Targetable and Inhalable Nanoerythrosomes Containing Fasudil, a Rho-kinase Inhibitor, for the Treatment of Pulmonary Arterial Hypertension

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ABSTRACT SUMMARY:
A novel lung homing peptide, CARSKNKDC (CAR), was conjugated with nanoerythrosomes (NERs) containing fasudil, a rho-kinase inhibitor, and tested for its potential in the treatment of pulmonary arterial hypertension (PAH). The results indicate that CAR peptide conjugated NERs specifically accumulate in lysophosphatidic acid (LPA) activated rat pulmonary arterial smooth muscle cells (PASMCs) and effectively inhibit rho-kinase expression. NERs were found to be safe for pulmonary administration.

INTRODUCTION:
NERs are nanosized ghosts derived from erythrocytes that have been investigated as drug delivery carriers for various small and large molecule drugs over the past few decades1. They offer several advantages such as ease of availability, biocompatibility and biodegradability, targetability, and prolongation of circulation half-life of drugs with varying physicochemical properties.

PAH is a rare but seriously life threatening disorder of pulmonary circulation affecting individuals of all ages. Histopathological features of PAH include extensive remodeling of pulmonary vasculature due to intimal fibrosis which eventually leads to right heart accumulation in PAH lungs3. CAR binds exclusively to heparan sulfate, a proteoglycan overexpressed in PAH. Further, inhalational delivery will result in local deposition of the drug in lungs and thus minimize systemic side effects.

In the present study, we hypothesize that fasudil containing NERs functionalized with CAR (Fig.1), when administered intratracheally, produces prolonged pulmonary vasodilation and reduces the symptoms of PAH.

EXPERIMENTAL METHODS:
NERs containing fasudil were prepared by hypotonic osmotic lysis + extrusion method (Fig.2).

NERs were characterized for size, entrapment and stability. Nebulization stability was determined by a microsprayer which disperses the formulation into fine mist. CAR peptide was conjugated onto the NERs surfaces by using SPDP (N-Succinimidyl 3-[2-pyridyldithio]-propionate) and formulations were purified using PD10 column. CAR-NERs containing fasudil were characterized for size and entrapment efficiency. Cellular uptake was performed using rat PASMCs which were also activated by LPA to compare the binding efficiencies of plain NERs and Table 1: Nebulization stability of nanoerythrosomes

<table>
<thead>
<tr>
<th>Nebulization</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>EE (%)</th>
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<tbody>
<tr>
<td>Before</td>
<td>147.24±3.12</td>
<td>0.17±0.01</td>
<td>48.9±2.63</td>
</tr>
<tr>
<td>After</td>
<td>148.09±2.46</td>
<td>0.19±0.03</td>
<td>48.7±1.92</td>
</tr>
</tbody>
</table>

CAR-NERs. Briefly, normal and activated cells were incubated with formulations, fixed, stained for nucleus and actin and visualized under a fluorescence microscope.

Formulations were investigated for safety by in vitro cytotoxicity test on Calu-3 and rat PASM cells for 24 hrs. Also, bronchoalveolar lavage (BAL) fluid analysis was performed for detecting lung injury by the inhalation of formulations and biomarkers such as protein content; wet lung weight and, alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were measured5.
RESULTS AND DISCUSSION:

NERs, prepared by hypotonic lysis + extrusion method, were spherical in shape (Fig.3a) having a size of ~150 nm and an entrapment efficiency of 48.9±2.63%. NERs displayed stability upon centrifugal stress, turbulence shock and storage at 4°C for at least 3 weeks. NERs were completely stable after nebulization with a microsprayer as shown by minimal changes in size and entrapment efficiency (Table 1). CAR conjugation was confirmed by a characteristic spectroscopic peak at 343 nm and further by microscopy (Fig.3b). Plain CAR peptide and CAR conjugated NERs showed increased internalization by activated rat PASMCs as compared to plain NERs (Fig.4). Size of CAR conjugated NERs was ~190 nm and entrapment efficiency was ~48%. CAR-NERs inhibited rho-kinase expression by more than ~15% when compared to plain NERs as determined by a rho-kinase Elisa assay (Fig.5). Pharmacokinetic analysis revealed that intratracheal administration of fasudil containing NERs resulted in remarkable extension of elimination t1/2 (~ 13.64 hr) which was significantly higher than plain fasudil which has half-life of 30-45 min. In vitro cytotoxicity studies show that formulations produced no cytotoxicity after 24 hrs. treatment (Fig.6). BAL fluid analysis suggests that formulations were completely safe after pulmonary delivery as demonstrated by low protein content, wet lung weight and ALP/LDH levels (Fig.6). In vivo efficacy studies are underway to compare the hemodynamic efficacy of plain NERs and CAR conjugated NERs containing fasudil in a monocrotaline induced PAH model.

CONCLUSIONS:

Overall, this study investigates and suggests the potential of a targeted and inhalable controlled release formulation of fasudil for the treatment of PAH. In vitro experiments show the efficacy of CAR conjugated NERs in inhibiting rho kinase expression. Efficacy studies are underway to further validate the delivery system.

REFERENCES:
2. Gupta and Ahsan; Crit Rev Ther Drug Carrier Syst. 27:313-370 (2010).