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Research paper

Cell permeable peptide conjugated nanoerythrosomes of fasudil prolong pulmonary arterial vasodilation in PAH rats

Nilesh Gupta, Brijeshkumar Patel, Kamrun Nahar, Fakhrul Ahsan *

Department of Pharmaceutical Sciences, School of Pharmacy, Texas Tech University Health Sciences Center, Amarillo, USA

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ABSTRACT

In this study, we tested the hypothesis that a cell permeable peptide, CARSKNKDC (CAR), conjugated nanoerythrosomes (NERs) containing fasudil, a rho-kinase (ROCK) inhibitor, produces prolonged pulmonary preferential vasodilation. CAR conjugated NERs containing fasudil were prepared by hypotonic lysis and extrusion method, and optimized for various physicochemical properties *in-vitro*. The formulations were then used to study the hemodynamic efficacy in a monocrotaline-induced rodent model of pulmonary arterial hypertension (PAH). CAR-NERs-Fasudil was spherical in shape with an average vesicle size and entrapment efficiency of 161.3 ± 1.37 nm and 48.81 ± 1.96%, respectively. Formulations were stable for ~3 weeks when stored at 4 °C and the drug was released in a controlled fashion for >48 h. The uptake of CAR-NERs-Fasudil by TGF-β activated pulmonary arterial smooth muscle cell was ~1.5-fold greater than the uptake of NERs-Fasudil. CAR-NERs-Fasudil inhibited ROCK activity and 5-hydroxytryptamine induced cell proliferation. In terms of reduction of pulmonary arterial pressure, intratracheal administration of CAR-NERs-Fasudil was ~2-fold more specific to the lungs compared with plain fasudil. Overall, CAR peptide grafted nanoerythrosomes offers a new platform for improving the therapeutic efficacy of a rho-kinase inhibitor, fasudil, without affecting peripheral vasodilation.

1. Introduction

The RhoA/Rho-kinase signaling pathway influences the pathogenesis of a number of diseases related to cardiovascular, nervous and metabolic systems [1]. Endogenous vasoconstrictors, including endothelin-1, angiotensin, and 5-hydroxy tryptamine, activate Rho-kinase (ROCK) and downstream effector molecules. ROCK, a potent downstream effector of RhoA, inhibits myosin phosphatase and causes the cells to differentiate, proliferate, adhere, migrate and contract. ROCK mediated vasoconstriction and vascular remodeling play a major role in the development and progression of PAH, a deadly chronic disorder. Inhibition of ROCK activity dilates pulmonary arteries and arterioles, and reduces arterial remodeling [2]. Of the ROCK inhibitors, fasudil (HA-1077) ameliorates the chief PAH symptom, elevated pulmonary arterial pressure, both in animals and human patients. But lack of pulmonary vascular selectivity, drug induced peripheral vasodilation, and drug's short biological life are major deterrents for fasudil's transition from an investigational drug to a clinically viable therapeutic agent [3]. The drawback of short half-life can be addressed by putting the drug in carriers with an ability to modulate drug release and extend plasma circulation time. The problem of pulmonary selectivity and peripheral vasodilation can be overcome by means of homing device-empowered carriers that can position the formulation over the lung vasculature.

The approaches to extend the biological half-life and deploy drugs at a disease site include the use of polymeric, lipidic, inorganic, and cellular micro- and nano-sized chariots equipped with different homing devices such as antibodies, aptamers, enzymes, and peptides [4,5]. Particulate drug chariots armed with homing devices have been used to deliver drugs for the treatment of many disorders localized in the respiratory system. Liposomes modified with an epidermal growth factor receptor binding peptide, for example, enhanced the accumulation of doxorubicin in lung tumor tissue by 2.2-fold compared with unmodified liposomes [6].

Abbreviations: ALP, alkaline phosphatase; BAL, bronchoalveolar lavage; CAR, CARSKNKDC; 5-HT, 5-hydroxytryptamine; LDH, lactate dehydrogenase; LPA, lysophosphatidic acid; LTI, lung targeting indices; MCT, monocrotaline; MPAP, mean pulmonary arterial pressure; MSAP, mean systemic arterial pressure; NERs, nanoerythrosomes; OD, optical density; PAH, pulmonary arterial hypertension; PASM, pulmonary arterial smooth muscle; PBS, phosphate buffered saline; ROCK, rho-kinase; SD, Sprague Dawley; SDS, sodium dodecyl sulfate; SPDP, N-succinimidyl 3-[2-pyridyldithio]-propionate; TGF-β, tissue growth factor-beta.

* Corresponding author at: Department of Pharmaceutical Sciences, School of Pharmacy, Texas Tech University Health Sciences Center, 1300 Coulter Drive, Amarillo, TX 79106, USA. Tel.: +1 806 414 9235; fax: +1 806 356 4034.
E-mail address: fakhrul.ahsan@ttuhsc.edu (F. Ahsan).

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Modified nanoparticles prolonged the residence time of dexamethasone in deep lung tissue and helped overcome steroid resistance in asthma [7], and promoted the localization of nanoparticles in respiratory syncytial viral infection site [8].

Similar to the above approaches, we and others have used nanoparticles to deliver anti-PAH drugs such as fasudil, iloprost, pitavastatin and adrenomedullin directly to the lung [9–11]. We have shown that nanoerythrosomes (NERs), an erythrocyte based biomimetic system, extend the biological half-life of fasudil and are safe for intratracheal administration [12]. NERs exhibit reduced aggregation, minimal drug loss, biodegradability, longer circulation time, and superior targeting efficiency [12]. Being natural in origin, NERs are endowed with biological and chemical senses; thus NERs can deliver enzymes, peptides, toxins, contrast agents. Proteins and small molecule drugs can also be conjugated to NERs surface [13]. Importantly, unlike polymeric particles, NERs can be functionalized without involving complex chemical modification [14]. However, these cell based carriers have not yet been used to deliver pharmacologically active drug via inhalational route for the treatment of a debilitating respiratory disease, PAH.

Since the therapeutic limitations of fasudil stem from the short half-life and lack of pulmonary selectivity, the pharmacological efficacy and site specificity can be improved and fine-tuned by loading the drug in cell based carriers, NERs, modified with a homing device that can specifically recognize molecular structures not present in normal tissues or vasculature but are expressed in diseased sites. Recent studies report that CARSKNKDC (CAR), a cyclic peptide, specifically accumulates in PAH lesions by binding heparan sulfate at PAH sites [15]. CAR also exhibits cell penetrating properties and has bystander effect when given along with several vasodilators [16]. However, inhalational delivery of CAR peptide decorated cellular nanoparticles containing anti-PAH drugs have not yet been tested for their pulmonary selectivity and hemodynamic efficacy in a rodent model of PAH. Thus, in this study, we propose to use nanoerythrosomes equipped with CAR for delivery of fasudil into the lung vasculature and test the hypothesis that CAR-conjugated NERs produce an extended pulmonary preferential vasodilation in PAH rats. To test this assumption, we prepared and characterized CAR-NERs-Fasudil (Fig. 1) and evaluated the pulmonary vasodilatory effects in PAH rats.

2. Methods and materials

2.1. Preparation and characterization of CAR conjugated NERs containing fasudil

NERs containing fasudil were prepared by a combination of hypotonic osmotic lysis and extrusion method as reported by us previously (Fig. 2) [12]. Briefly, Sprague–Dawley® (SD) rat erythrocytes were hemolyzed by incubating sequentially in hypotonic solution of different strengths (50–30 mOsm) for 15 min. Hypotonic solutions were prepared by diluting isotonic PBS solution (~300 mOsm) with distilled water. White unsealed ghosts obtained after several rounds of hemolysis were separated from hemoglobin by centrifugation. To load the drug, we added fasudil (LC Labs Inc., Woburn, MA) at a concentration of 20 mg/ml in the hypotonic solution along with the unsealed ghosts and incubated for 30 min at low temperature. Drug loaded erythrocyte ghosts were resealed by adjusting the isotonicity with hypertonic solution (10× PBS) followed by incubation at 37 °C for 60 min. Resealed erythrocytes, erythrosomes, were washed 3 times with isotonic PBS and un-encapsulated fasudil was removed by gel permeation chromatography using a Sephadex-G-25 PD-10 pre-packed column (GE Healthcare Biosciences, Piscataway, NJ). Erythrosomes were stored at 4 °C until further use.

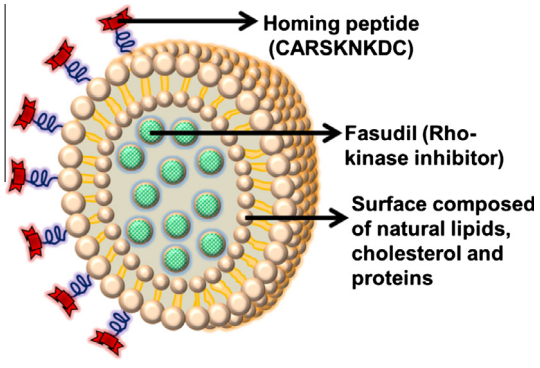

Fig. 1. Architecture of targeted nanoerythrosomes containing fasudil. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

FAM-labeled CAR (LifeTein LLC, South Plainfield, NJ) was grafted onto the surface of NERs-Fasudil by a simple linker chemistry [17]: The amine groups on the surface of NERs-Fasudil were first activated by N-succinimidyl 3-[2-pyridyldithio]-propionate (SPDP) to convert them to sulfhydryl-reactive pyridyldisulfide groups; the unreacted SPDP was removed using a PD-10 column. SPDP-activated NERs-Fasudil was then incubated with a solution of CAR peptide in PBS for crosslinking between the free amine groups of NERs-Fasudil and thiol groups of cyclic CAR peptides. This reaction released pyridine-2-thione as a byproduct, which exhibits absorbance maxima at 343 nm. CAR conjugated erythrosomes were then purified using a PD-10 column to remove unconjugated peptides and extruded sequentially through polycarbonate membranes (800–400–200 nm) to obtain CAR-NERs-Fasudil. The particle size, polydispersity and zeta potential of CAR-NERs were determined using a Malvern Zetasizer® (Malvern® Instruments Limited, Worcestershire, UK). The preparation process of CAR-NERs-Fasudil from pure erythrocytes was observed in a fluorescence microscope (IX-81, Olympus, Center Valley, PA). The entrapment efficiency was determined by lysing the CAR-NERs in methanol ($n = 3$) and assaying the drug content at 320 nm using a UV spectrophotometer (UV/Vis 918, GBC Scientific Equipment, Hampshire, IL). A standard curve of fasudil in methanol was prepared to assay the amount of entrapped drug; blank NERs were used as a control in all measurements. The entrapment efficiency was calculated using the following equation: *Percent entrapment efficiency* = (*Amount of drug loaded*/*Amount of drug added*) × 100.

2.2. In-vitro release studies

The *in-vitro* release studies for plain and CAR conjugated NERs containing fasudil were performed in dialysis cassettes (Slide-A-Lyzer, 3500 MWCO, 0.1–0.5 ml, Thermo-Scientific, Waltham, MA) for a period of 48 h at 37 °C in a temperature-controlled incubator as reported previously [18]. Plain fasudil and blank NERs were used as controls to rule out the influence of dialysis cassettes on drug release. Briefly, pre-hydrated cassettes were loaded with formulations (500 µl) using a $23^{1/2}$ gauze needle attached with an 1-ml syringe. The sink condition was maintained by immersing the cassettes in a beaker containing 100 ml PBS (1×, pH 7.4) under moderate stirring. Samples were withdrawn at predetermined time intervals for 48 h and replaced with an equal volume of fresh PBS. The amount of drug in the samples ($n = 3$) was quantified spectrophotometrically at 320 nm as described above; blank NERs were also used as a control to take into account the interfering absorbance from the carrier.

2.3. Evaluation of in-vitro stability of formulation

The storage stability of CAR-NERs containing fasudil was evaluated for 21 days. Briefly, 500 µl formulation was stored at 4 °C and