Published in final edited form as:

Eur J Pharm Biopharm. 2014 November ; 88(3): 1046–1055. doi:10.1016/j.ejpb.2014.10.012.

Cell Permeable Peptide Conjugated Nanoerythroosomes of Fasudil Prolong Pulmonary Arterial Vasodilation in PAH Rats

Nilesh Gupta¹, Brijeshkumar Patel¹, Kamrun Nahar¹, and Fakhrul Ahsan^{1,*}

¹Department of Pharmaceutical Sciences, School of Pharmacy, Texas Tech University Health Sciences Center, 1300 Coulter Drive, Amarillo, TX-79106, USA

Abstract

In this study, we tested the hypothesis that a cell permeable peptide, CARSKNKDC (CAR), conjugated nanoerythroosomes (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, 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 $48.81 \pm 1.96\%$, respectively. Formulations were stable for ~3 weeks when stored at 4°C and the drug was released in a controlled fashion for >48 hrs. 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 nanoerythroosomes offers a new platform for improving the therapeutic efficacy of a rho-kinase inhibitor, fasudil, without affecting peripheral vasodilation.

Keywords

Targeted delivery; rho-kinase; fasudil; nanoerythroosomes; controlled release; pulmonary arterial hypertension; pulmonary delivery; safety

© 2014 Elsevier B.V. All rights reserved.

*Corresponding Author: Tel.: +1 806-414-9235; Fax: 806-356-4034, fakhrul.ahsan@ttuhsc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

CONFLICT OF INTEREST

Authors report no conflict of interest.

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]. 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 carrier, NERs, modified with 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 nanoerythrocytes equipped with CAR for delivery of fasudil in 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 hemolysed 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 (10X PBS) followed by incubation at 37°C for 60 min. Resealed erythrocytes, erythrocytes, 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). Erythrocytes were stored at 4°C until further use.

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 erythrocytes 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* = $(\text{Amount of drug loaded}/\text{Amount of drug added}) \times 100$.

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 hrs 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 (1X, pH 7.4) under moderate stirring. Samples were withdrawn at predetermined time intervals for 48 hrs 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 samples (n=3) were withdrawn on 0, 7, 14 and 21 days, respectively, followed by measurement of vesicle size and drug entrapment as described above. As the formulations were intended for pulmonary administration, the nebulization stability was assessed using a PennCentury Microsprayer® (Model IA-1B, PennCentury, PA). For this, CAR-NERs-Fasudil was loaded in the syringe attached with the aerosolizer and sprayed for at least ten times. The nebulized droplets were collected and analyzed for changes in size, polydispersity index, and entrapment efficiency that occurred due to aerosolization.

2.4. Cellular uptake studies

The *in-vitro* cellular uptake studies were performed in rat pulmonary arterial smooth muscle (PASM) cells as described previously [12]. Briefly, rat PASM cells were cultured to 90% confluency in a 75 cm² corning flask using DMEM F-12 medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% 200 mM glutamine (ATCC, Manassas, VA), and placed in a humidified incubator maintained at 37°C with 5% CO₂. Cells were seeded on a cover slip placed in 24-well plate at a density of 5×10³ cells/mL and left overnight for attachment. To compare the binding efficiencies of plain and CAR conjugated NERs-Fasudil, cells were divided into two groups: normal and activated cells. Tissue growth factor-beta (TGF-β, 10 ng/mL) were used to activate and increase the expression of heparan sulfate, the target of CAR peptide, prior to performing uptake studies.

Cells received the following two treatments (n=3): (i) NERs-Fasudil and (iii) CAR-NERs-Fasudil equivalent to 100 μ M fasudil for 60 min at 37°C. Both NERs and CAR-NERs were stained red with CellMask™ (Molecular Probes Inc., Eugene, OR), a plasma membrane labeling stain. Following incubation, cells were washed to remove the formulations and fixed with an equal mixture of acetone and methanol. To prevent non-specific binding, fixed cells were incubated with a blocking solution. Cells were incubated sequentially with monoclonal anti- β -actin primary antibody (Sigma-Aldrich, St. Louis, MO) and Alexa Fluor® 495 goat anti-mouse IgG antibody (Invitrogen, Grand Island, NY). Finally, glass coverslips were transferred carefully to glass slides and the cellular uptake was observed under fluorescence microscope.

The flow cytometric studies were performed to confirm cellular uptake data. Rat PASM cells were grown as described above and seeded on a 6-well plate at a density of 1 $\times$ 10⁶ cells/ml. Cells were divided in two major groups, normal and activated, to compare the uptake of NERs-Fasudil and CAR-NERs-Fasudil. Both NERs and CAR-NERs were stained red as described above. After 60 min incubation at 37°C, the cells were washed, trypsinized, resuspended in PBS, and analyzed using a BD Accuri C6 cytometer (BD Biosciences, San Jose, CA). A total of 10,000 gated cells per sample (n=3) were counted. Plain cells without any treatment were used as control to rule out any self-fluorescence.

2.5. *In-vitro* inhibition of 5-hydroxytryptamine (5-HT) induced proliferation of rat PASM cells

Rat PASM cells were grown as described above and seeded into 96-well plates at a density of 5 $\times$ 10⁴ cells/well. The cells were then incubated in medium containing the vehicle (10% FBS+DMEM F-12) and 5-HT (1 μ M) for 24 h with the following treatments (n=8): (i) no treatment, (ii) fasudil (100 μ M), (iii) NERs (100 μ M fasudil) and (iv) CAR-NERs (100 μ M fasudil). The cells were treated with test and control formulations 60 min before addition of 5-HT. At the end of the treatment period, MTT was added to each well, incubated at 37°C for 4 hrs, the medium was removed, and the formazan crystals were solubilized in dimethyl sulfoxide. The optical density of each well was measured at 570 nm using a SynergyMX microplate reader (Biotek, Winnoski, VT) and the percent proliferation for each treatment was plotted to compare the efficacy. We also studied the dose-response of plain fasudil (25–200 μ M) to evaluate whether doses above the therapeutic concentration produce any toxic effects as described above.

2.6. Rho-kinase activity inhibition assay

The *in-vitro* efficacy of the formulations in inhibiting ROCK activity was evaluated in rat PASM cells. Briefly, cells were cultured as discussed above in a 24-well plate and divided into 4 groups to receive the following treatments for 4 hrs (n=6): (i) no treatment, (ii) plain fasudil (100 μ M), (iii) NERs and (iv) CAR-NERs containing 100 μ M fasudil. The ROCK activity was increased by activating the cells with lysophosphatidic acid (LPA, 0.5 μ g/ml) for 2 hrs prior to the treatment. Cells with no treatment were used as the negative control. The inhibition of ROCK activity was directly measured using a commercial Rho-associated protein kinase activity EIA kit (EMD Millipore Corporation, Billerica, MA) as per the manufacturer's instructions. In brief, following the treatment period, the cells were lysed and

diluted with the assay buffer. An aliquot of lysed cells were then added to the wells pre-coated with recombinant myosin phosphatase target subunit-1 (rMYPT1) and incubated for 30 min. Next, horseradish peroxidase (HRP) conjugated primary and secondary antibodies for MYPT1 were added sequentially followed by 1 hr incubation. The amount of phosphorylation of the substrate was determined by addition of a chromogenic substrate, tetramethylbenzidine (TMB), which turns the solution color to blue. Finally the stop solution was added that turned the solution to yellow. The relative amount of ROCK activity in the lysed cells was determined by measuring the absorbance at 450 nm.

2.7. The acute hemodynamic efficacy studies in monocrotaline (MCT)-induced PAH rats

The *in-vivo* efficacy of the formulations was evaluated in MCT-induced PAH rats as reported previously [18, 19]. Briefly, male SD rats (250–300 g) were injected a single subcutaneous injection of MCT (50 mg/kg body weight, Sigma-Aldrich Inc., St. Louis, MO) and housed for 28 days for development of PAH. After 4 weeks, PAH rats were anesthetized by an intramuscular injection of a cocktail of ketamine (90 mg/kg) and xylazine (10 mg/kg) and two separate catheters were placed in the pulmonary (via jugular vein and right ventricle) and right carotid artery. The mean pulmonary (MPAP) and systemic arterial pressure (MSAP) were measured with Memscap SP844 physiological pressure transducers (Memscap AS, Scoppum, Norway) and bridge amplifiers connected to a PowerLab 16/30 system with LabChart Pro 7.0 software (AD Instruments, Inc., Colorado Springs, CO). Rats were divided into four groups (n=6–8) to receive 3 mg/kg fasudil as (i) plain intravenous (IV) fasudil, (ii) plain intratracheal (IT) fasudil, (iii) NERs containing fasudil, and (iv) CAR-NERs containing fasudil via the IT route. IV formulations were administered into the penile vein and IT administration was performed using the PennCentury Microsprayer[®]. The animals were maintained under anesthesia throughout the procedure, and MPAP and MSAP were recorded for at least 6 hrs. At the end of the experiment, rats were sacrificed by exsanguination.

All animal studies were performed in accordance with NIH Guidelines for the care and use of Laboratory Animals under a protocol approved by TTUHSC Animal Care and Use Committee (AM-10012).

2.8. Safety studies

The cell viability assay was performed to determine the detrimental effects of the formulations on the native cells of bronchial respiratory system. For this, we performed an MTT assay in two different cell lines, human airway epithelial (Calu-3) and rat PASM cells, as reported previously. Briefly, cells were seeded in a 96-well plate at a density of 5×10^4 cells/well and left overnight for adherence. Then, cells were treated with (n=12): (i) saline (negative control), (ii) 0.1% sodium dodecyl sulfate (SDS, positive control), (iii) plain fasudil (100 μ M), and (iv) CAR-NERs (equivalent to 100 μ M fasudil) for 24 hrs. Post treatment, cells were washed twice with saline and incubated with 100 μ l MTT (0.5 mg/ml in sterile PBS) for 4 hrs. The formazan crystals were solubilized in dimethyl sulfoxide along by moderate shaking for 1 hr, and the absorbance was measured at 570 nm. The cell viability was calculated using the following equation: % *Cell viability* = $(OD_{sample} -$

$OD_{blank}) / (OD_{control} - OD_{blank}) \times 100$; OD is optical density, sample is test compound, control is saline and blank is no treatment.

Further, the safety of the formulations was evaluated *in-vivo* by measuring the levels of injury markers in bronchoalveolar lavage (BAL) fluid after the pulmonary administration. Briefly, male SD rats (250–300 g) were anesthetized using a cocktail of ketamine and xylazine and divided into three groups (n=4) to receive following treatments via the intratracheal route: (i) saline (negative control), (ii) CAR-NERs (equivalent to 6 mg/kg fasudil), (iii) 0.1% SDS (positive control). 12 hrs post-administration, animal weights were recorded, lungs were surgically removed, and weighed separately. The wet lung weight was reported as g/100 g body weight. The BAL procedure was performed and fluid was collected to measure the total protein concentration (mg/ml) by a Bradford assay. The levels of two lung injury markers, lactate dehydrogenase (LDH) and alkaline phosphatase (ALP), in BAL fluid were measured using commercial kits (Pointe Scientific, Canton, MI) and reported in IU/L.

2.9. Data analysis and statistics

The data are presented as mean $\pm$ standard deviation and were analyzed by one-way ANOVA followed by Tukey post-hoc analysis using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA). *p* value < 0.05 was considered as statistically significant.

3. RESULTS AND DISCUSSION

In this study, we have developed a targeted biomimetic system for inhalational delivery of fasudil. Previously, we have shown that it is possible to encapsulate fasudil, a small molecular weight rho-kinase inhibitor, in erythrocyte based systems [12]. As a continuation to our published study, we conjugated a homing peptide, CAR, onto the surface of NERs-Fasudil, performed a series of *in-vitro* studies, and evaluated the efficacy in a rodent model of PAH.

3.1. Preparation and characterization of CAR-NERs containing fasudil

Fasudil loaded NERs were prepared according to our previously established procedure to achieve a maximum entrapment and favorable physiochemical properties. The transition of erythrocytes from erythrocytes was captured by microscopy. NERs-Fasudil was completely resealed and was spherical with smooth texture as opposed to binconcave discoidal erythrocytes. Formulations showed no aggregation over time.

A major drawback of the current PAH therapy is the lack of site specificity, and thus causes systemic vasodilation. We aimed to address this issue by conjugating a homing peptide onto the NERS surface. CAR peptide was grafted on NERs surface utilizing the SPDP linker chemistry (Fig. 3A); the absence of peak at 343 nm before addition of CAR peptide but appearance of a characteristic peak after addition of CAR suggest conjugation of peptide with activated amine groups of NERs-Fasudil (Fig. 3B). CAR-NERs-Fasudil showed green fluorescent peptide (FAM-labeled CAR) under a fluorescent microscope, suggesting that CAR was grafted onto NER surface (Fig. 4). The average size of CAR-NERs-Fasudil was

161.3±1.37, slightly higher than that of plain NERs-Fasudil as reported earlier. The slight increase in size may be the result of peptide accumulation over the erythrocyte surface. Published reports suggest that particles less than 250 nm can escape lungs' natural clearance mechanisms and stay longer in the lungs [20]. The zeta potential of the formulation was -19.83 ± 1.66 mV that is optimal for colloidal stability of erythrocytes. Some studies suggest negatively charged particles accumulate more efficiently in the lungs than positively charged ones [21]. CAR-NERs-Fasudil exhibited homogenous size distribution with a PDI of 0.159 ± 0.21 . The drug entrapment was $48.81 \pm 1.96\%$ and the extrusion process did not reduce the drug entrapment, indicating that NERs-Fasudil are flexible and can travel via the small and distal pulmonary vasculature. This also confers stability to the formulation because a slight change in the flexibility of erythrocytes may lead to their clearance by macrophages. Our previously published studies suggest that NERs-Fasudil prevents the premature release of fasudil, and consequently increases drug's biological half-life. CAR peptide has a biological half-life of ~27 hrs and thus we anticipate that it will guide the carrier to accumulate on PAH sites and produce localized action for an extended period of time.

3.2. *In-vitro* release studies of NERs-Fasudil containing fasudil

The *in-vitro* release studies of fasudil from NERs and CAR-NERs were performed for 48 hrs in PBS buffer at 37°C (Fig. 5A). About 100% of the plain drug was released within 2 hrs which suggested that cassettes were not the rate limiting factors for drug release. In contrast, only $33.9 \pm 1.71\%$ and $28.9 \pm 1.92\%$ of drug was released from plain NERs and CAR-NERs, respectively, which showed the controlled release property of the formulations. Also, a small amount of drug (10–12%) was released in 15 min from both formulations, which may be due to disruption of some cells caused by stirring or release of surface associated drug. A slower and prolonged release behavior may stem from the compact structure of NERS surface that is composed of natural lipids, cholesterol and surface proteins [22]. The *in-vitro* release data strongly suggest that CAR-NERs could be used as controlled and continuous release carriers for delivery of fasudil. However, this release data may not reflect the drug release in the lung, since lung fluid is composed of surfactants and various ions.

3.3. *In-vitro* stability of CAR-NERs-Fasudil

The storage stability of CAR-NERs-Fasudil was evaluated for a period of three weeks at 4°C. The drug loaded CAR-NERs were resistant to aggregation and showed no significant changes in vesicle size (Fig. 5B). As NERs are composed of endogenous phospholipids, proteins and cholesterol, and have a very compact structure, drug leakage from NER was minimal (~6%). Large negative zeta potential also contributed to the stability of formulations because oppositely charged NERs created repulsion among the particles, reduced aggregation, and minimized drug loss. Based on this short term stability studies, we expect that CAR-NERs-Fasudil would be suitable for long term use. However, NERs are not stable at low pH [22], thus storage at 4°C in a neutral pH is recommended. Alternatively, NERS surface should be modified with polyethylene glycol to prevent premature degradation.

We have used a microsyringe to spray instill the formulations to the animals' lungs. The aerosolizer we have used has an atomizer at the tip that spray the solution into smaller and uniform droplets; but such spraying may rupture the formulation and prematurely release the drug. This prompted us to evaluate the effect of nebulization on the stability and integrity of CAR-NERs-Fasudil. No significant differences in the vesicle size, homogeneity, and entrapment efficiency before and after nebulization of the CAR-NERs (Fig. 5C) were observed, indicating that formulations can withstand the force of nebulization.

3.4. Cellular Uptake of the formulations

Fasudil causes smooth muscle relaxation by inhibiting ROCK and reduces mean pulmonary arterial pressure. We expect that our formulations will produce the intended therapeutic effects on arterial smooth muscle cells after crossing the air-blood barrier. So we have performed cellular uptake studies in normal and activated PASM cells and compared the binding efficiencies of plain drug with that of CAR conjugated NERs-Fasudil. Cells were activated with TGF- β because it is a potent modulator of heparan sulfate expression, which is mediated by an increase in steady state levels of mRNA for heparan sulfate proteoglycan [23]. In agreement with the published report, CAR-NERs-Fasudil (red color, TRITC channel) was internalized more efficiently by activated PASM cells (green color, FITC channel) compared with plain NERs-Fasudil that showed moderate internalization (Fig. 6A). Enhanced uptake can be explained by cell penetrating property of the peptide, which is evident from the enhanced cytoplasmic and nuclear localization of CAR-NERs-Fasudil in 60 min.

In addition to qualitative microscopic studies, we performed flow cytometry to quantitate the cellular uptake. Compared with plain NERs-Fasudil, cellular uptake of CAR-NERs-Fasudil was higher in activated cells (Fig. 6B). Mean fluorescence intensity (Fig. 6C) shows that CAR-NERs-Fasudil was internalized ~1.5-fold more than plain NERs-Fasudil by TGF- β activated cells. This agrees with the microscopy data which shows enhanced localization of peptide conjugated formulation in the cells. Also, CAR peptide has cell penetrating properties which, we believe, is also contributing to enhance the cellular uptake. Further transport studies using co-culture of cells lining the pulmonary vasculature would provide more information regarding the transport mechanism of CAR-NERs-Fasudil from respiratory epithelial side to the vascular side.

3.5. Inhibition of 5-HT induced proliferation and rho-kinase activity of rat PASM cells

5-HT, a potent vasoconstrictor, is mitogenic for PASM cells and their proliferation influences the pulmonary arterial remodeling in PAH [24]. We examined the effect of our formulation in inhibiting 5-HT induced proliferation of PASM cells (Fig. 7A). Pretreating the cells with fasudil or formulations reduced the proliferation of PASM cells. Although the formulations produced similar effect as that of plain fasudil, we believe that the controlled release property and targeting potential of the formulation would be beneficial for PAH therapy. Furthermore, we also performed dose-response studies for fasudil to differentiate drug's pharmacological effect from toxic effects. A dose-dependent decrease in 5-HT induced cell proliferation was observed but no cytotoxicity was observed when the drug was applied at 200 μ M concentration (Fig. 7B). Based on this observation, we assume that

accumulation of drug in lungs during multiple dosing would not adversely affect the viability of lung cells.

Further, we tested the efficacy of formulations in inhibiting ROCK activity by an antibody capture ELISA assay. ROCK inactivates myosin phosphatase, increases phosphorylation of myosin light chain that induces smooth muscle cell contraction. Rho-kinase mediated vasoconstriction and smooth muscle proliferation play an important role in the pathogenesis of PAH [25]. In this experiment, we exposed cells to LPA to increase ROCK activity [26] and treated with either plain fasudil or formulations (NERs-Fasudil or CAR-NERs-Fasudil). No drug treatment exhibited high levels of ROCK, as evidenced by near zero percent inhibition (Fig. 7C). In contrast, plain fasudil inhibited the ROCK activity by $65.57 \pm 7.89\%$ and in case of NERs-Fasudil and CAR-NERs-Fasudil, the percent inhibition was $53.39 \pm 8.99\%$ and $58.76 \pm 10.04\%$, respectively. A reduced level ROCK inhibition by the formulations may have resulted from slow release property of the formulation, which restricted the amount of drug available to act against ROCK.

3.6. Acute hemodynamic efficacy of formulations

The pharmacological efficacy of the formulations was tested in a widely accepted model, MCT-induced PAH [27]. The basal MPAP in MCT induced PAH rats was 45.17 ± 4.23 mm Hg, which was ~ 12 – 16 mm Hg in healthy rats. In agreement with the previously published studies, single IV fasudil (3 mg/kg) produced a $33.47 \pm 7.95\%$ reduction in MPAP, which peaked within 30–40 min but the vasodilatory effect disappeared within 60 to 80 min (Fig. 8A). But MSAP started to go down within 10–20 min because of the availability of free drug in the circulation. When plain fasudil was administered via IT, MPAP level fell rapidly within 10–20 min, but lasted a little longer than IV fasudil (Fig. 8B). MSAP did not fall immediately after the administration perhaps because the drug was administered as an aerosol. However, plain NERs-Fasudil and CAR-NERs-Fasudil produced vasodilatory effect for >200 min; the maximal reduction in MPAP produced by NERs and CAR-NERs was $49.32 \pm 2.52\%$ and $52.46 \pm 2.37\%$, respectively, which was ~ 1.5 -fold higher than that produced by plain fasudil administered as IT or IV bolus (Figs 8C and 8D). MSAP was minimally affected by CAR-NERs-Fasudil ($\sim 17\%$), whereas plain NERs-Fasudil reduced the MSAP to $28.68 \pm 7.82\%$. A point-by-point comparison of hemodynamic data suggests that the reduction in MSAP produced by aerosolized CAR-NERs was consistently lower than that produced by plain fasudil or NERs administered via the either route (Fig. 8). Fasudil encapsulated in CAR-NERs is likely to reduce the frequency of administration and minimize the fluctuations in MSAP due to IV bolus or continuous infusion that leads to serious side effects including syncope and cardiovascular collapse. To further validate pulmonary selectivity, we calculated the lung targeting indices (LTI) for all treatments. CAR-NERs-Fasudil showed the highest lung targeting index of 8.83 ± 0.69 , a ~ 2 fold increase compared with plain fasudil and NERs-Fasudil (Fig. 8E). This supports our claim that CAR-NERs accumulate on the diseased site, release the payload of drug, and show prolonged localized effects. Further, the extended vasodilatory action reflects the absorption profile of the formulation, where the $t_{1/2}$ of nanoerythrosomal fasudil was significantly longer than that of plain fasudil [12]. To calculate the duration of pulmonary vasodilatory effects, we used at least 12–15% reduction in MPAP as therapeutically relevant. All animals

were kept under observation until their pressure values return to ~90% of the original values after the administration of plain drugs or formulations. CAR-NERs-Fasudil showed the strongest and most extended vasodilatory duration (291.71 ± 18.62) compared with plain drug or NERs-Fasudil (Fig. 8F). Thus, CAR enabled the formulation to accumulate more on the PAH sites which resulted in significant reduction in MPAPs and controlled release nature of the formulation sustained the therapeutic effects. Overall, the hemodynamic efficacy study results strongly support the feasibility of an inhaled and targeted controlled release biomimetic formulation of fasudil for the treatment of PAH but chronic studies are warranted to assess changes in histopathology and various biomarkers in the pulmonary vasculature.

3.7. Safety studies of formulation

Plain drug and formulations were also evaluated for their safety. *In-vitro* cell viability was determined on two cell lines, Calu-3 and rat PASM cells, by an MTT assay. Saline treated cells were considered as negative control and the cell survival was assumed to be 100% (Fig. 9A). Only ~15–16% cell viability was observed after SDS treatment which was the positive control in this experiment. 100 μ M plain fasudil or equivalent concentration in the form of CAR-NERs was incubated with both cell lines for 24 hrs. The viability of CAR-NERs-Fasudil treated Calu-3 and rat PASM cells was $87.79 \pm 5.62\%$ and $86.09 \pm 11.29\%$, respectively, which is comparable with that obtained with the pure drug. Based on this data we assume that formulation is not toxic to the cells of pulmonary vasculature and bronchio-respiratory system. This is consistent with the earlier study that NERs-Fasudil composed of endogenous materials such as phospholipids and cholesterol, the main constituents of lung tissue and surfactant pool, are unlikely to cause undesirable side effects; CAR peptide has been reported to exhibit no preclinical side-effects [12]. Recently, inhaled liposomal amphotericin B (AmBisome), amikacin (ARIKACE), and ciprofloxacin (Lipoquin and Pulmaquin), have been tested in humans with no report of significant adverse effects even after one month of multiple administrations [28]. Since NERs resemble liposomes in composition, we anticipate that our formulation will be safe to administer via the pulmonary route.

The *in-vivo* safety was evaluated by determining wet lung weight and analyzing BAL fluid after intratracheal administration. Wet lung weight (gm/100 gm body wt.) was determined prior to performing lavage. Lungs of CAR-NERs-Fasudil treated rats showed a little higher weight (0.41 ± 0.09) than that of saline treated ones (0.38 ± 0.07) but significantly lower than 0.1% SDS treated rat lungs (0.72 ± 0.03). The formulation did not cause any extensive lung injury (Fig. 9B). The total protein content in BAL fluid of CAR-NERs-Fasudil treated rats did not change much, suggesting minimal inflammation in the lungs (Fig. 9C). Neither LDH nor ALP levels increased in CAR-NERs-Fasudil treated rats (Fig. 9D). But total protein content, ALP, and LDH levels went up in SDS treated rats. The cyclic structure of CAR peptide with disulfide connection offers resistance to degradation by proteases and the pulmonary route has reduced enzymatic activity compared with other routes of administration [16]. While this acute safety data are encouraging, evaluation of pathological changes after repeated administrations may provide more useful data regarding the long-term safety of the formulations.

4. CONCLUSIONS

In this study, we have evaluated CAR conjugated NERs as inhalable carriers of fasudil for the treatment of PAH. *In-vitro* studies such as cellular uptake, rho-kinase inhibition and 5-HT induced proliferation showed favorable results which was translatable to *in-vivo* efficacy. We demonstrated that CAR-NERs-Fasudil were effective in pulmonary selective vasodilation with little effect on MSAP. This study suggests that CAR-NERs based formulation of fasudil would be clinically significant because they prolong vasodilation in the pulmonary circuit. Chronic efficacy studies are underway to evaluate the long term effects of formulation in alleviating the symptoms of PAH.

ACKNOWLEDGEMENTS

The authors acknowledge Drs. E. Nozik-Grayck and K. Stenmark at the University of Colorado, Denver for providing PASM cell lines. This work was supported in part by an American Recovery and Reinvestment Act Fund, NIH 1R15HL103431, to F. Ahsan.

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	nanoerythroosomes
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

REFERENCES

1. Guan R, Xu X, Chen M, Hu H, Ge H, Wen S, Zhou S, Pi R. Advances in the studies of roles of Rho/Rho-kinase in diseases and the development of its inhibitors. *Eur J Med Chem.* 2013; 70:613–622. [PubMed: 24211637]
2. Barman SA, Zhu S, White RE. RhoA/Rho-kinase signaling: a therapeutic target in pulmonary hypertension. *Vasc Health Risk Manag.* 2009; 5:663–671. [PubMed: 19707285]
3. Schwenke DO, Pearson JT, Sonobe T, Ishibashi-Ueda H, Shimouchi A, Kangawa K, Umetani K, Shirai M. Role of Rho-kinase signaling and endothelial dysfunction in modulating blood flow distribution in pulmonary hypertension. *J Appl Physiol* (1985). 2011; 110:901–908. [PubMed: 21212241]
4. Majumdar S, Siahaan TJ. Peptide-mediated targeted drug delivery. *Med Res Rev.* 2012; 32:637–658. [PubMed: 20814957]
5. Dassie JP, Giangrande PH. Current progress on aptamer-targeted oligonucleotide therapeutics. *Ther Deliv.* 2013; 4:1527–1546. [PubMed: 24304250]
6. Cheng L, Huang FZ, Cheng LF, Zhu YQ, Hu Q, Li L, Wei L, Chen DW. GE11-modified liposomes for non-small cell lung cancer targeting: preparation, ex vitro and in vivo evaluation. *Int J Nanomedicine.* 2014; 9:921–935. [PubMed: 24611009]
7. Kim H, Park HT, Tae YM, Kong WH, Sung DK, Hwang BW, Kim KS, Kim YK, Hahn SK. Bioimaging and pulmonary applications of self-assembled Flt1 peptide-hyaluronic acid conjugate nanoparticles. *Biomaterials.* 2013; 34:8478–8490. [PubMed: 23932502]
8. Tripp RA, Alvarez R, Anderson B, Jones L, Weeks C, Chen W. Bioconjugated nanoparticle detection of respiratory syncytial virus infection. *Int J Nanomedicine.* 2007; 2:117–124. [PubMed: 17722519]
9. Kleemann E, Schmehl T, Gessler T, Bakowsky U, Kissel T, Seeger W. Iloprost-containing liposomes for aerosol application in pulmonary arterial hypertension: formulation aspects and stability. *Pharm Res.* 2007; 24:277–287. [PubMed: 17211729]
10. Mosgoeller W, Prassl R, Zimmer A. Nanoparticle-mediated treatment of pulmonary arterial hypertension. *Methods Enzymol.* 2012; 508:325–354. [PubMed: 22449934]
11. Wernig K, Griesbacher M, Andreae F, Hajos F, Wagner J, Mosgoeller W, Zimmer A. Depot formulation of vasoactive intestinal peptide by protamine-based biodegradable nanoparticles. *J Control Release.* 2008; 130:192–198. [PubMed: 18601963]
12. Gupta N, Patel B, Ahsan F. Nano-Engineered Erythrocyte Ghosts as Inhalational Carriers for Delivery of Fasudil: Preparation and Characterization. *Pharm Res.* 2014
13. Gutierrez Millan C, Colino Gandarillas CI, Sayalero Marinero ML, Lanao JM. Cell-based drug-delivery platforms. *Ther Deliv.* 2012; 3:25–41. [PubMed: 22833931]
14. Ravi Kumar MN. Nano and microparticles as controlled drug delivery devices. *J Pharm Pharm Sci.* 2000; 3:234–258. [PubMed: 10994037]
15. Urakami T, Jarvinen TA, Toba M, Sawada J, Ambalavanan N, Mann D, McMurtry I, Oka M, Ruoslahti E, Komatsu M. Peptide-directed highly selective targeting of pulmonary arterial hypertension. *Am J Pathol.* 2011; 178:2489–2495. [PubMed: 21549345]
16. Toba M, Alzoubi A, O'Neill K, Abe K, Urakami T, Komatsu M, Alvarez D, Jarvinen TA, Mann D, Ruoslahti E, McMurtry IF, Oka M. A novel vascular homing peptide strategy to selectively enhance pulmonary drug efficacy in pulmonary arterial hypertension. *Am J Pathol.* 2014; 184:369–375. [PubMed: 24401613]
17. Yu MK, Park J, Jon S. Targeting strategies for multifunctional nanoparticles in cancer imaging and therapy. *Theranostics.* 2012; 2:3–44. [PubMed: 22272217]
18. Gupta V, Gupta N, Shaik IH, Mehvar R, McMurtry IF, Oka M, Nozik-Grayck E, Komatsu M, Ahsan F. Liposomal fasudil, a rho-kinase inhibitor, for prolonged pulmonary preferential vasodilation in pulmonary arterial hypertension. *J Control Release.* 2013; 167:189–199. [PubMed: 23353807]
19. Gupta V, Gupta N, Shaik IH, Mehvar R, Nozik-Grayck E, McMurtry IF, Oka M, Komatsu M, Ahsan F. Inhaled PLGA particles of prostaglandin E(1) ameliorate symptoms and progression of

- pulmonary hypertension at a reduced dosing frequency. *Mol Pharm*. 2013; 10:1655–1667. [PubMed: 23485062]
20. Chono S, Tanino T, Seki T, Morimoto K. Uptake characteristics of liposomes by rat alveolar macrophages: influence of particle size and surface mannose modification. *J Pharm Pharmacol*. 2007; 59:75–80. [PubMed: 17227623]
 21. Fidler IJ, Raz A, Fogler WE, Kirsh R, Bugelski P, Poste G. Design of liposomes to improve delivery of macrophage-augmenting agents to alveolar macrophages. *Cancer Res*. 1980; 40:4460–4466. [PubMed: 7002293]
 22. Pouliot R, Saint-Laurent A, Chypre C, Audet R, Vitte-Mony I, Gaudreault RC, Auger M. Spectroscopic characterization of nanoErythrosomes in the absence and presence of conjugated polyethyleneglycols: an FTIR and (31)P-NMR study. *Biochim Biophys Acta*. 2002; 1564:317–324. [PubMed: 12175913]
 23. Dodge GR, Kovalszky I, Hassell JR, Iozzo RV. Transforming growth factor beta alters the expression of heparan sulfate proteoglycan in human colon carcinoma cells. *J Biol Chem*. 1990; 265:18023–18029. [PubMed: 1698783]
 24. Song D, Wang HL, Wang S, Zhang XH. 5-Hydroxytryptamine-induced proliferation of pulmonary artery smooth muscle cells are extracellular signal-regulated kinase pathway dependent. *Acta Pharmacol Sin*. 2005; 26:563–567. [PubMed: 15842774]
 25. Kolozsvari B, Bako E, Becsi B, Kiss A, Czikora A, Toth A, Vamosi G, Gergely P, Erdodi F. Calcineurin regulates endothelial barrier function by interaction with and dephosphorylation of myosin phosphatase. *Cardiovasc Res*. 2012; 96:494–503. [PubMed: 22869619]
 26. van Nieuw Amerongen GP, Vermeer MA, van Hinsbergh VW. Role of RhoA and Rho kinase in lysophosphatidic acid-induced endothelial barrier dysfunction. *Arterioscler Thromb Vasc Biol*. 2000; 20:E127–E133. [PubMed: 11116077]
 27. Gomez-Arroyo JG, Farkas L, Alhussaini AA, Farkas D, Kraskauskas D, Voelkel NF, Bogaard HJ. The monocrotaline model of pulmonary hypertension in perspective. *Am J Physiol Lung Cell Mol Physiol*. 2012; 302:L363–L369. [PubMed: 21964406]
 28. Cipolla D, Gonda I, Chan HK. Liposomal formulations for inhalation. *Ther Deliv*. 2013; 4:1047–1072. [PubMed: 23919478]

HIGHLIGHTS

CAR-NERs-Fasudil was successfully prepared by hypotonic lysis+extrusion method.

CAR-NERs were spherical in shape containing ~49% encapsulated fasudil.

Formulations showed controlled release behavior and were stable at 4°C.

CAR-NERs were ~1.5 fold more internalized by cells as compared to plain NERs.

CAR-NERs showed pulmonary preferential and prolonged vasodilation in PAH rats.

Fig. 1.
Architecture of targeted nanoerythrocytes containing fasudil.

Fig. 2.
The preparation method of nanoerythroosomes from erythrocytes by hypotonic lysis–extrusion method.

Fig. 3.
(A) Conjugation of CAR peptide on the surface of nanoerythrocytes (NERs) utilizing linker chemistry. (B) UV spectrophotometric monitoring for release of pyridine-2-thione at 343 nm before and after conjugation.

Fig. 4.

The fluorescent microscopic images of erythrocytes (inherently red colored due to hemoglobin), erythrosomes (colorless erythrocyte ghosts resealed after complete hemolysis) and CAR conjugated erythrosomes (green color is due to FAM-labeled CAR peptide). The lower panel shows the physicochemical properties of CAR-NERs containing fasudil after extrusion and purification. PDI here refers to polydispersity index. Data represent mean $\pm$ standard deviation (n=3).

Fig. 5.

(A) The *in-vitro* release profiles of plain fasudil and formulations in PBS (pH 7.4) at 37°C. (B) The storage stability of the formulations at 4°C for 3 weeks. (C) Changes in vesicle size and entrapment efficiency of the formulations before and after nebulization through the microsprayer. Data represent mean $\pm$ standard deviation (n=3).

Fig. 6. Comparative cellular uptake of plain and CAR conjugated NERs (stained red with plasma membrane dye CellMask) by normal and TGF- β activated rat PASM cells (actin stained green). (A) Fluorescent microscopic images. (B) Flow cytometric analysis. (C) Quantitative analysis of uptake of formulations. Data represent mean $\pm$ standard deviation ($n=3$).

Fig. 7.

In-vitro inhibition of 5-hydroxy tryptamine induced cell proliferation (n=8) by (A) same dose of plain drug and formulations, and (B) different doses of plain fasudil. (C) Rho-kinase inhibition by plain fasudil and formulations (n=6). Data represent mean $\pm$ standard deviation.

Fig. 8. The hemodynamic efficacy of plain drug and formulations in MCT-induced PAH rats. (A) plain intravenous fasudil (IV); (B) plain intratracheal fasudil (IT); (C) plain NERs IT; (D) CAR-NERs IT; (E) lung targeting indices and (F) duration of vasodilatory effects. Data represent mean $\pm$ standard deviation (n=6–8).

Fig. 9.

Safety of the formulations: (A) Viability of Calu-3 and rat PASM cells upon incubation with plain drug or CAR-NERs for 24 hrs (n=12). Effect of the formulation on (B) wet lung weight; (C) total protein content, and (D) levels of injury markers in bronchoalveolar lavage fluid (n=4). Data represent mean $\pm$ standard deviation.