Lipid nanoparticles with minimum burst release of TNF-α siRNA show strong activity against rheumatoid arthritis unresponsive to methotrexate

Abdulaziz M. Aldayel[a,b], Hannah L. O'Mary[a], Solange A. Valdes[a], Xu Li[a], Sachin G. Thakkar[a], Bahar E. Mustafa[a], Zhengrong Cui[a,*]

[a] *Division of Molecular Pharmaceutics and Drug Delivery, College of Pharmacy, The University of Texas at Austin, Austin, TX 78712, United States*
[b] *King Abdullah International Medical Research Center/King Saud bin Abdulaziz University for Health Sciences (KSAU-HS), King Abdulaziz Medical City (KAMC), NGHA, Riyadh 11426, Saudi Arabia*

A B S T R A C T

TNF-α siRNA has shown promising therapeutic benefits in animal models of rheumatoid arthritis. However, there continues to be a need for siRNA delivery systems that have high siRNA encapsulation efficiency and minimum burst release of TNF-α siRNA, and can target inflamed tissues after intravenous administration. Herein we report a novel acid-sensitive sheddable PEGylated solid-lipid nanoparticle formulation of TNF-α-siRNA, AS-TNF-α-siRNA-SLNs, prepared by incorporating lipophilized TNF-α-siRNA into solid-lipid nanoparticles composed of biocompatible lipids such as lecithin and cholesterol. The nanoparticles are approximately 120 nm in diameter, have a high siRNA encapsulation efficiency (> 90%) and a minimum burst release of siRNA (< 5%), and increase the deilvery of the siRNA in chronic inflammation sites in mouse models, including in a mouse model with collagen-induced arthritis. Importantly, in a mouse model of collagen antibody-induced arthritis that does not respond to methotrexate therapy, intravenous injection of the AS-TNF-α-siRNA-SLNs significantly reduced paw thickness, bone loss, and histopathological scores. These findings highlight the potential of using this novel siRNA nanoparticle formulation to effectively treat arthritis, potentially in patients who do not respond adequately to methotrexate.

1. Introduction

Inflammation is an acute, signal-mediated process that occurs in response to harmful stimuli. It involves the infiltration of immune cells and soluble mediators, such as tumor necrosis factor alpha (TNF-α), to the site of inflammation, which is highly elevated in many chronic inflammation-related diseases [1]. Chronic inflammation-related diseases, such as rheumatoid arthritis (RA), may develop in response to failure to resolve acute inflammation [1, 2].

Anti-TNF-α therapies (e.g., Humira) have proven effective in treating arthritis [3–7]. In the past decade, there has been a growing interest in using TNF-α small interfering RNA (siRNA) to selectively reduce the production of the pro-inflammatory TNF-α cytokine to treat arthritis (16–21). Small interfering RNA has been formulated into nanoparticles to address issues related to siRNA's short half-life, poor extravasation from blood vessels to target tissues, poor cellular uptake, and potential immunogenicity [8, 9]. Data from several studies showed that TNF-α siRNA-loaded nanoparticles or nanocomplexes, prepared with polymers (e.g., chitosan, poly (lactic-*co*-glycolic) acid (PLGA)) or

lipids, are effective against RA in mouse models [10–15]. Various methods and compositions have been used to formulate nanoparticles with high siRNA encapsulation efficiency [15–18]. Previously, we developed acid-sensitive sheddable PEGylated PLGA nanoparticles that increase the distribution and retention of siRNA in chronic inflammation sites in a mouse model [19]. However, the nanoparticles suffer from low encapsulation efficiency and high burst release of siRNA [19]. In fact, high burst release of siRNA (20% or more within two days) is a common problem for siRNA formulations that have high encapsulation efficiencies [16, 20]. Therefore, a need persists for siRNA formulations that maintain high siRNA encapsulation efficiency but with minimum siRNA burst release [21].

In the present paper, we report the development of such a TNF-α siRNA nanoparticle formulation by complexing TNF-α siRNA with a biocompatible cationic lipid and then incorporating the nanocomplexes into solid-lipid nanoparticles prepared using lecithin, cholesterol, and a previously reported acid-sensitive stearic acid-polyethylene glycol (2000) hydrazone conjugate (PHC) [22]. Previously, we have shown that nanoparticles PEGylated with PHC have increased distribution and

retention in chronic inflammation sites in a mouse model, likely due to the relatively lower pH microenvironment in chronic inflammation sites [19, 23]. Herein, we have also evaluated the biodistribution of the new TNF-α siRNA nanoparticles in mouse models of chronic inflammation, including a mouse model of collagen-induced arthritis (CIA), and tested their efficacy in a mouse model of collagen antibody-induced arthritis (CAIA). Both CIA and CAIA models develop clinical features representative of RA in humans, including increased capillary permeability, accumulation of white blood cells, and severe joint damage and bone erosion [24–26]. Importantly, it is known that the CAIA model does not respond to methotrexate treatment [26]. Methotrexate is the first-line therapy for patients with early RA or low disease activity. However, some patients do not respond adequately to methotrexate, and biologics such as anti-TNF-α antibodies are combined with methotrexate (or other disease-modifying antirheumatic drugs) to manage the disease [24, 27].

2. Materials and methods

2.1. Materials

The polyethylene glycol (2000)-hydrazone-stearic acid (C18) derivative (PHC) and polyethylene glycol (2000)-amide-stearic acid (C18) derivative (PAC) were synthesized following our previously published methods [22]. Cholesterol, chloroform, tetrahydrofuran (THF), Lugol's solution, Tris-EDTA (TE), sodium dodecyl sulfate, Triton X-100, *N,N*-dimethyl-9,9-biacridinium dinitrate (Lucigenin), lipopolysaccharides (LPS) from *Salmonella enterica* serotype enteritidis, MISSION® siRNA Fluorescent Universal Negative Control #1 (Cyanine 5), Amicon Ultra centrifugal filter units Ultra-15 (MWCO 30 kDa) were from Sigma-Aldrich (St. Louis, MO). TopFluor cholesterol and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were from Avanti Polar Lipids (Alabaster, AL). Lecithin was from Alfa Aesar (Ward Hill, MA). BLOCK-iT™ Fluorescent Oligo siRNA (labeled with fluorescein) was from Life Technologies (Grand Island, NY). Negative control siRNA (Medium GC Duplex), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and streptomycin/penicillin were from Invitrogen (Carlsbad, CA). TNF-α siRNA (5′-GUCUCAGCCUCUUCUCAUUCCU GCT-3′) was synthesized by Integrated DNA Technologies (Coralville, IA). Methotrexate was from MP Biomedicals (Santa Ana, CA). Bio-Rad Bradford Protein Assays was from Bio-Rad Laboratories (Hercules, CA).

2.2. Preparation of siRNA-incorporated nanoparticles

The siRNA was first lipophilized by complexing it with DOTAP in a monophase [28]. Briefly, a 100 μl solution of 20 μM siRNA in TE buffer (10 mM Tris–HCl and 1 mM EDTA in water, pH 7.5) was added to 400 μl of RNase-free water. DOTAP in chloroform (1.25 mg in 680 μl) was then added drop-wise to the siRNA solution while stirring. The mixture was sonicated briefly in a water bath sonicator and mixed with 1.36 ml of methanol to form a monophase. After 1 h of incubation at room temperature, the siRNA/DOTAP complexes were extracted into chloroform by phase separation. At the siRNA/DOTAP ratio used, > 99% of the siRNA partitioned into the chloroform phase (by measuring the fluorescence intensity in the water phase and using fluorescently labeled siRNA).

Lecithin (3.2 mg) and cholesterol (1.6 mg), dissolved in chloroform, were added drop-wise to the siRNA/DOTAP complexes in chloroform while stirring. PHC or PAC (2 mg) dissolved in chloroform was then added drop-wise to the siRNA-lipids mixture. The resultant mixture was dried under nitrogen gas and then dissolved in 500 μl of THF, which was then added drop-wise into water while stirring to form nanoprecipitates. The resultant nanoparticle suspension was stirred at room temperature for 6 h to faciliate the evaporation of THF, subjected to ultrafiltation (MWCO, 30 KDa), washed once with water, and reconstituted in diethylpyrocarbonate (DEPC)-treated water (Invitrogen).

Nanoparticles prepared with PHC were named AS-siRNA-SLNs, where AS indicates that the nanoparticles were PEGylated with acid-sensitive sheddable PEG(2000) (i.e., PHC) [19, 29]. Nanoparticles prepared with PAC were named AI-siRNA-SLNs, where AI indicates that the nanoparticles were PEGylated with the PAC, which is acid-insensitive. Fluorescently labeled nanoparticles were prepared by using fluorescently labeled siRNA or TopFluor cholesterol (62.5% of total cholesterol) in the preparation.

2.3. Characterization of siRNA-incorporated nanoparticles

The particle size, polydispersity index (PDI), and zeta potential of the siRNA-incorporated nanoparticles were determined using a Malvern Zeta Sizer Nano ZS (Westborough, MA). To determine the encapsulation efficiency of the siRNA in the nanoparticles, nanoparticles were prepared with fluorescein-labeled siRNA to measure the fluorescence intensity of the siRNA in the (ultra)filtrate using a BioTek Synergy HT Multi-Mode Microplate Reader (Winooski, VT, Ex = 485 nm, Em = 528 nm).

2.4. Transmission electron microscopy (TEM)

The morphology of the AS-TNF-α siRNA-SLNs was examined using an FEI Tecnai Transmission Electron Microscope in the Institute for Cellular and Molecular Biology (ICMB) Microscopy and Imaging Facility at The University of Texas at Austin (Austin, TX). Carbon-coated 400-mesh grids were activated for 1–2 min. One drop of the nanoparticle suspension was deposited on the grids and incubated overnight at room temperature before examination.

2.5. In vitro release of siRNA from the nanoparticles

The release of siRNA from the nanoparticles was measured using nanoparticles prepared with fluorescein-labeled siRNA. Briefly, about 9 mg of AS-siRNA-SLNs were suspended in 1 ml PBS (10 mM, pH 7.4) inside a dialysis device (MWCO 50 kDa, Spectrum Laboratories, Rancho Dominguez, CA), which was then placed into 50 ml PBS (10 mM, pH 7.4) and maintained in a shaker incubator (100 rpm, 37 °C) (MAQ 5000, MODEL 4350, Thermo Fisher Scientific, Waltham, MA). At given time points (1, 24, 48, 96, 192, 450 and 720 h), the amount of siRNA in the release medium was determined by measuring the fluorescence intensity using a BioTek Synergy HT Multi-Mode Microplate Reader. The percent of siRNA released was calculated using the following equation: % released = 100 X (fluorescence intensity in the release medium/total fluorescence intensity of the encapsulated siRNA).

2.6. Effect of pH on the shedding of PEG from the nanoparticles and in vitro binding/uptake of siRNA-incorporated nanoparticles by macrophages

Murine macrophage J774A.1 cells (American Type Culture Collection, Manassas, VA) were seeded in a 12-well plate (2×10^5 cells/well). To study the effect of the acid-sensitive sheddable PEGylation of the nanoparticles on their uptake and/or binding by the cells, the AS-siRNA-SLNs or AI-siRNA-SLNs were pre-incubated in PBS (200 mM, pH 6.8 or 7.4) for 6 h to facilitate the shedding of PEG before the nanoparticles were added into the cell culture medium. After 50 min of co-incubation, the cells were washed with PBS (10 mM, pH 7.4) and lysed with a lysis solution that contained 2% (v/v) sodium dodecyl sulfate and 1% Triton X-100. The fluorescence intensity in the cell lysate was measured using a plate reader (Ex = 485 nm, Em = 528 nm). Bradford protein assay did not show any significant difference in the total protein concentrations in the lysates among the groups.

The shedding of PEG from the the nanoparticles was tested using an iodide staining method with Lugol's solution [19]. Briefly, ~4.5 mg of nanoparticles were incubated in PBS (pH 6.8 or 7.4) for 6 h,