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Cocktail of Superoxide Dismutase and Fasudil Encapsulated in Targeted Liposomes Slows PAH Progression at a Reduced Dosing Frequency

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Abstract

Currently, two or more pulmonary vasodilators are used to treat pulmonary arterial hypertension (PAH), but conventional vasodilators alone cannot reverse disease progression. In this study, we tested the hypothesis that a combination therapy comprising a vasodilator plus a therapeutic agent that slows pulmonary arterial remodeling and right heart hypertrophy is an efficacious alternative to current vasodilator-based PAH therapy. Thus, we encapsulated a cocktail of superoxide dismutase (SOD), a superoxide scavenger, and fasudil, a specific rho-kinase inhibitor, into a liposomal formulation equipped with a homing peptide, CAR. We evaluated the effect of the formulations on pulmonary hemodynamics in monocrotaline-induced PAH rats (MCT-induced PAH) and assessed the formulation's efficacy in slowing the disease progression in Sugen-5416/hypoxia-induced PAH rats (SU/hypoxia-induced PAH). For acute studies, we monitored both mean pulmonary and systemic arterial pressures (mPAP and mSAP) for 2 to 6 h after a single dose of the plain drugs or formulations. In chronic studies, PAH rats received plain drugs every 48 h and the formulations every 72 h for 21 days. In MCT-induced PAH rats, CAR-modified liposomes containing fasudil plus SOD elicited a more pronounced, prolonged, and selective reduction in mPAP than unmodified liposomes and plain drugs did. In SU/hypoxia-induced PAH rats, the formulation produced a >50% reduction in mPAP and slowed right ventricular hypertrophy. When compared with individual plain drugs or combination, CAR-modified-liposomes containing both drugs reduced the extent of collagen deposition, muscularization of arteries, increased SOD levels

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Notes

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in the lungs, and decreased the expression of pSTAT-3 and p-MYPT1. Overall, CAR-modified-liposomes of SOD plus fasudil, given every 72 h, was as efficacious as plain drugs, given every 48 h, suggesting that the formulation can reduce the total drug intake, systemic exposures, and dosing frequency.

Graphical abstract

Keywords

pulmonary arterial hypertension; liposomes; fasudil; super oxide dismutase; inhalation

INTRODUCTION

The pathophysiology of pulmonary arterial hypertension (PAH) is entwined with multiple signaling pathways, and thus, the mainstay of current therapy involves the use of a single or a combination of drugs that work on three major pathways: endothelin, nitric oxide, and prostacyclin pathways.^{1,2} Indeed, a complex treatment algorithm comprising various add-on therapies or sequential combinations of two or more drugs are used to improve patient outcomes in PAH.^{3–5} However, existing anti-PAH drugs function primarily as vasodilators that do not effectively lower pulmonary arterial pressure and are unable to reduce right heart enlargement and dysfunction or to prevent occlusions in pulmonary arteries or arterioles. As a result, the long-term benefits from conventional vasodilator-based combination therapies in PAH have been disappointing. Combinations of two or more vasodilators produce only incremental improvement in clinical deterioration and pulmonary hemodynamics;⁶ patient mortality is no better in the combination arms than in monotherapy arms.⁷

To address the limitations of current vasodilators-only combination therapy in PAH, we posit that a combination therapy comprising a more potent vasodilator plus a therapeutic agent that can reverse the chief underlying causes of disease progression, vascular remodeling, and right heart dysfunction may improve patient outcome, regress the disease, and extend progression free survival of PAH patients. One of those agents, we believe, is superoxide dismutase (SOD), an enzyme responsible for converting superoxide radical (O_2^-) to molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). SOD reduces elevated pulmonary arterial pressure and arterial occlusion and slows right heart enlargement.^{8–10} SOD levels decline in both PAH patients and PAH animals,^{8–11} and thus, reactive oxygen species (ROS) levels rise and cause oxidative stress and metabolic dysfunction.^{1,2,11,8–10} An excess of superoxide anions uncouples endothelial nitric oxide synthase (eNOS), disrupts the redox signaling, and causes vascular remodeling that leads to right heart enlargement and failure,

the chief reason for death from PAH.^{12–14} Further, ROS inactivates nitric oxide (NO), an important anti-inflammatory and vasodilating agent, and converts NO to a strong oxidant, peroxynitrite, which causes endothelial and mitochondrial dysfunction.¹⁵ Encouragingly, delivery of exogenous SOD, either as gene therapy or administering SOD mimetics, can supplement the SOD levels in PAH animals and reduce elevated pulmonary arterial pressure, arterial occlusion, and right heart enlargement.^{9,16–22}

In PAH development, the Rho-kinase pathway, in addition to the above three pathways, is now known to play a dominant role. The activation of Rho-kinase, a downstream effector enzyme of the small GTPase–RhoA, causes pulmonary arterial constriction and increases pulmonary vascular resistance.^{23–26} Rho-kinase also reduces eNOS expression in endothelial cells and makes vascular smooth muscle cells hyper-reactive to vasoconstrictors.^{26,27} We and others have reported that oral or intratracheal administration of fasudil, a potent and selective inhibitor of Rho-kinase, causes pulmonary arterial vasodilation and reduces vascular resistance in both PAH animals and PAH patients.^{28–31} Further, fasudil suppresses myosin phosphatase, increases eNOS expression, and decreases migration of inflammatory cells.^{32–34}

Considering the above roles of SOD and Rho-kinase in PAH, we hypothesize that an inhalable combination therapy consisting of a vasodilator (fasudil) and a ROS scavenger (Cu/Zn SOD), formulated in liposomes equipped with a homing peptide, will ameliorate pulmonary vasoconstriction, reverse pulmonary arterial remodeling, and abate right heart enlargement in PAH. To test this hypothesis, we packaged the two drugs into liposomes equipped with CARSKNKDC (CAR), the homing peptide, with the assumption that CAR will guide the particles to concentrate in hypertensive pulmonary arteries/arterioles (Figure 1). We investigated the acute vasodilatory efficacy in monocrotaline (MCT)-induced PAH rats and the long-term effects of the formulation in SUGEN-5416 (SU)/hypoxia-induced PAH rats. We determined the efficacy in reducing chronic symptoms such as pulmonary hypertension, right ventricular hypertrophy, degree of pulmonary arterial occlusion, extent of collagen deposition, and expression of biomarkers for vascular dysfunction.

MATERIALS AND METHODS

Preparation and Characterization of CAR-Liposomes Containing SOD and Fasudil

Liposomes were prepared by solvent evaporation, thin film formation, and extrusion methods as described by us previously.^{29,35} Lipids, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol (CHOL), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide(polyethylene glycol)-2000] (DSPE-PEG-MAL) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Briefly, lipids (DPPC/CHOL/DSPE-PEG-MAL, 6:3:1) were dissolved in a mixture of chloroform and methanol and left overnight in a rotary evaporator (Buchi Rotor Evaporator R-114; BUCHI Labortechnik AG, Switzerland) so that methanol/chloroform evaporates completely leaving a thin homogeneous film. Next day, the dried lipid film was hydrated with PBS buffer containing 20 mg of SOD (EMD Millipore, Billerica, MA) and 30 mg of fasudil (LC Laboratories Inc., Woburn, MA) at 65 °C for 2 h, followed by four freeze–thaw cycles (10 min each at –196 and 65 °C). To prepare small unilamellar vesicles, the liposomes were extruded through

polycarbonate membranes (100 nm pore size). Untrapped drugs were separated by size-exclusion chromatography using a Sephadex based gel filtration column (PD10; GE Healthcare, Piscataway, NJ). By using Amicon Ultra centrifugal filter units (MWCO-3000, Millipore Inc., Billerica, MA), we further purified and concentrated the liposomes by centrifugation for 45 min at 300g (Centrifuge 5702-R, Eppendorf AG, Hamburg, Germany). The homing device, CAR, is a small cyclic peptide with the amino acid sequence CARSKNKDC (Synthesized by LifeTein LLC, South Plainfield, NJ; licensed by Vascular Bioscience, Durham, NC). CAR peptide preferentially binds to unchained heparan sulfate at PAH-afflicted vascular endothelium that initiates micropinocytosis mediated internalization of the CAR-conjugated drug moieties into the endothelial cells.³⁶ To conjugate the homing peptide, a solution of CAR peptide in PBS was incubated with liposomes in dark for an hour to form thiol linkage between maleimide group of the lipid (DSPE-PEG-MAL) and sulfhydryl group of the peptide. The liposomes were then purified as described previously to remove the unconjugated peptide. Liposomes were prepared in triplicate and stored at 4 °C until further characterization and use. The particle size, polydispersity index (PDI), and zeta potential of liposomes were measured in a Malvern Zetasizer (Malvern Instruments Limited, Worcestershire, U.K.). Encapsulation of both drugs was determined by disrupting liposomes in methanol and quantifying the fasudil content using a UV spectrophotometer at 320 nm and SOD using a commercial kit (EpiGentek Group Inc., Farmingdale, NY). Blank liposomes (liposomes without the drugs) dissolved in methanol was used as control for fasudil quantitation but not liposomal SOD because SOD does not show absorbance at 320 nm. The entrapment efficiency was calculated from the following equation: entrapment efficiency (%) = (amount of drug in liposomes/amount of drug originally added) × 100.

Acute Hemodynamic Studies in MCT-Induced PAH Rats

The *in vivo* efficacy of a single dose of liposomes was evaluated in MCT-induced PAH rats as described previously.^{29,37} Briefly, PAH was induced in male Sprague–Dawley (SD) rats (250–300 g) by injecting a single subcutaneous dose of MCT (50 mg/kg body weight, Sigma-Aldrich Inc., St. Louis, MO) and housing for 28 days (Figure 2A). At the end of 28 days, PAH rats were anesthetized by an intramuscular injection of a cocktail of ketamine (90 mg/kg) and xylazine (10 mg/kg). As reported in our earlier publications,^{29,37} two catheters, one in pulmonary artery, by maneuvering via jugular vein and right ventricle, and the other in right carotid artery, were placed and sutured for simultaneous measurements of mPAP and mSAP, respectively. Pressures were recorded 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). We normalized the baseline mPAP and mSAP recorded in mmHg to 100% and presented the data in percent reduction from baseline mPAP or mSAP before administration of any drugs.

Rats were divided into six groups to receive six treatments, which were then again divided into three subgroups (Figure 3A). The first two subgroups of rats received sequential treatment of (i) intravenous (IV) SOD and fasudil ($n = 3$) and (ii) intratracheal SOD and fasudil ($n = 4$). To these two groups of rats, SOD was administered first, and fasudil was administered 30 min after SOD administration. The second two groups of rats received a

mixture of SOD and fasudil, given either (iii) intratracheally (IT) ($n = 4$) or (iv) intravenously ($n = 4$). The third subgroup received liposomal formulation of SOD and fasudil administered intratracheally: (v) unmodified-liposomes containing SOD and fasudil ($n = 4$) and (vi) CAR-modified-liposomes containing SOD and fasudil ($n = 3$). For all six treatments, the doses of SOD and fasudil, chosen based on published studies,^{18,29} were 5 and 3 mg/kg, respectively. When the baseline hemodynamic values were stabilized, IV dose was given via the penile vein and IT administration was performed using the PennCentury Microsprayer as described earlier.³⁷ We chose the dose of fasudil based on our earlier publications^{29,38} and a clinical trial that used fasudil in PAH patients.²⁸ Similarly, we selected SOD dose based on a study that gave SOD to hypoxia induced lung injury in baboons at a dose of 1–10 mg/kg.³⁹ The equivalent dose for rats, converted following US FDA guidance,⁴⁰ is 3.4–34 mg/kg. The dose of SOD (5 mg/kg) used in this study falls within the range reported earlier and did not show any interactions with fasudil when administered concurrently via pulmonary route.¹⁹ Animals were maintained on anesthesia throughout the procedure, and pressures were recorded for at least 6 h. At the end of the experiment, rats were euthanized 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).

Chronic Studies in SU/Hypoxia-Induced PAH Rats

The long-term efficacy studies were conducted in SU/hypoxia-induced PAH rats as described previously^{17,41} (Figure 2B). Briefly, male SD rats (150–200 g), injected with subcutaneous 20 mg/kg SU5416 (R&D Systems Inc., Minneapolis, MN), were kept inside a hypoxic chamber (BioSpherix, Lacona, NY) at 10% oxygen for 3 weeks. On the 22nd day, rats were removed from the hypoxic chamber and housed in normoxia for 3 weeks. During the normoxic period, rats received, every 48 h, the following four treatments via the intratracheal route: (i) saline ($n = 4$), (ii) plain fasudil ($n = 4$), (iii) plain SOD ($n = 3$), and (iv) a mixture of plain SOD plus fasudil. Additional two groups received two treatments intratracheally every 72 h: (v) unmodified liposomes containing SOD plus fasudil ($n = 3$), and (vi) CAR modified liposomes containing SOD plus fasudil ($n = 4$) (Figure 3B). The dose of both drugs was the same as that of acute studies. One group of healthy rats ($n = 3$) was used as sham group. After 3 weeks of treatment, rats were catheterized, and mPAP and mSAP (mmHg) were recorded for a period of 30 min using the PowerLab system as described above and data are presented in percent reduction from baseline mPAP or mSAP before administration of any drugs.

Following the hemodynamic measurements, the animals were euthanized by exsanguination, and lung tissues were collected as described previously.³⁷ Briefly, right lung was separated and flash-frozen in liquid nitrogen for assaying SOD, levels of phosphorylated myosin phosphatase-1 (pMYPT-1), and phosphorylated signal transducer and activator of transcription 3 (pSTAT-3). The heart and left lung were inflation fixed at 30 cm H₂O pressure with 4% paraformaldehyde for 30 min, dissected from the chest cavity, placed in 4% paraformaldehyde at 4 °C overnight, transferred to 70% ethanol, and paraffin embedded for further immunohistochemistry and morphometric analysis.

Right Ventricular Hypertrophy (RVH) Measurements

RVH analysis was performed according to our published protocol.³⁷ Briefly, the hearts stored at 4 °C in 70% ethanol were resected, atria and great vessels were detached, and the right and left ventricle, including the septum, were separated and weighed. RVH was determined from the ratio of the right ventricular/left ventricular + septum weights (RV/LV + S).

Immunohistochemistry

Paraffin-embedded lung sections were deparaffinized and rehydrated with Citrisolv (Thermo Fisher Scientific, Waltham, MA) and graded ethanol, treated with antigen retrieval solution (Vector Lab, Burlingame, CA), then incubated with 3% hydrogen peroxide, and blocked with 2.5% horse serum in Tris-buffered saline and Tween 20 (TBST) prior to incubation with primary antibodies. For dual fluorescent staining, sections were incubated overnight with mouse monoclonal antimouse α -smooth muscle actin antibody (α -SMA, 1:100, clone 1A4; Sigma, St. Louis, MO) and rabbit polyclonal von-Willebrand Factor (FVIII, 1:500; Sigma, St. Louis, MO). Next day, sections were treated with Alexa 594 (1:500) and Alexa 488 (1:2000) fluorescent secondary antibodies (Invitrogen, Carlsbad, CA). Slides were counterstained with methyl green (Vector Laboratories, Burlingame, CA) to stain the nuclei. Additional slides stained for α -SMA were incubated with an anti IgG antibody followed by the ABC reagent (Vectastatin kit; Vector Laboratories, Burlingame, CA), developed with ImmPact DAB diluent (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin for morphometric analysis. Tissue sections were examined by light microscopy and photographed on a Zeiss Axiovert S100 (Carl Zeiss LLC, Thornwood, NY) at 100 $\times$ magnification, and images were analyzed using AxioVision software.

Determination of Muscularization, Fractions of Occluded Arteries, and Medial Wall Thickness

By counting α -SMA positive vessels, which are <50 μ m in diameter, in 10 fields of view (10 $\times$ magnification), we evaluated the muscularized pulmonary arteries and arterioles and then calculated the fraction of muscularized vessels that were occluded. We assumed arteries to be occluded when the intimal layer occupied >50% of the diameter of the lumen and intima of the vessels. For medial wall thickness (MWT), four measurements of the perpendicular lumen radius and medial wall were taken in small muscularized arteries (<50 μ m). MWT was expressed as the average MWT divided by the average vessel radius. The α -SMA staining was evaluated by an investigator blinded to the treatment groups.

Collagen Analysis

Collagen deposition around pulmonary arteries was determined by trichrome staining with collagen stained blue for better visualization as described previously.³⁷ Briefly, lung sections (100 μ m) were deparaffinized with Citrisolv (Thermo Fisher Scientific, Waltham, MA) and rehydrated in graded ethanol followed by deionized water. Sections were then sequentially treated with hematoxylin, acid fuchsin, and aniline blue. Extra stain was removed by dipping the slides in hydrochloric acid followed by dehydration in graded ethanol and finally drying

and putting coverslips along with the mounting media. Stained sections were imaged by light microscopy by an investigator blinded to treatment groups.

SOD Content in Lungs after Chronic Treatment

We determined the levels of SOD in the PAH rat lungs as a marker for reduction in oxidative stress in pulmonary vasculature. For this, we homogenized a small portion of flash-frozen lung in PBS and analyzed the SOD content by a commercial kit (EpiQuick SOD Assay Kit, Epigentek Group Inc., Farmingdale, NY). Briefly, reaction mixture was prepared by mixing lung tissue homogenate, dilution buffer, assay buffer, substrate, indicator solution, and reaction enzyme, and incubated for 1 h in dark. The plates were read at 470 nm using a SynergyMX microplate reader (Biotek, Winnoski, VT).

Western Blot Analysis

Lysates were prepared by homogenizing the flash-frozen tissues in PBS supplemented with a protease inhibitor cocktail. Total protein content was determined by using Bradford's reagent (Biorad, Hercules, CA). Then, an equal amount of protein was denatured with beta-mercaptoethanol for Western blot analysis. Protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the segregated protein was transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked using fat-free milk and incubated overnight at 4 °C with primary antibodies specific to pMYPT-1 (Thr-850) (EMD Millipore, Billerica, MA; 1:700), pSTAT-3 (Y705) (Abcam, Cambridge, MA; 1:1000), and actin (Sigma-Aldrich, St. Louis, MO; 1:10,000). Next day, PVDF membranes were washed with TTBS followed by incubation with antirabbit and antimouse secondary antibodies (1:2000 in fat free milk), respectively. Membranes were developed using chemiluminescence detection reagent (Bio-Rad, Hercules, CA). The blots were quantitated with UN-SCAN-IT graph digitizing software (Silk Scientific, Orem, UT) using Actin as an internal control.

Data Analysis and Statistics

The data are presented as mean $\pm$ standard deviation and were analyzed by one-way ANOVA followed by Tukey's posthoc test using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA). The data presented in Figure 4 were analyzed by two-way repeated-measure ANOVA followed by Bonferroni's posthoc test. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Preparation and Characterization of CAR-Liposomes Containing SOD and Fasudil

We have prepared and characterized CAR-liposomes containing both SOD and fasudil by thin-film formation, hydration, and extrusion methods. We have used PEGylated lipid because of its ability to protect liposomes in biological environment and prevent clearance by macrophages.⁴² The average size of liposomes was ~ 150 nm with a polydispersity index of 0.119 ± 0.02 , a size optimal for avoiding alveolar clearance because particles < 250 nm can escape lungs' natural clearance mechanism, deposit in the deep lung, and reside longer in the lungs.²⁹ We have previously shown that CAR-conjugated liposomal combination was

safe to administer intratracheally to rats and was not cytotoxic to the pulmonary arterial smooth muscle cells.¹⁹ Entrapment efficiencies of SOD and fasudil were $54.91 \pm 1.66\%$ and $39.22 \pm 3.41\%$, respectively. The formulation was stable against aggregation as shown by zeta potential value (-28.17 ± 1.77 mV). In vitro release study shows that ~60% of SOD and fasudil was released in first 12 h and ~75% by 24 h. The physical and release characteristics of these formulations are consistent with our previous studies^{19,38} and are acceptable for intratracheal delivery to rat lungs.

Acute Hemodynamic Efficacy Studies in MCT-Induced PAH Rats

We have shown previously that fasudil^{29,38} and fasudil plus SOD formulated in liposomes,¹⁹ when administered for an acute effect, produces pulmonary preferential vasodilation. As a continuation of our previous studies, here we have evaluated the chronic effect of two drugs, when given as a combination therapy. Because the goal of this project is to assess the chronic effects of two drugs and their formulations in ameliorating the underlying pathophysiology of PAH, here we compared the efficacy of plain drug combination with that of the two drugs formulated in plain liposomes or CAR-modified liposomes, when administered for 21 days.

However, before conducting chronic studies, we evaluated the acute hemodynamic efficacies of the drugs and liposomal formulations containing both drugs in MCT-induced PAH rats, a classical animal model that has been used extensively to evaluate the effect of anti-PAH drugs in reducing mPAP.^{38,43–45} The MCT rat model of PAH produces characteristic clinical features of PAH such as increased mPAP, right ventricular hypertrophy, pulmonary vascular remodeling, and diminished luminal circumference of small pulmonary arterioles.^{41,46–48} Generally, healthy rats show an mPAP of ~12–16 mmHg, which elevates to >25 mmHg in PAH rats. In this study, we observed an average mPAP of 44.89 ± 3.94 mmHg in MCT-treated rats, pointing to development of PAH. Cu/Zn SOD treatment, by scavenging ROS and inhibiting peroxynitrite formation, increases the bioavailability of nitric oxide and hence promotes vasodilation.^{49,50} Pretreatment with SOD was reported to increase the bioavailability of inhaled nitric oxide.⁴⁹ Thus, we posit that SOD pretreatment may increase NO availability and produce a synergistic effect when used in combination with fasudil. However, fasudil pretreatment may not have any effect on SOD because fasudil predominantly functions as a vasodilator. Based on this assumption and published studies, we gave SOD first and then fasudil in acute studies.

When SOD alone was administered, as expected, mPAP did not change because SOD is not a strong vasodilator. However, as soon as we gave fasudil 30 min after SOD administration, both mPAP and mSAP started to decline due to fasudil's potent vasodilatory effects (Figure 4A,B), as observed in our earlier studies.^{29,51} Although intravenous sequential administration, SOD first and fasudil 30 min later, showed a similar pattern in reducing mPAP and mSAP, intratracheal administration showed a 14% less reduction in mSAP than the IV administration did. This pulmonary selective reduction of mPAP in intratracheal-SOD-then-fasudil-treated rats was significantly different from mSAP for a period of 70 min (Figure 4B), which may have resulted from a higher local fasudil concentration, and the delay in the availability of drugs in the systemic circulation, pointing to the advantages of

pulmonary route over intravenous route of administration. These data suggest that IT route gives rise to a higher drug concentration in the lung vasculature; thus, IT drugs elicited greater reduction in mPAP than IV drugs did. Likewise, IV drug produced a more pronounced reduction in mSAP than IT drugs because IV drug distributes instantaneously throughout the body, but IT drugs undergo an absorption phase before entering the systemic circulation. The total amount of a drug, administered via IT, in the body in a given time is less than the amount when the drugs are given via IV, and the net result is reduced peripheral vasodilation.

When we administered a mix of the two drugs to the rats, both mPAP and mSAP reduced to 40–50% of the initial values (Figure 4C,D). For the first 60 min, the reduction in mSAP was 50%, which was much greater than that of mPAP in animals treated with an intravenous cocktail of SOD plus fasudil (Figure 4C), but the opposite was true when PAH rats received the mix of SOD plus fasudil via the intratracheal route: the reduction in mSAP was smaller than the reduction in mPAP, supporting our hypothesis that preferential pulmonary vasodilation can be achieved by intratracheal administration (Figure 4D).

When we gave unmodified-liposomes containing SOD plus fasudil intratracheally, the pattern of reduction in mPAP and mSAP was very similar to that of the intratracheally administered mixture of plain SOD plus fasudil. During the first 60 min, mPAP and mSAP declined concomitantly; after that period, the reduction in mPAP was greater than that in mSAP (Figure 4E). However, when CAR-equipped liposomes containing SOD plus fasudil were given intratracheally, mPAP declined dramatically: a 50% reduction in mPAP for more than 250 min (Figure 4F), but only a 20% reduction in mSAP. The differences between mPAP and mSAP were statistically significant for ~180 min. CAR-modified liposomes selectively and continuously reduced mPAP because of the propensity of the homing device, CAR, to bind to the hypertensive blood vessels, which express heparan sulfate, the binding site for CAR peptide.⁵² Because of long vasodilatory duration, drugs loaded in CAR-modified liposomes can be administered less frequently with minimal reductions in mSAP. Thus, CAR-modified-liposomes of SOD and fasudil, when administered via the pulmonary route, can circumvent the limitations of currently approved oral or intravenous anti-PAH therapies that produce serious side effects such as systemic hypotension and cardiovascular collapse.^{53,54}

Assuming that 12–15% reduction in mPAP is therapeutically relevant and recording the pressure until mPAP and mSAP returned to ~90% of baseline, we calculated the duration for vasodilation. The vasodilatory duration for CAR-modified-liposomes was ~2.5-fold longer than that observed in plain drug combination and unmodified liposome treated groups (Figure 5A). The presence of CAR on the liposomes caused the formulations to accumulate more on the PAH-afflicted blood vessels and thus reduced mPAP selectively. Moreover, the drugs were released from the liposomes in a continuous fashion that maintained therapeutic concentration of the drug for a longer period and thus would reduce the dosing frequency.

To determine the pulmonary selectivity of the formulations, we calculated lung targeting indices (LTI) from the ratio of the area above the pressure–time curve (AAC) for mPAP to that of mSAP curves ($LTI = AAC_{mPAP}/AAC_{mSAP}$). The LTI for inhaled CAR-modified-

liposomes was 6-fold greater than for plain drugs or their combination, indicating that CAR-equipped-liposomes were lung-selective and caused greater pulmonary vasodilation (Figure 5B). Overall, acute hemodynamic studies in MCT-induced PAH rats strongly support the feasibility of an inhaled and targeted controlled release formulation of SOD plus fasudil for the treatment of PAH, which we have further evaluated by administering the formulations for 21 days to SU/hypoxia-induced PAH rats.

Chronic Hemodynamics and Right Ventricular Hypertrophy

To evaluate the chronic effect of the formulations on PAH progression, we have used the SU/hypoxia-induced PAH model, which develops occlusive neointimal lesions akin to the pulmonary arteriopathy in human PAH.⁵⁵ The MCT-induced PAH rat gives useful data on the hemodynamic efficacy of a therapy, but the SU/hypoxia-induced PAH rat is a better alternative for evaluating whether a given drug or formulation ameliorates the more severe form of PAH with occlusive remodeling of small pulmonary arteries/arterioles.

In this study, we aimed to establish that the dosing frequency of plain drugs can be reduced by putting them in liposomal formulation. Based on our previously published studies, we assumed that dosing plain drugs every 72 h would not be relevant to chronic effect because the half-lives of SOD and fasudil after IT administration are 2.24 and 1.49 h, respectively,¹⁹ and the vasodilatory duration of plain drugs is rather short.^{29,38} In contrast, liposomal formulations showed a prolonged vasodilatory effect in previous studies. However, we observed no side effects when plain drugs or formulations were administered every 48 or 72 h, respectively.

In chronic studies, saline treated PAH rats had mPAPs ~50–55 mmHg, after 3 weeks of normoxia. Interestingly, although not a vasodilator, plain SOD, when administered alone, reduced mPAP after chronic administration, perhaps by counteracting high ROS generation and increasing the levels of NO, an endogenous vasodilator and inhibitor of cell proliferation.⁵⁶ When compared with saline treated PAH rats, the reduction in mPAP in plain fasudil or SOD treated groups, which was 39.1% and 27.2%, respectively, was statistically significant (Figure 6A). Treatment with the combination of plain fasudil and SOD resulted in a 48.9% reduction of mPAP, which was greater than either drug alone. Treatment with unmodified liposomal combination also produced a statistically significant and more pronounced reduction (51.9%) in mPAP than that of either drug alone. However, of all the experimental groups, the greatest reduction in mPAP (59.6%) occurred in the CAR-modified-liposome treated group. After 3 weeks of treatment, the mPAP in CAR-modified-liposome treated group reduced to 21.3 ± 2.8 mmHg, which is below the lower threshold of mPAP (25 mmHg) for positive diagnosis of PAH.⁵⁷ This reversal to the normal mPAP can be attributed to the preferential and prolonged action of the CAR-modified liposomes on the diseased vasculature.

We also measured the effect of the formulations in reducing right ventricular hypertrophy in PAH. The RV/LV + S ratio in saline treated PAH was about 2-fold greater than that in healthy animals (Figure 6B). While plain fasudil or plain SOD caused a moderate reduction (16.7% and 37.7%, respectively) in RV/LV + S ratio, when compared with that in the saline treated controls, the combination of plain drugs caused a 6.7% more reduction in RV/LV + S

ratio. Similar to mPAP data in Figure 6A, unmodified and CAR-modified liposomes reduced RV/LV + S ratio by 48.3% and 51.7%, respectively. CAR-modified liposomes containing fasudil plus SOD produced the lowest RV/LV + S ratio (0.31), which was close to that observed in control rats (0.29). However, the differences among the plain combination, unmodified liposomes, and CAR-modified liposomes were not statistically significant, suggesting that these combinations were equally effective in reducing right heart enlargement.¹⁴ The additive effect of two drugs was likely the result of their role as anti-inflammatory, antiproliferative, and antioxidant effects.^{13,18,19,21,28,51,58} Altogether, these data suggest that fasudil and SOD, packed in unmodified or CAR-modified liposomes, prevent hemodynamic deterioration and right ventricular remodeling in PAH, even when administered at a longer dosing interval.

Morphometric Analysis of Lung Sections

Two characteristic features of PAH are the muscularization and endothelial dysfunction of the small pulmonary arterioles that lead to narrowing of the arterioles and increase in pulmonary vascular resistance.⁵⁹ Because development of PAH involves endothelial dysfunction and subsequent proliferation of both endothelial and smooth muscle cells in the pulmonary arteries,^{60–62} we have assessed the expression of vWF, an endothelial cell marker, and α -SMA, a marker for pulmonary arterial smooth muscle cells, to visualize and to quantitate the extent of arterial medial wall thickening in PAH.^{62–64} Counting the number of small muscularized vessels in high power field, we measured the extent of arterial muscularization and the thickening of the endothelial layer, respectively.^{62,65}

The fraction of occluded arteries in saline treated rats was ~ 0.387 , which reduced to ~ 0.127 , a ~ 3 -fold reduction, after administration of CAR-modified-liposomes containing fasudil plus SOD (Figure 7A). Both formulations, administered every 72 h, were more effective than plain drugs given every 48 h, suggesting that the formulations can be administered at a longer dosing interval without compromising the effect on occlusion of arteries, but no statistically significant difference in the fraction of occluded arteries was observed in rats that received either of the drugs alone. The medial wall thickness of the pulmonary arteries of rats treated with the formulations (Figure 7B) is consistent with the representative micrographs of the lung sections (Figure 8).

Thus, these quantitative morphometric analyses and the representative photomicrographs of the PAH afflicted lungs point to the formulation's potency in improving pulmonary vascular remodeling, pulmonary hypertension, and right ventricular hypertrophy. Sustained and selective vasodilation and reduced muscularization, elicited by the CAR-modified liposomes of fasudil and SOD in the hypertensive pulmonary arterioles, may have slowed arterial remodeling process.

The development of PAH also involves the deposition of several extracellular matrix proteins such as collagen fibers, fibronectin, and elastin.^{66,67} From the extent of collagen deposition within the adventitia, we assessed the efficacy of the formulations in reversing pulmonary arterial remodeling. As shown in Figure 9, saline treated rats have extensive collagen deposition along the small pulmonary arteries. However, little or no collagen was deposited in rats treated with plain drugs or formulations. In the rats treated with CAR-liposomes, the

perivascular collagen layer was as thin as that of the vehicle treated control group. In PAH patients, the high pulmonary vascular resistance and hemodynamic stress triggers an increase in collagen synthesis in the diseased arteries.⁶⁸ Moreover, an increase in oxidative stress and hyperactivity of the Rho-kinase pathway augments the severity of the disease and reduces extracellular SOD.^{8,11,13,26} Long-term inhibition of RhoA/ROCK pathway by fasudil has previously been reported to decrease collagen deposition.^{69–71} Overexpression of extracellular SOD reduces lung collagen content and thereby slows pulmonary vascular remodeling.^{16,72} Thus, we believe both fasudil and SOD released from CAR-modified liposomes have contributed to reduced collagen deposition.^{71–73} In addition, the reduction in pulmonary vascular resistance, replenishment of the SOD levels, and subsequent prevention of superoxide mediated NO inactivation by this liposomal combination may have also contributed to reduced collagen synthesis and vascular remodeling.^{16,17,74} However, quantitation of collagen deposition would have given further credence to trichrome staining-based data.

SOD Content and Expression of Biomarkers

Lung SOD levels in PAH rats treated with CAR-liposomes were ~3.5-fold greater than that in saline treated rats, suggesting an increase in antioxidant activity in the lungs (Figure 10A). In fact, extracellular SOD is a major antioxidant in the lung, which catalyzes the dismutation of superoxide anion to oxygen and hydrogen peroxide and protects from damage at cellular levels.^{9,12} These high antioxidant levels in the lungs, as a result of chronic pulmonary administration of SOD in the form of plain drug or in liposomes, may have delayed the progression of PAH in these rats.

Increased pulmonary vascular resistance, a hallmark of PAH, is the result of constitutive activation of Rho-kinase that causes vasoconstriction and lumen obliterating lesions in small pulmonary arteries.⁷⁵ So, we evaluated the expression of biomarkers that are downstream to Rho-kinase and are responsible for Rho-kinase-mediated vasoconstriction and arterial remodeling. Activation of the Rho-kinase pathway inhibits the dephosphorylation of the myosin light chain by myosin phosphatase-1 (MYPT-1). Dephosphorylation of myosin light chain by MYPT-1 initiates smooth muscle relaxation process. Thus, constitutively active Rho-kinase, as observed in case of PAH, favors the inactive and phosphorylated form of MYPT-1 and thus transmits continuous vasoconstrictive response.⁷⁵ STAT-3 is another important biomarker in PAH development, the phosphorylated form of STAT-3 (pSTAT-3) produces proliferative and antiapoptotic phenotype of pulmonary arterial smooth muscle cells (PASMCs).⁷⁶ Role of STAT-3 in smooth muscle proliferation in response to various cytokines, growth factors, and vaso-constrictors has recently been highlighted.⁷⁷ STAT-3 integrates not only upstream signals but also redistributes them downstream to propagate inflammation and downregulate NO synthesis.⁷⁶ The Western blot data of lung samples showed that CAR-liposomes significantly reduced the phosphorylated levels of STAT-3 and MYPT-1 at a reduced dosing frequency (Figures 10B,C). Reduced expression of pMYPT-1 is orchestrated by fasudil, which inhibits Rho-kinase activity, increasing MYPT-1 levels, and reducing smooth muscle contraction.⁷⁸

Although there were no statistically significant differences among various treatment groups in terms of mPAP reduction (Figure 6A), RV hypertrophy (Figure 6B), fraction of occluded arteries and MWT (Figure 7), SOD content, and biomarker expression (Figure 10), liposomal formulations will elicit the same effect as that of plain drugs when administered less frequently. Treatment with CAR-modified liposome would be preferred over unmodified liposome because CAR-modified liposome group showed the highest reduction in mPAP, fraction of occluded vessels, MWT, SOD content, and pMYPT-1/pSTAT3 levels.

CONCLUSION

Overall, our results show that CAR-modified liposomes containing fasudil plus SOD produced hemodynamic relief and prevented PAH progression, even when we administered the formulation at a dosing interval (72 h) longer than that of plain drugs (48 h). Further, targeted formulation reduced the muscularization and adventitial thickening, increased antioxidant levels, and modulated expression of biomarkers such as pSTAT-3 and pMYPT1 to the same extent as those elicited by plain drugs and their combination. However, successful clinical translation of this combination therapy requires thorough evaluation of long-term safety in preclinical settings. Altogether, our preclinical study suggests that SOD plus fasudil may prove to be a novel and promising combination approach for the effective treatment of PAH.

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Figure 1. Hypothetical structure of PEGylated liposomes equipped with the homing peptide, CAR, containing two drugs, fasudil and SOD, in the core of the construct.

Figure 2.
Animal models of PAH: (A) monocrotaline-induced PAH for the acute efficacy study and (B) SUGEN-5416/hypoxia-induced PAH for the chronic efficacy study.

Figure 3.

Treatment protocol for (A) acute efficacy studies in monocrotaline-induced PAH model. (B) chronic efficacy studies in SUGEN-5416/hypoxia-induced PAH model. The doses of super dismutase (SOD) and fasudil were 5 and 3 mg/kg, respectively. In case of formulations, formulations containing 5 mg/kg SOD and 3 mg/kg fasudil were administered.

Figure 4.

Hemodynamic efficacy of (A) sequential intravenous administration of plain SOD and fasudil, (B) sequential intratracheal administration of plain SOD and fasudil, (C) cocktail of intravenous plain fasudil plus SOD, (D) cocktail of intratracheal plain SOD plus fasudil, (E) intratracheal administered unmodified liposomes containing SOD plus fasudil, and (F) intratracheally administered CAR-modified liposomes containing SOD plus fasudil. Data represent mean $\pm$ standard deviation ($n = 3-4$). * $p < 0.05$, § $p < 0.005$, Z $p < 0.001$, ¥ $p < 0.0001$.

Figure 5.

(A) Duration of vasodilatory effects and (B) lung targeting indices after administration of plain combination of SOD plus fasudil, unmodified liposomes, or CAR-modified liposomes containing SOD plus fasudil to MCT-induced PAH rats. Data represent mean $\pm$ standard deviation ($n = 3-4$).

Figure 6. Efficacy of plain drugs or formulations in reducing (A) mean pulmonary arterial pressure (mPAP) and (B) right ventricular hypertrophy in SU5416/hypoxia-induced PAH rats. Data represent mean $\pm$ standard deviation ($n = 3-4$).

Figure 7. Efficacy of plain drugs or formulations in reducing (A) fractions of occluded blood vessels and (B) arterial medial wall thickness in SU5416/hypoxia-induced PAH rats. Data represent mean $\pm$ standard deviation ($n = 3-4$).

Figure 8. Representative photomicrographs of pulmonary arteries stained with α -SMA and counterstained with hematoxylin for the determination of muscularization and medial wall thickness upon chronic administration of plain drugs or combination of formulations in SU5416/hypoxia-induced PAH rats. About 15 images were analyzed for each lung ($n = 3-4$ animals).

Figure 9. Chronic efficacy of plain drugs or combination or formulations in reducing pulmonary arterial adventitial collagen (blue) deposition in SU5416/hypoxia-induced PAH rats. About fifteen images were analyzed for each lung ($n = 3-4$ animals).

Figure 10.

Influence of chronic administration of plain drugs or combination or formulations on (A) total SOD content, (B) pSTAT-3 expression, and (C) pMYPT-1 expression in the lungs of SU-5146/hypoxia-induced PAH rats. Total SOD concentration was determined by a commercial kit, and levels of pSTAT-3 and pMYPT-1 were analyzed by Western blot. Data represent mean $\pm$ standard deviation ($n = 3-4$).