Peptide conjugated liposomes for the targeted and localized delivery of fasudil for the treatment of pulmonary arterial hypertension

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ABSTRACT SUMMARY

In this study we sought to prepare a targeted delivery system for fasudil, a rho-kinase inhibitor, to treat pulmonary arterial hypertension. Fasudil liposomes were prepared by film hydration and extrusion method using a fixed composition of three lipids: Dipalmitoyl phosphatidylcholine (DPPC), cholesterol (CH), 1,2-distearyl-sn-glycero-3-phosphoethanolamine-N-(polyethylene glycol)-2000 (DSPE-PEG2000), while alternately using 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)]-2000 (DSPE-Mal2000), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). The surfaces of the liposomes were decorated with CAR (CARSKNKDC), a cyclic peptide, which preferentially accumulates on the pulmonary arterial smooth muscle cells of PAH animals. Liposomes were characterized for size, PDI, zeta potential, stability, in-vitro release profile and peptide conjugation.

INTRODUCTION

Pulmonary arterial hypertension is a progressive fatal disease characterized by remarkable proliferation and contraction of pulmonary arterial smooth muscle cells, which results in decrease in the size of the pulmonary arterial lumen, increase in pulmonary vascular resistance, decreased reactivity of the vascular bed and increased pressure in the pulmonary circulation. All these contribute to overload induced progressive right ventricular dilatation and low cardiac output

We proposed a delivery approach that will allow formulation to remain in the lungs for a prolonged period and produce a continuous release of fasudil. Recently, various therapeutic agents have been used to treat PAH. Of the various therapeutic pathways, Rho Kinase pathway has drawn a lot of attention because this pathway plays an important role in the pathogenesis of the disease. Therefore, we chose a rho kinase inhibitor, fasudil, as a drug for our delivery system. This drug has a short half-life and hence requires repeated administration and thus produces systemic hypotension. To reduce off target effects of the drug and provide prolonged pulmonary arterial vasodilation, we have decorated the surface of fasudil loaded liposomes with a cyclic peptide sequence, CAR. This peptide has shown preferential accumulation in PAH lung and binds to cell surface peptidoglycan heparin sulphate (HS)

Thus, we hypothesize that inhalable and peptide targeted nanoparticles are an efficacious and patient-compliant therapy for the management of pulmonary arterial hypertension.

EXPERIMENTAL METHODS

Preparation of CAR conjugated fasudil liposomes: We prepared liposomes using 50 µM of total lipids DPPC: CH: DSPE-Peg2000: DSPE/DSPE-Mal2000/DPPE with a molar composition of 70:30:5:5. First, we dissolved the lipids in a mixture of chloroform/methanol and developed a lipid film using a rotary evaporator which was later rehydrated by 250 mM ammonium sulfate solution. Liposomes were formed by extrusion and fasudil was encapsulated by creating ammonium sulfate gradient. CAR peptide was conjugated on the surface of the liposomes using SPDP chemistry.

Characterization of the CAR-fasudil liposomes: Physical characterization of the particles: The particle size and surface charge of the liposomal formulations were measured in a Malvern Zetasizer (ZS 90 Nano). To measure size and surface charge, liposomes were diluted with PBS and de-mineralized water.

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<th>Table 1: Composition of the formulations</th>
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<td>Formulation</td>
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Drug entrapment: The amount of entrapped drug was determined by breaking the liposomes formulation by sonication in methanol. Drug was separated by centrifugation and the concentration of the drug was measured using a UV-spectrophotometer at 320 nm. The amount of fasudil was calculated from a standard curve.

Stability study: Liposomes were evaluated for stability by measuring size and entrapped drug. Liposomes were stored at 4°C and periodically examined for size and entrapped drug according to the above described method.

In-vitro drug release: The in-vitro release study of different formulations was performed using Slide-A-Lyzer dialysis cassettes (MWCO 3500, ThermoScientific, MA) in phosphate buffered saline (10 mM, pH 7.4) at 37°C for 5 days. Samples were withdrawn at different time points and were replaced with new PBS to maintain sink condition. The
concentration of the drug released was determined using a UV-spectrophotometer.

RESULTS AND DISCUSSION

**Characteristics of CAR-liposomes:** Liposomes have shown a size range of 102 to 116 nm with a poly dispersity index (PDI) between 0.058 and 0.084, indicating a homogenous distribution of the colloidal system. Surface charge of liposomes prepared with DSPE-Mal2000, DSPE and DPPE was ranged from -33.6 to -42.7 mV. However, liposomes prepared by DOPE showed a positive charge of 6.65 mV (Table 2).

**Drug entrapment:** Liposomes prepared with DSPE and DPPE resulted in an entrapment efficiency of 72 to 78% whereas the other two formulations exhibited an entrapment efficiency of <50% (Table 2).

**Stability study:** Stability study of CAR conjugated fasudil liposomes at 4°C demonstrated that liposomes prepared with DSPE (F-2) and DPPE (F-3) are the most stable formulations and showed no aggregation of liposomes over the period of 28 days. Furthermore, entrapment efficiency data showed leakage of the drug from all formulation but F-3 (Fig 1).

**In-vitro drug release**
Upon studying the stability of different formulations, we performed in-vitro release study of F-2 and F-3 for 120 h. A cumulative release of 66% and 79% was observed for F-2 and F-3 formulations, respectively (Fig 2).

**CONCLUSIONS:**
The result of this study demonstrate that it is feasible to prepare peptide conjugated lipidic formulation that can be administered via intra-tracheal route directly to the PAH lung. The surface decorating cyclic peptide is likely bind to the over-expressed heparan sulfate. This delivery approach will be a good choice for PAH treatment since it will provide both targeted and sustained delivery of fasudil in diseased area of small vasculature of PAH without producing systemic hypotension.

**Future study:** Future studies will be directed toward qualitative (by fluorescent microscope) and quantitative (HPLC) analysis of cellular uptake of CAR-liposomes by heparan sulfate expressed pulmonary arterial smooth muscle cells. We will also study pharmacokinetic behavior, lung distribution and efficacy of the optimized formulation in healthy and monocrotaline induced PAH animal model.

**References:**