merck-Co., Germany. Dimethyldioctadecylammonium bromide (DDAB) were purchased from Sigma-Aldrich Co., USA. All other chemicals were of analytical grade and used as received. Ultrapure water was used in all stages needed.

2.2. Methods

2.2.1. Vector preparation

SLNs as gene delivery vectors were prepared by the melt-emulsification process [33]. Firstly, oil in water (o/w) microemulsion system was formed. For this stage, microemulsion was prepared by using Compritol ATO 888 as internal oil phase; Tween 80 as surfactant (S); ethanol as co-surfactant (CoS) and ultra-pure water as the continuous water phase. Pseudo ternary phase diagram was drawn by water titration with components of microemulsion over lipid melting temperature (~80 °C). The o/w microemulsion formation area on the phase diagram was determined due to transparency of the mixture. To impart cationic character to the SLNs, DDAB was incorporated into the oil phase of the microemulsion. Following microemulsion formation, obtained o/w microemulsion was dispersed in cold ultra-pure distilled water (0-4 °C) at a ratio of 1:10 (w/v) under magnetic stirring at 1000 rpm. SLNs were formed when hot microemulsion droplets were met with cold water.

2.2.2. Gel retardation assay and DNase I protection analysis

Gel retardation assay was performed to evaluate the complex formation ability and protection capability of SLNs against serum nucleases [34]. SLN: p5α-Red vectors were obtained by mixing the constant amount of p5α-Red (0.1 μg) with the increasing amount of obtained SLNs (10–100 μg) under agitation for 30 min at room temperature, which allows the formation of electrostatic interactions between the positive charges of SLNs and the negative charges of p5α-Red. The resultant complexes were characterized by gel retardation assay.

In order to show the capacity of the SLNs to protect p5α-Red, SLN:p5α-Red vectors were incubated with DNase I (0.4 IU DNase I/1 μg pDNA) at 37 °C for 30 min, then decomplexed in presence of SDS 1% and further subjected by gel retardation assay [35]. The bands were observed by a UV transilluminator with a digital imaging system (Vilber Lourmat, France) and the integrity of the vector was compared with a control of untreated p5α-Red. All agarose gel electrophoresis studies (1% agarose/1xTAE, w/v) were carried out under a voltage of 100 V for 60 min.

2.2.3. Particle size and zeta potential measurements

For particle size measurements, SLN formulations and SLN: p5α-Red vectors were diluted 5-fold with ultrapure water. Measurements were performed on Zetasizer Nano ZS instrument (Malvern Instruments Ltd., England) using non-invasive back scattering mode with the detector positioned at 173° from the incident beam. Samples were filled in disposable polystyrene microcuvettes. At least three measurements were performed for each sample. Cumulative analysis was employed by the software in order to analyze the autocorrelation function, and Z-average particle size and polydispersity index (PDI) were reported. Electrophoretic mobility of the dispersions was measured in standard zeta cuvettes (Malvern Instruments Ltd., England), and the zeta potential was calculated by the software using Smoluchowsky equation. The data are presented as mean ± S.D.

2.2.4. Stability of SLNs

The particle size of SLNs were measured to evaluate their stability for 3 months. Samples were stored at 4 °C and analyzed at days 0, 7, 14, 30, 60, and 90.

2.2.5. In vitro membrane permeation assay

Dialysis membrane method was used for the in vitro penetration study of SLN:p5α-Red vectors. In these systems, cellulose membrane was used as barrier between donor and receptor compartment and nanosized dosage form itself can diffuse through the dialysis membrane to the outer compartment [36,37]. To determine the in vitro permeation of SLN:p5α-Red vectors, a suitable apparatus was used that allows the evaluation of dialysis in a very small donor volumes (Fig. 1).

For this purpose, 0.75 cm^2 holes as diffusional surface area were opened on the lid of 1.5 mL microcentrifuge tubes. Complexes which contain 12.5 μg p5α-Red in 0.5 mL volume were loaded into the tubes

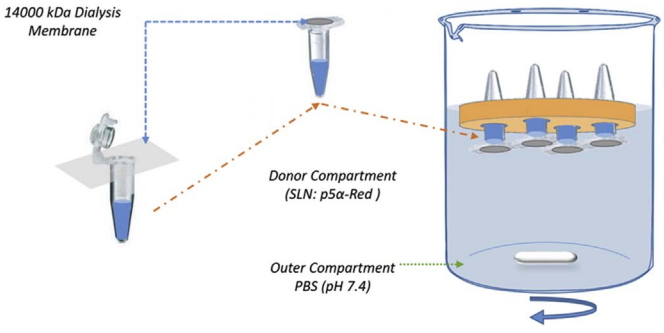

Fig. 1. Schematic illustration of the apparatus used for dialysis membrane method.