Encapsulation of beraprost sodium in nanoparticles: analysis of sustained release properties, targeting abilities and pharmacological activities in animal models of pulmonary arterial hypertension

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Prostaglandin I₂ (PGI₂) and its analogues (such as beraprost sodium, BPS) are beneficial for the treatment of pulmonary arterial hypertension (PAH). The encapsulation of BPS in nanoparticles to provide sustained release and targeting abilities would improve both the therapeutic effect of BPS on PAH and the quality of life of patients treated with this drug.

BPS was encapsulated into nanoparticles prepared from a poly(lactic acid) homopolymer and monomethoxy poly(ethyleneglycol)-poly(lactide) block copolymer. The accumulation of nanoparticles in damaged pulmonary arteries was examined using fluorescence-emitting rhodamine S-encapsulated nanoparticles. The monocrotaline-induced PAH rat model and the hypoxia-induced mouse model were used to examine the pharmacological activity of BPS-encapsulated nanoparticles.

A nanoparticle, named BPS-NP, was selected among various types of BPS-encapsulated nanoparticles tested; this was based on the sustained release profile *in vitro* and blood clearance profile *in vivo*. Fluorescence-emitting rhodamine S-encapsulated nanoparticles were prepared in a similar manner to that of BPS-NP, and showed accumulation and prolonged residence in monocrotaline-damaged pulmonary peripheral arteries. Intravenous administration of BPS-NP (once per week, 20 µg/kg) protected against monocrotaline-induced pulmonary arterial remodeling and right ventricular hypertrophy. The extent of this protection was similar to that observed with oral administration (once per day, 100 µg/kg) of BPS alone. The once per week intravenous administration of BPS-NP (20 µg/kg) also exhibited an ameliorative effect on hypoxia-induced pulmonary arterial remodeling and right ventricular hypertrophy.

The beneficial effects of BPS-NP on PAH animal models seem to be mediated by its sustained release and tissue targeting profiles. BPS-NP may be useful for the treatment of PAH patients due to reduced dosages and frequency of BPS administration.

KEY WORDS: prostaglandin I₂; encapsulation; biodegradable nanoparticle; beraprost sodium; pulmonary arterial hypertension.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a rare but life-threatening disease characterised by progressive increases in pulmonary vascular resistance, vasoconstriction and arterial pressure, which lead to right ventricular (RV) hypertrophy and failure, and subsequently to death [1-3]. Although several classes of drugs have been approved for the treatment of PAH (such as endothelin receptor antagonists and phosphodiesterase-5 inhibitors), the prognosis for this disease remains poor (median survival, 2.8 years) [4, 5]. While the primary cause of PAH is unclear, it is believed that pulmonary vasoconstriction, endothelial cell proliferation, inflammation, smooth muscle cell proliferation, and thrombosis play key roles in the pathogenesis of PAH [1, 2, 6].

The progression of PAH is associated with a reduced production of prostaglandin I_2 (PGI_2) [7, 8], a potent vasodilator produced in the vascular endothelium, which also has antithrombotic effects and inhibitory effects on smooth muscle cell proliferation [5, 9]. Thus, PGI_2 and its analogues could be therapeutically beneficial for the treatment of PAH in affected patients [5, 10-16]. In fact, the intravenous administration of epoprostenol (PGI_2) is currently the most effective approach for the treatment of PAH, not only because it can ameliorate symptoms of severe PAH, but because it also prolongs life expectancy [16, 17]. One major drawback of PGI_2 is that it is very unstable in blood [18, 19], meaning that epoprostenol must be intravenously infused on a continuous basis, thereby drastically decreasing the quality of life (QOL) of patients due to problems associated with cosmetic appearance, transportation of an infusion pump and risk of infection [14, 20]. For these reasons, stable PGI_2 analogues such as beraprost sodium (BPS) formulated for various routes of administration (oral, subcutaneous and inhalation) have been developed [5, 18]. However, the half-life of these PGI_2 analogues in the blood remains short and their therapeutic efficacy in PAH patients is inferior to that of continuously infused epoprostenol [18, 21]. For example, the half-life of BPS in blood is less than 40 min following its oral administration, and it is not effective in the treatment of patients with severe PAH, even with frequent administration [5, 22, 23]. Therefore, a drug delivery system (DDS) that enables the sustained release of intravenously administered PGI_2 or its analogues would be clinically beneficial. On the other hand, various physiological activities of PGI_2 are related to adverse effects (such as hypotension) due to its whole-body distribution when administered systemically [24]. Furthermore, adverse events, such as headache, jaw pain, vasodilation, nausea, diarrhea,

leg pain, and foot pain were observed in patients treated with BPS [23, 25]. Therefore, a DDS that enables the specific targeting of PGI₂ or its analogues to the damaged pulmonary arteries is also important.

We recently established a new DDS that enables both the sustained release and specific targeting of encapsulated drugs [26-30]. By employing a nanotechnology approach, drugs are encapsulated in solid nanoparticles prepared from biodegradable and biocompatible poly(lactide) homopolymer (PLA) [31, 32]. This type of nanoparticle can be used to achieve the sustained release of encapsulated drug, which takes place concomitantly with the degradation of PLA [28, 31, 32]. To avoid the uptake of these nanoparticles by the mononuclear phagocyte system [32, 33], a monomethoxy poly(ethyleneglycol)-poly(lactide) block copolymer (PEG-PLA) was used in combination with PLA. Furthermore, the encapsulation of drug compounds in small-diameter nanoparticles (50-150 nm) would enhance the selective delivery of the drug to damaged blood vessels due to the enhanced permeability and retention (EPR) effect [34].

In the present study, we succeeded in encapsulating BPS into this type of nanoparticle (BPS-NP). BPS-NP showed a sustained release profile *in vitro* and a prolonged residence in the blood and accumulation in damaged pulmonary peripheral arteries *in vivo*. Compared to the oral administration of BPS alone, the intravenous administration of BPS-NP in animal models of PAH showed ameliorative effects even at lower doses and frequencies of administration. These results suggest that BPS-NP could serve as a useful option for the treatment of PAH patients.

MATERIALS AND METHODS

Materials and animals

BPS, monocrotaline, pentobarbital, fluorescein isothiocyanate (FITC)-labeled albumin and fetal bovine serum (FBS) were obtained from Sigma (St. Louis, MO). D,L-PLA (MW = 5,000 or 20,000), 1,4-dioxane, iron chloride and rhodamine S (RS) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-PLA (MW = 5,000 or 20,000) was from Taki Chemical Co., Ltd. (Kakogawa, Japan). PEG-D,L-PLA (average molecular weight of PEG and PLA are 5,600 and 9,200, respectively) or PEG-L-PLA (average molecular weight of PEG and PLA are 5,600 and 9,400, respectively) was synthesized and evaluated as described previously [28, 35]. Diethanolamine (DEA) was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Wistar rats (5 weeks old, male) and C57/BL6 mice (8 weeks old, male) were from Kyudo Co., Ltd. (Kumamoto, Japan) or Charles River Laboratories Inc. (Yokohama, Japan). The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and were approved by the Animal Care Committee of Kumamoto University and Keio University.

Determination of BPS and RS levels in samples

BPS and RS levels in samples were determined by analytical high-performance liquid chromatography (HPLC) with a reverse-phase column (L-column ODS (150 x 4.6 mm, 5 μ m, Chemicals evaluation research institute, Tokyo, Japan) or Eclipse XDB-C18 column (150 x 2.1 mm, 5 μ m, Agilent Technologies, Santa Clara, CA)). A Waters 2695 Alliance separation module, a Waters 2996 photodiode array detector, and a Waters Alliance system running Empower software (Waters, Milford, MA) were used for HPLC analysis.

For detection of BPS, solvent A (0.1% acetic acid in Methanol) and solvent B (0.1% acetic acid in Milli-Q water) were used at a flow rate of 0.5 ml/min. After injection of the sample (0 min), the mobile phase was changed as follows; 70% solvent A (20 min), a linear gradient of 70-100% solvent A (2 min), 100% solvent A (11 min) and a linear gradient of 100-70% solvent A (2 min). Detection was performed at an optical density of 285 nm.

For detection of RS, solvent A (0.1% acetic acid in acetonitrile) and solvent B (0.1% acetic acid in Milli-Q water) were used at a flow rate of 0.05 ml/min. After injection of the sample, 80% solvent A was used as a mobile phase in isocratic elution mode for 30 min. Detection was performed at an optical density of 530 nm.

Analysis of solubility of BPS and RS

BPS or RS (0.56 mM) was mixed with DEA (various concentrations) and iron chloride (10 mM) in ethanol. After incubation for 30 min at 25°C, the pH value of the solutions/suspensions was measured and they were then centrifuged at 13,000 rpm for 10 min to precipitate insoluble BPS or RS. The amount of BPS or RS in the supernatant was determined by analytical HPLC as mentioned above.

Preparation and characterization of nanoparticles

Nanoparticles were prepared by using the oil-in-water solvent diffusion method as described previously [27, 28]. We named nanoparticle types based on the chirality (L or DL) of PLA and PEG-PLA and the molecular weight of PLA (Table 1).

For preparation of L-L20, L-DL20, DL-L20 or DL-DL20, 26 mg of L-PLA or D,L-PLA in 300 µl of 1,4-dioxane, 24 mg of PEG-L-PLA in 300 µl of 1,4-dioxane or PEG-D,L-PLA in 300 µl of acetone, 2.5 mg of BPS in a mixture of 50 µl of methanol, 150 µl of acetone and 500 µl of 1,4-dioxane, DEA (4.75 mg) in 200 µl of acetone and iron chloride (2.43 mg) in 30 µl of acetone were mixed.

For preparation of L-L5, L-DL5, DL-L5 or DL-DL5, 26 mg of L-PLA or D,L-PLA in 300 µl of 1,4-dioxane, 24 mg of PEG-L-PLA in 300 µl of 1,4-dioxane or PEG-D,L-PLA in 300 µl of acetone, 2.5 mg of BPS in a mixture of 50 µl of methanol and 150 µl of acetone, DEA (4.75 mg) in 200 µl of acetone and iron chloride (2.43 mg) in 30 µl of acetone were mixed.

For preparation of RS-NP, 26 mg of D,L-PLA in 300 µl of 1,4-dioxane, 24 mg of PEG-L-PLA in 300 µl of 1,4-dioxane, 2.5 mg of RS in 500 µl of ethanol, DEA (9.5 mg) in 200 µl of acetone and iron chloride (2.43 mg) in 30 µl of acetone were mixed.

Samples described above were incubated for 10 min at room temperature and added to 25 ml of Milli-Q water stirred at 1000 rpm. After addition of 2.5 ml of 0.5 M EDTA (pH 7.0) and 12.5 µl of 200 mg/ml Tween 80, nanoparticles were purified and concentrated by centriprep YM-50 (Merck Millipore, Billerica, MA).

For determination of the BPS or RS content in nanoparticles, the nanoparticles were degraded by incubation with 50 mM EDTA (pH 7.0) and 1,4-dioxane (final concentration, 80%). After centrifugation, the amount of BPS or RS in the supernatant was determined by analytical HPLC as mentioned above. The drug content was defined as the ratio of BPS or RS weight to the total weight of nanoparticles.

Particle size distribution was determined by the dynamic light scattering method (ZETASIZER Nano-ZS, Malvern Instruments Ltd., Malvern, UK) and the average diameter was calculated by Marquadt's method.

BPS release from nanoparticles *in vitro*

BPS-encapsulated nanoparticles were dispersed in a 50% (v/v) solution of FBS in phosphate-buffered saline (PBS). After incubation at 37°C for the specified period, each suspension (100 µl) was diluted with 900 µl of 50 mM EDTA solution (pH7.0) and centrifuged at 35,000 rpm for 30 min. The BPS content in the precipitate was determined as described above.

Determination of BPS- or RS-encapsulated PLA-nanoparticles in blood

BPS-encapsulated nanoparticles were intravenously administered to animals via the tail vein (2 ml/kg). Blood samples were periodically collected from the tail vein into heparin-treated capillary tubes. Each sample (50 µl) was mixed with 150 µl of 1,4-dioxane. After centrifugation (13,000 rpm for 30 min), the BPS content of the supernatant was determined by analytical HPLC as described above.

Accumulation of BPS- or RS-encapsulated nanoparticles *in vivo*

Wistar rats anesthetized with pentobarbital were subcutaneously administered either monocrotaline (30 or 60 mg/kg) or saline once only. Two to three weeks after the administration, BPS, BPS-encapsulated nanoparticles, RS or RS-encapsulated nanoparticles were intravenously administered into the tail vein (2 ml/kg).

For determination of RS, lung homogenates were prepared and mixed with 1,4-dioxane (4 ml/g tissue). After centrifugation (13,000 rpm for 30 min), RS fluorescence intensity of the supernatant was determined by fluorescence spectrophotometry (Infinite 200 PRO, Tecan Group Ltd., Männedorf, Switzerland) at emission and excitation wavelengths of 550 nm and 580 nm, respectively.

For determination of BPS, lung homogenates were prepared in PBS (1 ml/g tissue) and mixed with 1,4-dioxane (4 ml/g tissue) and PBS (3 ml/g tissue). After centrifugation (15,000 rpm for 10 min), the BPS content of the supernatant was determined by analytical HPLC as described above.

For detection of RS, 24 h after the administration of RS or RS-encapsulated nanoparticles, lung sections were prepared and stained with Mayer's haematoxylin followed by 1% eosin alcohol solution (H & E staining). Samples were mounted with malinol and inspected with the aid of an Olympus BX51 microscope.

Assessment of vascular permeability in animal models of PAH

Vascular permeability assay was performed as described previously [36] with some modifications.

For rats, 3 weeks after the monocrotaline (60 mg/kg) administration, FITC-labeled albumin (10 mg/kg) was intravenously administered into the tail vein (2 ml/kg). Three hours after the administration, rats were killed and perfused with PBS. Lung homogenates were prepared in PBS (5 ml/g tissue).

For mice, after the hypoxia exposure (6 weeks), FITC-labeled albumin (50 mg/kg) was intravenously administered into the tail vein (10 ml/kg). Three hours after the administration, mice were killed and perfused with PBS. Lung homogenates were prepared in 500 µl of PBS.

After centrifugation (15,000 rpm for 10 min), FITC fluorescence intensity of the supernatant was determined by fluorescence spectrophotometry (Infinite 200 PRO) at emission and excitation wavelengths of 488 nm and 530 nm, respectively.

Development of animal models of PAH

Wistar rats anesthetized with pentobarbital were subcutaneously administered either monocrotaline (60 mg/kg) or saline once only at day 0. BPS-encapsulated nanoparticles or BPS alone were intravenously administered via the tail vein (2 ml/kg) once per week or orally administered (2 ml/kg) once per day, respectively. Pulmonary arterial remodeling and RV hypertrophy were assessed at day 21 as described below.

C57/BL6 mice were kept in a tightly sealed chamber under hypoxic (10% O₂) or normal (20% O₂) conditions for 7 weeks. The status of the hypoxia chamber was maintained using an hypoxic air generator (TEIJIN, Tokyo, Japan) and monitored with an

O₂ analyser (JIKO-25SIIR, JIKCO LTD., Tokyo, Japan) as described previously [37]. BPS-encapsulated nanoparticles were intravenously administered once per week via the tail vein (2 ml/kg). Pulmonary arterial remodeling and RV hypertrophy were assessed at week 7 as described below.

Assessment of pulmonary arterial remodeling and RV hypertrophy

Pulmonary arterial remodeling was assessed based on the wall thickness of the pulmonary peripheral arteries as described previously [38]. Lung tissue samples were fixed in 10% formalin neutral buffer solution for 24 h and embedded in paraffin before being cut into 4 µm-thick sections. Sections were subjected to Elastica van Gieson staining and inspected with the aid of an Olympus BX51 microscope (Olympus, Tokyo, Japan). The external diameter and medial wall thickness were measured for 10 peripheral arteries (rat, 50-200 µm external diameter; mouse, 25-100 µm external diameter) for each lung section and the average value was calculated. The relative medial wall thickness (% wall thickness) was calculated as follows: $[\text{medial wall thickness} \times 2] / [\text{external diameter} \times 100]$.

RV hypertrophy was assessed by the relative weight of the RV [38]. After animals had been sacrificed, RV was separated from the left ventricle (LV) and ventricular septum (S), and each of them was weighed. RV hypertrophy was calculated as follows: $[\text{RV} / (\text{LV} + \text{S})]$.

Statistical analysis

All values are expressed as the mean $\pm$ standard error of the mean (S.E.M.). One-way ANOVA followed by the Tukey test or the Student's *t*-test for unpaired results were used to evaluate differences between three or more groups or between two groups, respectively, which were considered to be significant for values of $P < 0.05$.

RESULTS

Preparation of BPS-encapsulated PLA-nanoparticles

We recently reported that both the molecular weight and chirality (racemic or optically active) of PLA are important factors for determining the stability of PLA-nanoparticles [28]. In order to obtain an appropriate sustained release profile for BPS and a prolonged residence time in the blood, we prepared various types of BPS-encapsulated nanoparticles by using different combinations of PLA (L-PLA (MW = 20,000), L-PLA (MW = 5,000), D,L-PLA (MW = 20,000) or D,L-PLA (MW = 5,000)) and PEG-PLA (PEG-L-PLA or PEG-D,L-PLA) (Table 1). In relation to PGE₁, which has a carboxyl group, we recently reported that insolubilization of PGE₁ by iron chloride under appropriate pH conditions is essential for its encapsulation in PLA-nanoparticles [26, 28]. Since BPS also has a carboxyl group, we used this strategy to encapsulate BPS in PLA-nanoparticles. Figure 1A shows the solubility of BPS in aqueous solutions with or without iron chloride under various conditions of pH, which was adjusted by the addition of DEA. In the presence of iron chloride, the residual amount of BPS in the supernatant after centrifugation varied with changing pH values (Fig. 1A). On the other hand, in the absence of iron chloride, the residual amount of BPS was not affected by the pH value (Fig. 1A). We used conditions that achieved maximum insolubilization of BPS (30 mM DEA and 10 mM iron chloride) for the encapsulation of BPS in PLA-nanoparticles.

Figure 1

We prepared the BPS-encapsulated nanoparticles from PLA and PEG-PLA by the solvent diffusion method [27, 28]. We confirmed that the conditions of 30 mM DEA that achieved the lowest solubility of BPS (Fig. 1A) is the optimum condition for efficient encapsulation of BPS in nanoparticles (supplemental Fig. S1). Figure 2A shows the size distributions of representative BPS-encapsulated PLA-nanoparticles (BPS-NP, see below). The average particle size was similar between different types of BPS-encapsulated PLA-nanoparticles (Table 1). The efficiency of encapsulation (BPS content in nanoparticles) was also determined (Table 1).

Figure 2

A

B

Stability of BPS-encapsulated nanoparticles *in vitro* and *in vivo*

The *in vitro* release profile of BPS from each PLA-nanoparticle type was examined in a 50% v/v solution of FBS in PBS at 37°C, with release determined by measuring the amount of BPS remaining in the nanoparticles. When PEG-D,L-PLA was used (left panel in Fig. 3), a faster release profile of BPS was observed in nanoparticles prepared from lower molecular weight PLA. Furthermore, nanoparticles prepared from D,L-PLA showed the fastest release profile of BPS with respect to those prepared from L-PLA (left panel in Fig. 3). These phenomena are consistent with what was observed for PGE₁-encapsulated nanoparticles in our previous studies [28, 29]. We also found here that similar phenomena are observed for nanoparticles prepared from PEG-L-PLA (right

panel in Fig. 3), and that the use of PEG-L-PLA instead of PEG-D,L-PLA stimulates or suppresses the release, depending on the PLA species used (Fig. 3).

Figure 3

We next examined the residence time of BPS in the blood (blood clearance profile) for each type of BPS-encapsulated PLA-nanoparticles; rats were intravenously administered nanoparticles of each type and the blood clearance profile was investigated by measuring the change in plasma concentration of BPS. To begin with, we confirmed that intravenously administered BPS alone was rapidly cleared from the blood (Fig. 4A), as reported previously [39]. For nanoparticles prepared from PEG-D,L-PLA (left panel in Fig. 4B), the clearance of BPS became faster as the molecular weight of the PLA used in the nanoparticles was reduced. Moreover, nanoparticles prepared from D,L-PLA exhibited faster BPS clearance times than those prepared from L-PLA (left panel in Fig. 4B). The use of PEG-L-PLA instead of PEG-D,L-PLA stimulates or suppresses the BPS release, depending on the PLA species used (Fig. 4B). Based on these results, we used BPS-encapsulated nanoparticles prepared from a combination of D,L-PLA ($M_w = 20,000$) and PEG-L-PLA (i.e., L-DL20) and subsequently refer to this type of BPS-encapsulated PLA-nanoparticles as “BPS-NP”.

Figure 4

Accumulation of BPS-NP-like nanoparticles in monocrotaline-damaged pulmonary peripheral arteries

To examine the distribution of BPS-NP in the PAH rat model (monocrotaline-administered rats), we prepared BPS-NP-like fluorescent RS-encapsulated nanoparticles. As shown in Fig. 1B, the solubility of RS decreased in a pH-dependent manner in the presence of iron chloride. In this way, we prepared RS-encapsulated nanoparticles from D,L-PLA (MW = 20,000) and PEG-L-PLA under similar conditions to those used in the preparation of BPS-NP and named the BPS-NP-like RS-encapsulated PLA-nanoparticles as “RS-NP”. The size distribution and efficiency of encapsulation of RS-NP were roughly similar to those of BPS-NP (Fig. 2 and Table 1).

We next compared the accumulation of RS in the pulmonary peripheral arteries after administration of RS-NP or RS alone in control and monocrotaline-administered rats. RS-NP or RS alone was intravenously administered 21 days after the administration of monocrotaline and the level of RS in the lung was monitored 1 day later by histopathological analysis (H & E staining). Pulmonary arterial remodeling (hypertrophy of pulmonary peripheral arterial walls) was clearly evident in monocrotaline-administered rats compared with control (Fig. 5A). Analysis of fluorescence patterns in micrographs showed RS accumulation in the pulmonary peripheral arteries of monocrotaline-treated rats administered RS-NP but not in other samples (Fig. 5A).

We also compared the amount of RS in the lung between monocrotaline-administered rats and control rats after the intravenous administration of RS-NP. As shown in Fig. 5B, compared to control rats, a higher level of RS in the lungs of monocrotaline-treated rats was detected from 1 to 5 days after the administration of RS-NP. These results suggest that RS-NP accumulates more efficiently and remains for longer in monocrotaline-damaged pulmonary peripheral arteries compared with that seen in those in control.

To test the idea that the accumulation of RS-NP shown in Fig. 5A and B is mediated through EPR effect, we compared the vascular permeability between monocrotaline-treated and control rats using FITC-labeled albumin. As shown in Fig. 5C, the level of FITC-labeled albumin at the lung of monocrotaline-treated rats was about 10 times higher than that in normal rats and the extent of accumulation is comparable to that seen in tumors in previous papers [40]. Such accumulation at the lung was also observed in mice exposed to hypoxia, however, the extent of accumulation was not so apparent as that seen in monocrotaline-treated rats (Fig. 5D). We also examined the accumulation of BPS at the lung of monocrotaline-treated rats after intravenous administration of BPS-NP or free BPS. As shown in Fig. 5E, the higher level of BPS at the lung of monocrotaline-treated rats, compared to control rats, was observed after administration of BPS-NP. On the other hand, we could not detect BPS at the lung after administration of free BPS even in monocrotaline-treated rats (Fig. 5E). Results in Fig. 5 suggest that BPS-NP accumulates more efficiently in monocrotaline-damaged pulmonary peripheral arteries compared with that seen in those in control rats through the EPR effect.

Figure 5

Pharmacological activity of BPS-NP in PAH animal models

We subsequently examined the effect of BPS-NP on monocrotaline-induced pulmonary arterial remodeling and RV hypertrophy. To begin with, we confirmed the ameliorative effect of the oral administration of BPS in this model. Rats were treated with monocrotaline once only at day 0 and BPS was orally administered once per day for 21 days. The pulmonary peripheral arteries were analysed on day 21. As shown in Fig. 6A, representative photomicrographs demonstrate that the monocrotaline-induced hypertrophy of arterial walls was suppressed by the BPS administration (100 $\mu\text{g}/\text{kg}$). Quantitative analysis revealed that the monocrotaline administration increased the

arterial wall thickness and that this change was suppressed by the oral administration of BPS (100 $\mu\text{g/kg}$) (Fig. 6B).

Monocrotaline-induced RV hypertrophy can be measured by weighing the RV, LV and S as previously described [41]. As shown in Fig. 6C, the administration of monocrotaline increased the ratio of the RV to (LV + S) weight, thus demonstrating RV hypertrophy; the simultaneous oral administration of BPS (30 or 100 $\mu\text{g/kg}$) suppressed this increase. We confirmed that once per week oral administration of BPS (up to 100 $\mu\text{g/kg}$) did not affect the monocrotaline-induced pulmonary arterial remodeling and RV hypertrophy (data not shown). These results are all consistent with previously reported findings [42].

Figure 6

We subsequently examined the effect of the intravenous administration of BPS-NP on monocrotaline-induced pulmonary arterial remodeling and RV hypertrophy in a similar way. As shown in Fig. 7, the intravenous administration of BPS-NP (20 or 200 μg BPS/kg) reduced the extent of monocrotaline-induced pulmonary arterial remodeling and RV hypertrophy even when the administration frequency was restricted to once per week. Intravenous administration of BPS-NP (once per week, 2 $\mu\text{g}/\text{kg}$) could not protect against monocrotaline-induced pulmonary arterial remodeling and right ventricular hypertrophy (supplemental Fig. S2). These results suggest that the encapsulation of BPS into PLA-nanoparticles can permit a reduction in the dose and frequency of BPS required to exert ameliorative effects against monocrotaline-induced disorders.

Figure 7

Hypoxia-induced pulmonary arterial remodeling and RV hypertrophy in mice forms the basis of another animal model of PAH, which we also used here to examine the effects of BPS-NP. As shown in Fig. 8, maintaining mice under hypoxic

conditions (10% O₂) for 7 weeks induced hypertrophy of the pulmonary peripheral arterial wall and RV hypertrophy, as described previously [37]. The intravenous administration of BPS-NP (20 or 200 µg BPS/kg, once per week) exerted ameliorative effects against these hypoxia-induced disorders (Fig. 8).

Figure 8

DISCUSSION

The underlying basis of DDS can be separated into three categories; targeting of lesioned tissues, sustained release, and increasing the efficiency of gastrointestinal absorption. Although a number of DDS carriers have been developed, most of those are developed to satisfy only one of these objectives. In contrast, we recently developed a new type of DDS, PEG-modified PLA-nanoparticles, that provides both sustained release and targeting abilities [26-30]. On the other hand, DDS carriers for the intravenous administration of PGI₂ and its analogues require both sustained release and targeting abilities, because frequent intravenous administration reduces the QOL of patients and the whole-body distribution of PGI₂ following its systemic administration results in adverse effects (see introduction section). Thus, we postulated that encapsulation of BPS in PEG-modified PLA-nanoparticles would provide a beneficial treatment option for PAH patients, for which reason we tested this idea here using two animal models.

A major obstacle to preparing drug-encapsulated PLA-nanoparticles is that relatively hydrophilic drugs, such as PGE₁ and BPS, are very hard to encapsulate in hydrophobic PLA-nanoparticles [43]. For PGE₁, we overcame this obstacle by taking advantage of its insolubilization in the presence of iron chloride under appropriate pH conditions [28, 29]. We used this same strategy here to insolubilize BPS and succeeded in encapsulating it into PLA-nanoparticles. This finding suggests that BPS is insolubilized by the formation of electrostatic complexes between the carboxyl group in the BPS and iron chloride, along with deprotonation of the carboxyl group and that ionic interaction through a metal bridge is a driving force to encapsulate ionizable BPS in solid PLA nanoparticles, in other words, iron colloid acted as an ionic bridge between the terminal carboxyl group of the PLA chain and the carboxyl group of BPS (Fig. 9).

Figure 9

Various combinations of PEG-PLA and PLA were tested to optimize the sustained release profiles of BPS *in vitro* and its residence period in blood *in vivo*. The results obtained suggested that a faster release profile and a faster clearance from blood could be achieved with nanoparticles prepared from low molecular weight PLA. Furthermore, nanoparticles prepared from D,L-PLA showed a faster release profile and clearance from blood than those prepared from L-PLA. These observations were similar to those reported for PGE₁-encapsulated PLA-nanoparticles [28, 29]. The mechanism by which nanoparticles containing D,L-PLA show a faster release profile and clearance from blood than those prepared only from L-PLA is unclear at present, because the release rate of drugs is regulated by complicated factors such as the thermodynamic stability, kinetic stability and degradation rate of the nanoparticles. Racemic D,L-PLA and isotactic L-PLA have different physicochemical properties [44]. For example, D,L-PLA is an amorphous polymer, but L-PLA is semi-crystalline [44]. The glass-transition temperature of L-PLA is higher than that of D,L-PLA, and hydrolysis of L-PLA is slower than D,L-PLA [44]. We consider that these differences may be involved in the mechanism by which nanoparticles containing D,L-PLA show a faster release profile and clearance from blood than those prepared only from L-PLA.

We also found here that, following monocrotaline treatment, accumulation of RS in the monocrotaline-damaged pulmonary peripheral arteries was observed in rats administered RS-NP, but not in those administered RS alone. We also observed the higher level of accumulation of FITC-labeled albumin at the lung in monocrotaline-treated rats, compared to control rats. These results suggest that RS-NP is

selectively delivered to monocrotaline-damaged arteries, probably as a consequence of the EPR effect. It was reported that inflammation was induced in pulmonary peripheral arteries not only in monocrotaline- or hypoxia-induced PAH animal models, but also in PAH patients [45]. Thus, the results of this study suggest that intravenously administered BPS-NP is likely to be selectively delivered to damaged arteries in PAH patients. This treatment approach would enable potent therapeutic effects to be obtained with a low associated risk of adverse effects.

Based on the ameliorative effects of BPS on monocrotaline-induced pulmonary arterial remodeling and RV hypertrophy, we compared the pharmacological activity of intravenously administered BPS-NP and orally administered BPS alone. The extent of amelioration with a once-weekly intravenous administration of BPS-NP (20 µg BPS/kg) was roughly similar to that obtained with daily oral administration of BPS alone (100 µg/kg). These results suggest that encapsulation of BPS into PLA-nanoparticles enables the effective dose of BPS to be decreased and a lower frequency of administration to be used. This lower targeted dose of BPS would be highly advantageous because it would avoid the adverse effects associated with the systemic administration of PGI₂ analogues. Since PAH has various causes, it is important to determine the effects of the candidate drug in numerous animal models. As such, the observed ameliorative effects of BPS-NP in the hypoxia-induced PAH mouse model add further value to its therapeutic potential in PAH patients.

Although various types of orally administered PGI₂ analogues are available, their efficacy in PAH patients is inferior to that obtained with continuous intravenous infusion of PGI₂. This would appear to be due to the fact that maintenance of a high concentration of PGI₂ around damaged pulmonary arteries is important to achieve its ameliorative effect. The findings of the present study suggest that BPS-NP is targeted to damaged pulmonary arteries and remains there for at least 5 days. Thus, if a similar scenario can be achieved in PAH patients, intravenously administered BPS-NP could be used to maintain high concentrations of BPS around damaged arteries and thus achieve therapeutic efficacy and improved QOL in PAH patients based on a lower frequency of administration.

CONCLUSION

Based on the results of this study, we propose that that improved sustained release properties and targeting abilities achieved with BPS-NP may be therapeutically beneficial for PAH patients as a consequence of the decreased BPS dose and frequency of administration that can be employed.

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TABLE

Table. 1. Characterization of BPS- or RS-encapsulated nanoparticles.

Nanoparticles encapsulated with BPS (A) or RS (B) were prepared by an oil-in-water solvent diffusion method. Particle size and BPS (A) or RS (B) content in the particles were determined by the dynamic light scattering method and HPLC, respectively. Values are mean $\pm$ S.E.M. ($n=3$).

A

NP code	PLA-PEG	PLA (Mw, kDa)	Diameter (nm)	BPS content (%)
L-L20	L-PLA-PEG	L-PLA ₍₂₀₎	121 $\pm$ 2	1.23 $\pm$ 0.02
L-DL20 (BPS-NP)	L-PLA-PEG	DL-PLA ₍₂₀₎	128 $\pm$ 3	1.08 $\pm$ 0.04
L-L5	L-PLA-PEG	L-PLA ₍₅₎	109 $\pm$ 2	0.86 $\pm$ 0.03
L-DL5	L-PLA-PEG	DL-PLA ₍₅₎	112 $\pm$ 1	0.88 $\pm$ 0.02
DL-L20	DL-PLA-PEG	L-PLA ₍₂₀₎	112 $\pm$ 3	1.15 $\pm$ 0.08
DL-DL20	DL-PLA-PEG	DL-PLA ₍₂₀₎	125 $\pm$ 3	0.86 $\pm$ 0.06
DL-L5	DL-PLA-PEG	L-PLA ₍₅₎	108 $\pm$ 2	2.39 $\pm$ 0.09
DL-DL5	DL-PLA-PEG	DL-PLA ₍₅₎	107 $\pm$ 1	2.16 $\pm$ 0.05

B

NP code	PLA-PEG	PLA (Mw, kDa)	Diameter (nm)	RS content (%)
RS-NP	L-PLA-PEG	DL-PLA ₍₂₀₎	107 $\pm$ 1	1.65 $\pm$ 0.04

FIGURE LEGENDS

Fig.1. Solubility of BPS and RS in aqueous solution.

An ethanol solution of BPS (A) or RS (B) was added to a 9-fold excess volume of solution containing 10 mM iron chloride (+FeCl₃) or 10 mM hydrochloride (-FeCl₃) at different pH values obtained by the addition of DEA. After centrifugation, residual BPS (A) or RS (B) in the supernatant was determined by analytical HPLC.

Fig.2. Size distribution of BPS-NP and RS-NP.

BPS-NP (A) and RS-NP (B) particle sizes were determined by the dynamic light scattering method. Values are mean $\pm$ S.E.M. ($n=3$).

Fig. 3. *In vitro* release profile of BPS from nanoparticles.

Each type of BSP-encapsulated nanoparticles was dispersed in 50% FBS in PBS and incubated at 37°C for the indicated number of days. The amount of BPS remaining in nanoparticles (NP) was determined by analytical HPLC. Values are mean $\pm$ S.E.M. ($n=3$).

Fig. 4. *In vivo* blood clearance profile of BPS alone and BSP-encapsulated nanoparticles.

BPS alone (A) or each type of BSP-encapsulated nanoparticles (B) was administered intravenously at the dose of 1.25 mg BPS/kg. BSP-encapsulated nanoparticles were degraded by 1,4-dioxane (B) and the amount of BPS in plasma was determined by analytical HPLC. Values are mean $\pm$ S.E.M. ($n=3$).

Fig. 5. Accumulation and prolonged residence of RS-NP and BPS-NP in the lung.

Rats were treated with monocrotaline (MCT; 30 mg/kg (A, B) or 60 mg/kg (C, E)) or saline (CTRL) once only on day 0. Mice were maintained in a hypoxic chamber (10% O₂, Hypoxia) or under normal conditions (CTRL) (D). On day 21 (A) or 14 (B), RS-NP (A, B) or RS (A) was injected intravenously at a dose of 0.6 mg RS/kg. On day 21 (C) or 45 (D), FITC-labeled albumin was injected intravenously at a dose of 10 (C) or 50 (D) mg/kg. On day 18, BPS alone or BPS-NP was injected intravenously at a dose of 0.9 mg BPS/kg (E). Lung sections were prepared 1 day after the injection of RS-NP or RS and subjected to histopathological examination (H & E staining and fluorescence microscopy) (A). The amounts of RS (B) or FITC (C, D) in the lung homogenates were determined by measuring the emission fluorescence intensity at 580 nm (excitation at 550 nm) (B) or at 530 nm (excitation at 488 nm) (C, D), respectively. The amount of BPS in the lung was determined by analytical HPLC (E). Values are mean $\pm$ S.E.M. ($n=3-11$) *** $P < 0.001$; ** $P < 0.01$; versus CTRL; n.d., not detected ($<0.66 \mu\text{g/g}$ tissue).

Fig. 6. Effects of BPS alone on monocrotaline-induced pulmonary arterial remodeling and RV hypertrophy.

Rats were treated with monocrotaline (MCT; 60 mg/kg) or saline (CTRL) once only on day 0. Indicated doses of BPS alone were orally administered once per day for 21 days (A-C). Sections of pulmonary tissue were prepared on day 21 and subjected to histopathological examination (Elastica van Gieson staining) (A). Pulmonary arterial remodeling (hypertrophy of the pulmonary arterial wall (% wall thickness)) was measured (B). RV hypertrophy (RV/(LV+S)) was measured on day 21 (C). Values are mean $\pm$ S.E.M. * P <0.05; ** P <0.01; versus CTRL. # P <0.05; ### P <0.01; versus MCT alone. Scale bar, 100 μ m.

Fig. 7. Effects of BPS-NP on monocrotaline-induced pulmonary arterial remodeling and RV hypertrophy.

Rats were treated with monocrotaline (MCT; 60 mg/kg) or saline (CTRL) once only on day 0. Indicated doses of BPS-NP were administered intravenously via the tail vein (2 ml/kg) once per week for 21 days (A-C). Sections of pulmonary tissue were prepared on day 21 and subjected to histopathological examination (Elastica van Gieson staining) (A). Pulmonary arterial remodeling (hypertrophy of the pulmonary arterial wall (% wall thickness)) was measured (B). RV hypertrophy (RV/(LV+S)) was measured on day 21 (C). Values are mean $\pm$ S.E.M. ** P <0.01 versus CTRL. # P <0.05; ### P <0.01; versus MCT alone. Scale bar, 100 μ m.

Fig. 8. Effects of BPS-NP on hypoxia-induced pulmonary arterial remodeling and RV hypertrophy.

Mice were maintained in a hypoxic chamber (10% O₂, Hypoxia) or under normal conditions (CTRL) for 7 weeks. Indicated doses of BPS-NP were administered intravenously via the tail vein (5 ml/kg) once per week for 7 weeks (A-C). Sections of pulmonary tissue were prepared at week 7 and subjected to histopathological examination (Elastica van Gieson staining) (A). Pulmonary arterial remodeling (hypertrophy of the pulmonary arterial wall (% wall thickness)) was measured (B). RV hypertrophy (RV/(LV+S)) was measured at week 7 (C). Values are mean $\pm$ S.E.M. ** P <0.01 versus CTRL. ### P <0.01 versus hypoxia alone. Scale bar, 100 μ m.

Fig. 9. Schematic illustration of BPS-encapsulated nanoparticles.

Graphical Abstract