Enhanced rapamycin delivery to hemangiomas by lipid polymer nanoparticles coupled with anti-VEGFR antibody

HAITAO LI[1*], YUNFEI TENG[1*], XIA XU[2] and JIANYONG LIU[1]

Departments of [1]Vascular Surgery and [2]Pathology, Wuhan Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430022, P.R. China

Received October 18, 2017; Accepted January 12, 2018

DOI: 10.3892/ijmm.2018.3518

Abstract. The most common tumors in children are infantile hemangiomas which could cause morbidity and severe complications. The development of novel alternative drugs to treat infantile hemangiomas is necessary, since Hemangeol is the only US Food and Drug Administration-approved drug for infantile hemangiomas. However, Hemangeol has several disadvantages, including a high frequency of administration and adverse effects. Rapamycin is a well-established antiangiogenic drug, and we have previously developed rapamycin lipid polymer nanoparticles (R-PLNPs) as a local sustained-release drug delivery system to achieve controlled rapamycin release and to decrease the frequency of administration and side effects of rapamycin. To improve the targeting of R-PLNPs to infantile hemangiomas in the present study, R-PLNPs were modified to include an antibody against vascular endothelial growth factor receptor (VEGF). The characteristics, and the anti-hemangioma activity of the resulting R-PLNPs coupled with the anti-VEGFR2 antibody (named R-PLNPs-V) were examined *in vitro* and *in vivo*. R-PLNPs-V possessed a small size (115 nm) and sustained drug release for 6 days. The anti-VEGFR2 antibody promoted the targeting and cytotoxic effect of R-PLNPs-V to human hemangioma endothelial cells and human umbilical vein endothelial cells. Using a subcutaneous infantile hemangioma xenograft in mice, the *in vivo* therapeutic effect (evaluated with hemangioma weight, volume, and microvessel density) of R-PLNPs-V was demonstrated to be superior compared with rapamycin alone and other non-targeted nanoparticles, without any total body weight loss. In summary, R-PLNPs-V could facilitate targeted delivery and sustained release of rapamycin to infantile hemangiomas, and thus may represent a promising candidate treatment for infantile hemangiomas.

Correspondence to: Dr Jianyong Liu, Department of Vascular Surgery, Wuhan Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefangdadao Road, Wuhan, Hubei 430022, P.R. China
E-mail: 350527508@qq.com

Dr Xia Xu, Department of Pathology, Wuhan Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefangdadao Road, Wuhan, Hubei 430022, P.R. China
E-mail: 19467809@qq.com

[*]Contributed equally

Key words: rapamycin, nanoparticles, controlled release, targeted delivery, hemangioma

Introduction

Infantile hemangiomas are the most common tumors in children, characterized by endothelial cell proliferation and disorganized blood vessels (1). Infantile hemangiomas are benign, but could result in severe complications, such as life-altering disfigurement or ulceration (2,3). Propranalol, a non-selective β-blocker, is a promising treatment for infantile hemangiomas (4). Hydrochloride propranalol (also known as Hemangeol) has become the first and only US Food and Drug Administration (FDA)-approved anti-infantile hemangioma drug (4). Although Hemangeol is effective and relatively safe, its frequency of oral administration is high, and could induce complications, such as aggravated respiratory tract infections (5). Thus, it is necessary to develop other candidate reagents for treating infantile hemangiomas.

Rapamycin is an inhibitor of the mammalian target of rapamycin (mTOR), and inhibits neovascularization (6,7). It is noteworthy that rapamycin inhibits the proliferation of hemangioma endothelial cells and secretion of vascular endothelial growth factor (VEGF), and rapamycin has been already used as a medication in renal transplantation, making it a promising candidate for treating infantile hemangiomas (8-10). Nevertheless, rapamycin also has the disadvantages of high frequency of administration and of several complications, including anemia and acute renal toxicity (11). Local controlled systems directly targeting diseased regions can reduce a body-wide distribution, leading to undesirable side effects, and can also reduce the high frequency of administration (12). Therefore, we previously fabricated rapamycin lipid polymer nanoparticles (R-PLNPs) to attain controlled rapamycin release locally, and to decrease the frequency of administration and side effects of rapamycin (13). Poly(lactic-co-glycolic acid) (PLGA) was selected as the core of R-PLNPs, since PLGA is a hydrophobic FDA-approved material for use in humans (13).

Targeted nanoparticles coupled with antibodies can promote the therapeutic effectiveness of conventional medications in various diseases (14-16). In order to target infantile hemangiomas with nanoparticles coupled with antibodies, the identification of suitable targets in infantile hemangiomas is pivotal. It has been reported that VEGF and its receptor (vascular endothelial growth factor receptor; VEGFR) is critical in the angiogenesis of infantile hemangiomas (17). VEGFR2, one of the three receptors of VEGF, is a critical receptor for blood vasculature development, and infantile hemangiomas are induced by elevated VEGF signaling through VEGFR2 (8,18). Thus, it can be hypothesized that VEGFR2 may be a suitable target for treating infantile hemangiomas. In fact, VEGFR2 has been previously targeted to promote urea-encapsulated liposomes to target hemangioma vascular endothelial cells (19). In the present study, it was hypothesized that an antibody targeting VEGFR2 could be utilized to promote the therapeutic effectiveness of the previously developed R-PLNPs towards infantile hemangiomas. Rapamycin lipid polymer nanoparticles coupled with an anti-VEGFR2 antibody (R-PLNPs-V) were constructed as a controlled and targeted release drug delivery system to treat infantile hemangiomas. It was hypothesized that R-PLNPs-V could release rapamycin lastingly, and promote the targeted delivery of rapamycin to infantile hemangiomas.

Materials and methods

Chemical reagents, antibody, kits, and cell culture. All analytical-grade organic reagents, PLGA (MW, 40-75 kDa; lactide: Glycolide, 50:50) and coumarin 6 were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Rapamycin and its standards were purchased from Dalian Meilun Biotech Co., Ltd. (Dalian, China). Soybean lecithin, DSPE-PEG2000 [1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxypolyethylene glycol)-2000)], and DSPE-PEG2000-Mal [1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(maleimide (polyethylene glycol)-2000)] were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). The Cell Counting Kit-8 (CCK-8 kit) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The complex protein mixture, Matrigel, was purchased from BD Biosciences (Franklin Lakes, NJ, USA). The mouse anti-human VEGFR2 monoclonal antibody (MAB3571; 1:1,000), and the basic fibroblast growth factor (bFGF) (DFB50) and VEGF-A (DVE00) ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The secondary antibody [goat anti-mouse immunoglobulin G (IgG) coupled with horseradish peroxidase] (sc-2005; 1:2,000), and β-actin antibody (sc-47778; 1:200) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell (Carlsbad, CA, USA). HUVECs were cultured in a humidified 5% CO_2 incubator at 37˚C, in completed endothelial cell medium (ECM; (ScienCell). The trypsinization of cells was performed with 0.15% trypsin-EDTA, and cells of passages 3-10 were used in the present study.

Human hemangioma endothelial cells (HemECs) isolated from the hemangiomas of a patient were obtained as described previously (13). The Research Ethics Committee of Wuhan Union Hospital (Wuhan, China) approved the present study, and written informed consent was obtained from the patient. The specimens were treated on the basis of the legal and ethical standards. Specimens of infantile hemangiomas were obtained from Wuhan Union Hospital (patient 2 years old, female, date of sample collection April, 2016), and when the cells reached confluence, they were subcultured at 1:3 ratio.

Fabrication of R-PLNPs. R-PLNPs were constructed using a one-step nanoprecipitation approach, as previously described (13) (Fig. 1). In brief, the two solutions (A and B solutions) were prepared as follows: Solution A was developed by dissolving rapamycin (2 mg) in acetonitrile solution dissolved with 1 mg/ml PLGA; Solution B was developed by dissolving DSPE-PEG2000 (0.2 mg) and soybean lecithin (0.5 mg) in 4% ethanol aqueous solution at 65˚C. Afterwards, A solution was mixed with B solution at a slow speed of 1 ml/min. Following mixing, the solution was vortexed vigorously for 5 min. The resulting solution was gently stirred for 6 h, and then dialysis to PBS (pH 7.4) with Spectra/pro 6 dialysis membrane (MWCO 3500) was performed. Amicon Ultra-4 centrifugal filter devices (Amicon Corporation; EMD Millipore, Billerica, MA, USA) were used to concentrate the nanoparticles and to achieve the desired concentration. Blank lipid polymer nanoparticles (PLNPs) and coumarin 6-loaded nanoparticles (C-PLNPs) were constructed similar to R-PLNPs.

Fabrication of rapamycin-encapsulated lipid polymer nanoparticles coupled with anti-VEGFR2 antibody (R-PLNPs-V). R-PLNPs-V were fabricated by coupling thiolated anti-VEGFR2 antibody to R-PLNPs as described before (8) (Fig. 1). In brief, 2-iminothiolane was adopted to thiolate the anti-VEGFR2 antibody at a molar ratio of 2-iminothiolane: VEGFR2 antibodies. R-PLNPs were fabricated as described above, using DSPE-PEG2000-Mal to replace DSPE-PEG2000. Then, 0.2 mg thiolated anti-VEGFR2 antibody was incubated with 4 mg R-PLNPs containing maleimide-terminated linker, and the mixed solution was incubated for 6 h at ambient temperature under nitrogen. The obtained R-PLNPs-V were washed and centrifuged (15,000 x g, 30 min) to eliminate any uncoupled antibody. As controls, blank lipid polymer nanoparticles coupled with anti-VEGFR antibody (PLNPs-V) and coumarin 6-encapsulated lipid polymer nanoparticles coupled with anti-VEGFR2 antibody (C-PLNPs-V) were also constructed.

Characteristics of nanoparticles. Following dispersion in deionized water, the nanoparticles were examined for their size and zeta potential using a Zetasizer Nano S (Malvern Instruments, Ltd., Malvern, UK). For analysis of morphology, the nanoparticles were stained by 2% phosphotungstic acid, and analyzed by Hitachi H-600 transmission electron microscopy (TEM; Hitachi Ltd., Tokyo, Japan).

Drug loading and encapsulation efficiency of rapamycin in nanoparticles. Reverse phase high performance liquid chromatography (HPLC) was utilized to analyze the rapamycin encapsulation efficacy and drug loading of nanoparticles, as

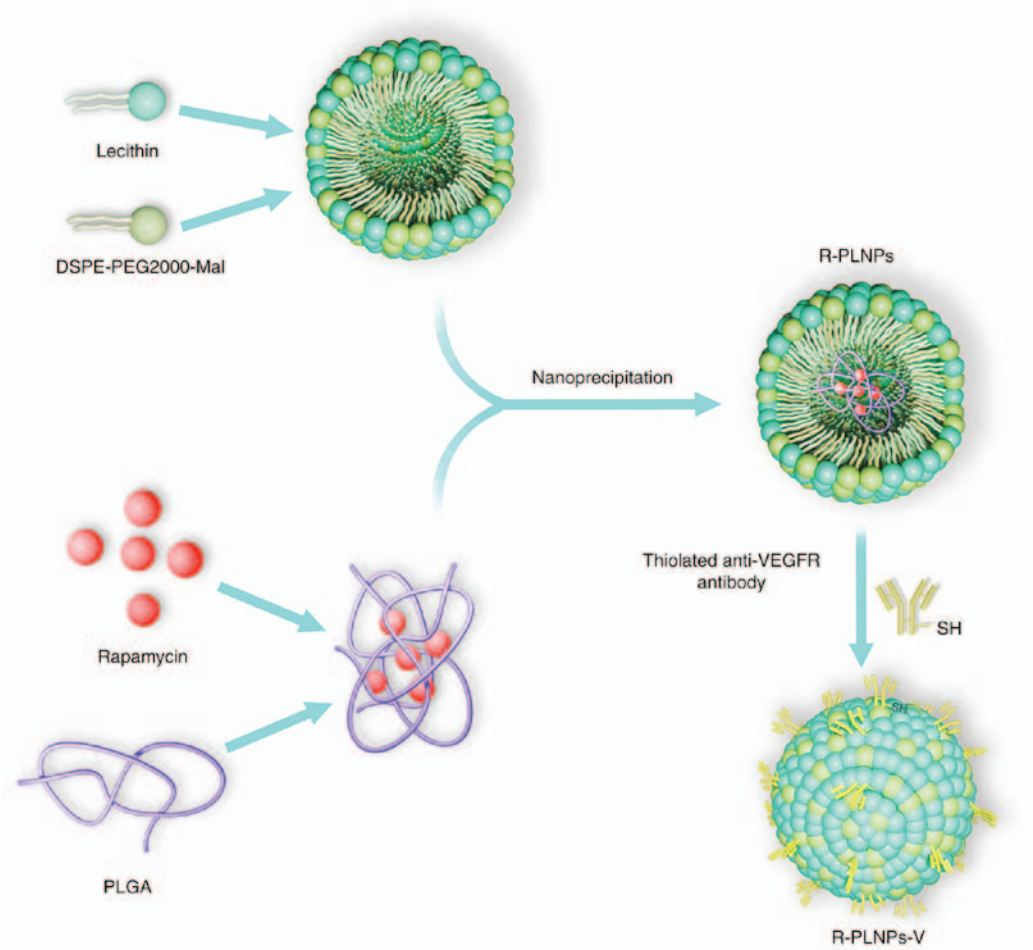

Figure 1. Fabrication of R-PLNPs-V. R-PLNPs were developed using a one-step nanoprecipitation approach. R-PLNPs-V were then developed by the conjugation of thiolated anti-VEGFR2 antibody to R-PLNPs via the reaction of thiol-maleimide. R-PLNPs, rapamycin-encapsulated lipid polymer nanoparticles; R-PLNPs-V, R-PLNPs coupled with anti-VEGR2 antibody; VEGFR, vascular endothelial growth factor receptor; PLGA, poly(lactic-co-glycolic acid); SH, thiol.

previously described (13). Briefly, the analysis was performed by HPLC, using Inertsil C-18 column (Octadecylsilane-3V, 4.6x250 mm in dimension) at 278 nm wavelength. The mobile phase was methanol-water (90:10 v/v). The flow rate was 1 ml/min. Drug encapsulation efficacy (EE) was calculated by the formula: amount of rapamycin loaded in nanoparticles/total rapamycin added initially. Drug loading was calculated by the formula: Rapamycin loaded in nanoparticles/total amount of nanoparticles. The coumarin 6 loading of coumarin 6-loaded nanoparticles was calculated by a coumarin-6 calibration curve.

Rapamycin release in vitro. A total of 2 mg nanoparticles were placed inside a membrane dialysis bag (MWCO, 3500; Spectra/pro 6 membrane). The bag was immersed in a vial containing PBS (200 ml; pH 7.4) with or without the addition of 10% FBS while being stirred at 100 rpm in a water bath at 37˚C. One ml of dialysate was removed at different time points for HPLC analysis, and 1 ml fresh solution was added instead.

In vitro cellular uptake. HUVECs or HemECs ($2x10^5$/well) were inoculated in 12-well cell culture plates at 37˚C, and incubated for 12 h. Free coumarin 6 or coumarin 6-loaded nanoparticles (an equivalent concentration of 20 ng/ml coumarin 6) were added to the cells, and incubated with the cells for 6 h. For competitive assays, 5 mg/m anti-VEGFR2 antibody was pre-incubated with the cells for 30 min. Following pre-incubation, the cells were rinsed with PBS to eliminate any unbound drugs. Following rinsing, the trypsinized cells were analyzed using a BD FACScan flow cytometer (Becton Dickinson, San Jose, CA). The data were analyzed using FlowJo software version 10 (FlowJo LLC, Ashland, OR, USA).

HUVECs or HemECs ($2x10^5$/well) were inoculated in 12-well cell culture plates at 37˚C, and incubated for 12 h. Rapamycin or rapamycin-loaded nanoparticles (an equivalent concentration of 200 ng/ml rapamycin) were added to the cells, and the cells were incubated with the drugs for 6 h at 37˚C. For competitive assays, 5 mg/ml anti-VEGFR2 antibody was pre-incubated with the cells for 30 min. Following pre-incubation, unbound drugs were eliminated by rinsing the cells with PBS. Then, the collected cells were homogenized by the lysis buffer consisting of 10% SDS. The rapamycin in the treated cells was quantitated by HPLC as described above. The % of rapamycin uptake was calculated by the following formula: Amount of internalized rapamycin/amount of rapamycin added initially x100%.

CCK-8 assay. HUVECs or HemECs were inoculated in 96-well culture plates (10,000/well) at 37˚C, and incubated for

Table I. Characteristics of nanoparticles.

	Size (nm)	Zeta potential (mv)	PDI	EE (%)	Drug loading (%)
R-PLNPs	110±21	-20±7	0.15±0.05	67.8±9.3	6.3±3.3
R-PLNPs-V	116±26	-24±9	0.16±0.06	62.9±8.1	5.8±3.7

Data are expressed as mean ± standard deviation (n=3). R-PLNPs, rapamycin-encapsulated lipid polymer nanoparticles; R-PLNPs-V, R-PLNPs coupled with anti-VEGR2 antibody; PDI, polydispersity; EE, encapsulation efficacy.

12 h. The cells were incubated with of the indicated concentrations of drugs for 72 h. The cell viability was determined by the CCK-8 assay, according to the manufacturer's protocols.

ELISA. HUVECs or HemECs ($2x10^5$/well) were inoculated in 12-well culture plates at 37˚C overnight. The cells were incubated with various concentrations of rapamycin and nanoparticles. After 72 h of treatments, the supernatant of the treated cells was isolated, and the cytokine levels were measured by ELISA kits, according to the manufacturer's protocols.

Western blot analysis. Total protein was extracted from the cells using ice-cold RIPA-Buffer (Beyotime Institute of Biotechnology, Jiangsu, China). The protein concentration was determined by the BCA assay with the BCA protein quantitative kit (Beyotime Institute of Biotechnology) and separated by 10% SDS-PAGE (20 μg of protein per lane). The protein was then transferred onto polyvinylidene fluoride membranes. The membrane was blocked with 10% BSA at 25˚C overnight. The primary antibody was the mouse anti-human VEGFR2 antibody (1:1,000) overnight at 4˚C. The secondary antibody was horseradish peroxidase-coupled goat anti-mouse IgG (1:2,000) 1 h at 25˚C). An Enhanced Chemiluminiscence kit (GE Healthcare) was used to detect the bands, and the bands were visualized with the ChemiDoc XRS$^+$ system with Image Lab™ image acquisition and analysis software version 4.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA).

In vivo anti-hemangioma activity of nanoparticles. A total of 40 BALB/c nude mice (6-8 weeks, female; 8 mice per group/5 groups) were provided by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The mice were housed under conditions of controlled temperature (22±2˚C), humidity (45-65%), and artificial light (12-h light/dark cycle) with free access to food and water. All the procedures in animal studies were performed in accordance with the guidelines of the Committee on Animals of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China).

The mice were inoculated at the flank with $7.5x10^6$ HemECs suspended in Matrigel. When the hemangioma had reached about 25 mm^3 on day 0, the mice received single intratumoral (i.t.) injections of R-PLNPs (2 mg rapamycin/kg), R-PLNPs-V (2 mg rapamycin/kg), rapamycin (2 mg rapamycin/kg) or PLNPs-V (40 mg/kg). Free rapamycin was first dissolved in dimethyl sulfoxide, and then dissolved in 5% polyethylene glycol, 0.2% carboxymethylcellulose, and 0.25% Tween-80 in H_2O. The i.t. injections were carried out on days 0, 5, 10, 15 and 20. The hemangioma volume of the mice, calculated as $V=(L \times W^2)/2$ (L indicates length, and W indicates width), was measured once every five days. On day 35, after the mice were euthanized, the weight of the excised hemangioma was measured. The excised hemangioma was stained by H&E. The analysis of the microvessel density (MVD) of the sections was done as previously described (8). Microvessels were quantified by counting lumens containing red blood cells. A total of 4 fields per slide were reviewed under a Zeiss Axiophot 2 microscope (Zeiss GmbH, Jena, Germany). MVD was calculated as vessels/mm^2.

Statistical analysis. Statistical analysis was performed with Student's non-paired t-tests or one-way analysis of variance followed by Newman-Keuls or Dunnett's test, using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference. Unless otherwise stated, all data were expressed as the mean ± standard deviation.

Results

VEGFR2 expression of endothelial cells and characteristics of nanoparticles. VEGFR2 was expressed in the endothelial cells (HUVECs and HemECs; Fig. 2A), suggesting that it could serve as a potential target in promoting the targeting of nanoparticles to the endothelial cells. The characteristics of nanoparticles are presented in Table I. The size of the nanoparticles was slightly >100 nm, with a narrow polydispersity <0.2. The drug encapsulation efficacy (EE) of R-PLNPs and R-PLNPs-V was 67.8 and 62.9%, respectively. The drug release profile of rapamycin from the nanoparticles was examined (Fig. 2B). The rapamycin release of both nanoparticles was faster in PBS with 10% FBS compared with PBS alone. In the early 48 h, both nanoparticles exhibited a burst release (~40% for PBS, and ~50% for PBS with 10% FBS). In the next 96 h, the cumulative release of both nanoparticles achieved ~65% in PBS and 80% in PBS with 10% FBS. Taken together, both nanoparticles exhibited a sustained release during a period of 192 h. The surface morphology of the nanoparticles was analyzed by TEM (Fig. 2C and D). TEM analysis demonstrated that both nanoparticles displayed smooth and round shape. The dim ring of the nanoparticles may reflect the structure of lipid layers which surround the polymer core.

Nanoparticle uptake by endothelial cells. First, coumarin 6 was used as the fluorescence probe to evaluate the nanoparticle uptake in endothelial cells (Fig. 3A). In HUVECs,

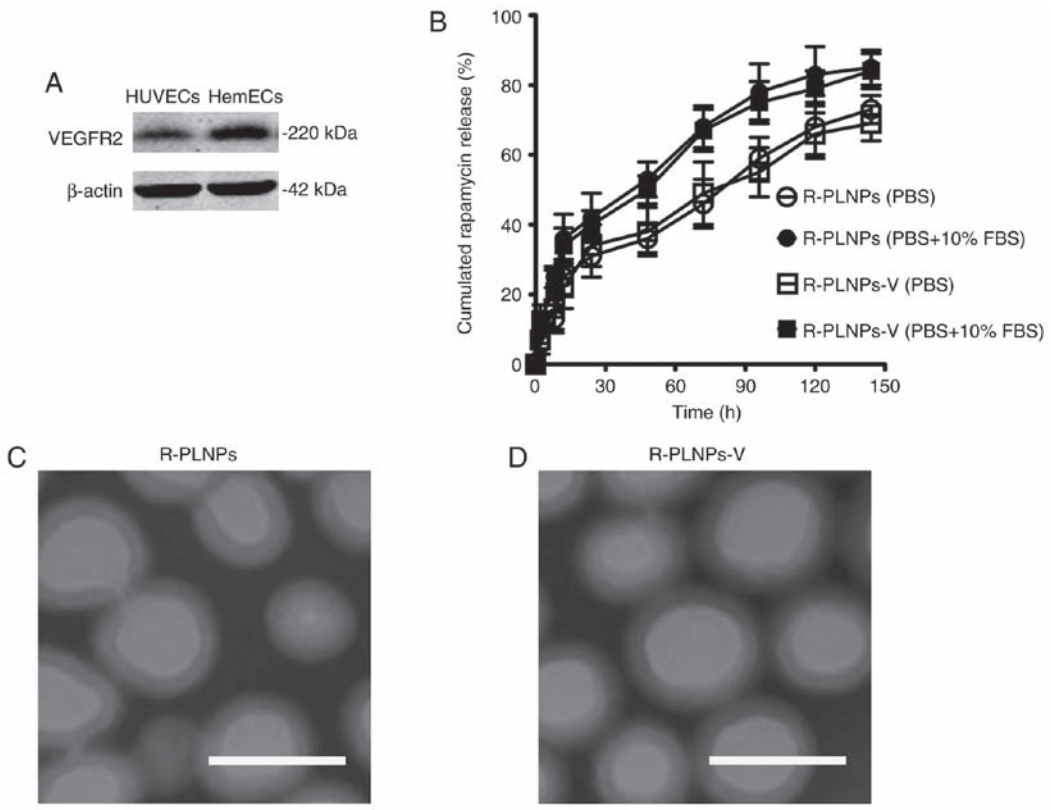

Figure 2. VEGFR2 expression in endothelial cell lines and characteristics of nanoparticles. (A) Protein expression of VEGFR2 was examined by western blot analysis. β-actin was used as the internal control. (B) The *in vitro* rapamycin release profile of the nanoparticles. Data are expressed as mean ± standard deviation (n=3). (C and D) Representative images of the nanoparticles morphology, as analyzed by transmission electron microscopy. Scale bar, 100 nm. VEGFR, vascular endothelial growth factor receptor; HUVECs, human umbilical vein endothelial cells; HemECs, hemangioma endothelial cells; R-PLNPs, rapamycin-encapsulated lipid polymer nanoparticles; R-PLNPs-V, R-PLNPs coupled with anti-VEGR2 antibody.

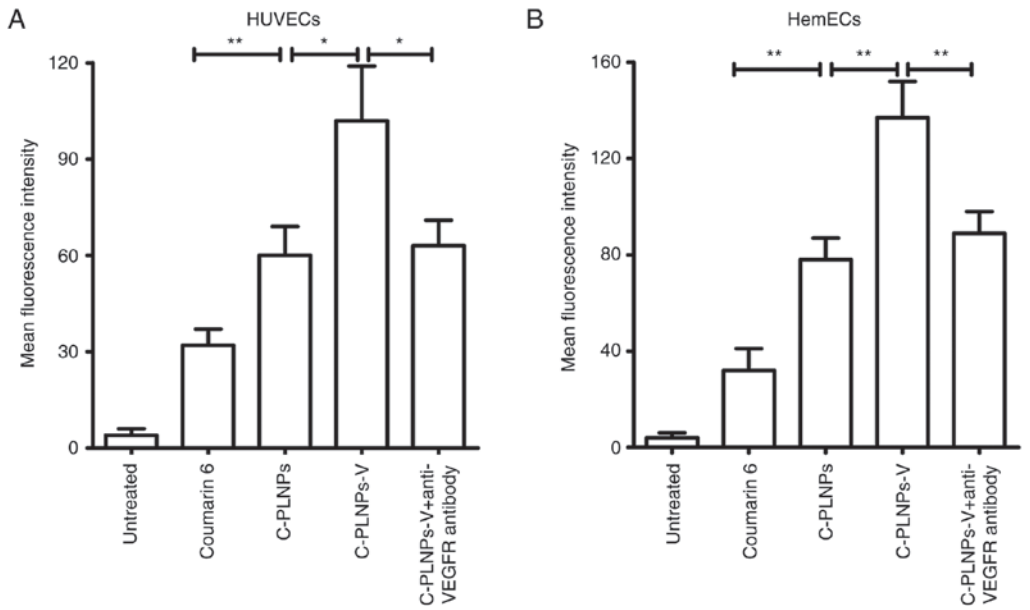

Figure 3. *In vitro* cellular uptake analyzed by flow cytometry in (A) HUVECs and (B) HemECs. The cells were treated with free coumarin 6 or coumarin 6-loaded nanoparticles (an equivalent concentration of 20 ng/ml coumarin 6) for 6 h. Data are expressed as mean ± standard deviation (n=3). *P<0.05, **P<0.01 and ***P<0.001, with comparisons indicated by brackets. HUVECs, human umbilical vein endothelial cells; HemECs, hemangioma endothelial cells; C-PLNPs, coumarin 6-loaded nanoparticles; C-PLNPs-V, C-PLNPs coupled with anti-VEGR2 antibody.

coumarin 6-loaded nanoparticles (C-PLNPs) exhibited higher fluorescence intensity than coumarin 6 alone (P<0.01; Fig. 3A), indicating that the formation of coumarin 6 in the nanoparticles formulation facilitated its uptake. After being coupled with the anti-VEGFR2 antibody, the fluorescence intensity of nanoparticles was further increased, as reflected

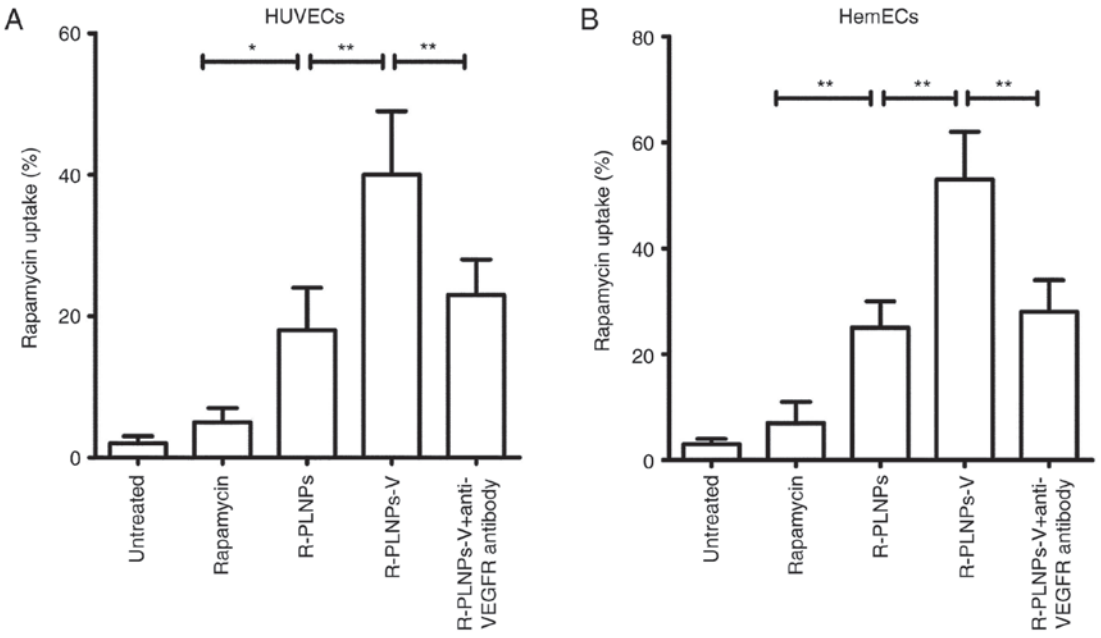

Figure 4. *In vitro* cellular uptake analyzed by HPLC in (A) HUVECs and (B) HemECs. The cells were treated with rapamycin or rapamycin-loaded nanoparticles (an equivalent concentration of 200 ng/ml rapamycin) for 6 h at 37˚C. For competitive assays, 5 mg/ml anti-VEGFR antibody was pre-incubated with the cells for 30 min. Data are expressed as mean ± standard deviation (n=3). *P<0.05 and **P<0.01, with comparisons indicated by brackets. HUVECs, human umbilical vein endothelial cells; HemECs, hemangioma endothelial cells; VEGFR, vascular endothelial growth factor receptor; R-PLNPs, rapamycin-encapsulated lipid polymer nanoparticles; R-PLNPs-V, R-PLNPs coupled with anti-VEGR2 antibody.

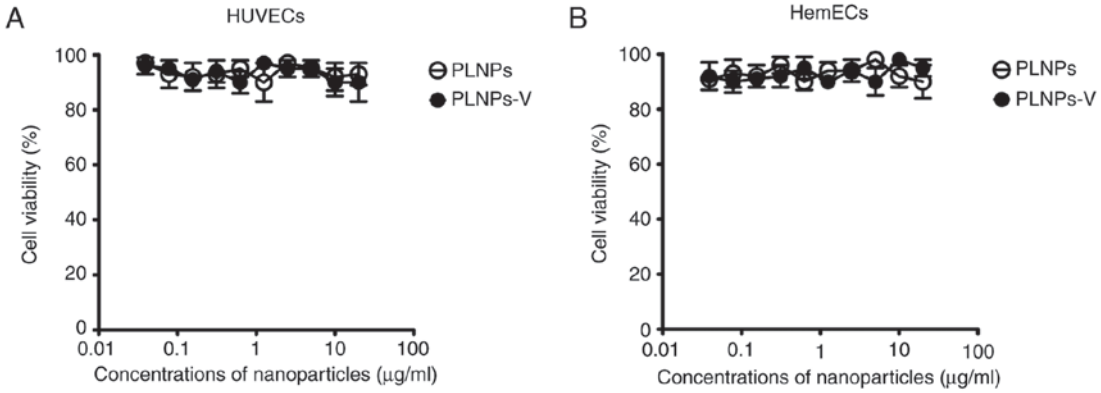

Figure 5. (A and B) Cytotoxicity of nanoparticles analyzed by the CCK-8 assay. The cells were incubated for 72 h with various concentrations of nanoparticles, and the cell viability was evaluated by the CCK-8 assay. Data (mean ± standard deviation) are representative of three independent experiments. CCK, Cell Counting Kit; HUVECs, human umbilical vein endothelial cells; HemECs, hemangioma endothelial cells; VEGFR, vascular endothelial growth factor receptor; PLNPs, blank lipid polymer nanoparticles; PLNPs-V, PLNPs coupled with anti-VEGR2 antibody.

by the higher fluorescence intensity of C-PLNPs-V compared with C-PLNPs (P<0.05; Fig. 3A). By contrast, the fluorescence intensity of C-PLNPs-V was significantly decreased after pre-incubation with the anti-VEGFR2 antibody (P<0.05; Fig. 3A), indicating that the conjugated anti-VEGFR2 antibody promoted the binding of C-PLNPs-V to HUVECs. Similar results were obtained in HemECs (Fig. 3B).

Subsequently, the rapamycin uptake of nanoparticles in HUVECs and HemECs was explored (Fig. 4). Similar results, as the fluorescence assay described above, were obtained. In HUVECs, nanoparticles significantly increased the uptake of rapamycin, as reflected by the fact that ~20% of rapamycin uptake was observed for R-PLNPs, whereas only ~5% of rapamycin uptake was observed for rapamycin alone (Fig. 4A). Once again, R-PLNPs-V exhibited significant rapamycin uptake (~40%), significantly higher compared with R-PLNPs (~20%; P<0.01; Fig. 4A). However, the uptake of R-PLNPs-V was decreased significantly to ~20% following anti-VEGFR2 antibody pretreatment (P<0.01; Fig. 4A), suggesting that the anti-VEGFR antibody mediated the efficient rapamycin uptake of R-PLNPs-V. Similar results were obtained in HemECs (Fig. 4B).

Effect of nanoparticles in cell viability by CCK-8 assay. The biocompatibility of nanoparticles is an important issue, since their toxicity may pose potential damage to humans. As illustrated in Fig. 5, the blank nanoparticles, PLNPs, and the PLNPs-V, did not result in any significant toxicity to HUVECs and HemECs at 72 h at nanoparticles concentrations of 0.04-20 μg/ml.

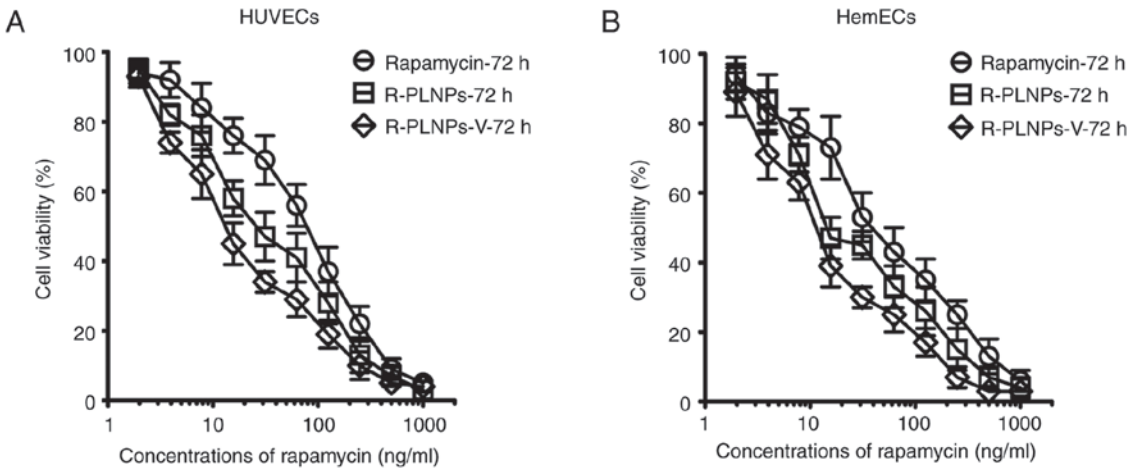

Figure 6. (A and B) Cytotoxicity of rapamycin and rapamycin-loaded nanoparticles analyzed by the CCK-8 assay. The cells were incubated for 72 h with various concentrations of rapamycin and rapamycin-loaded nanoparticles, and the cell viability was evaluated by the CCK-8 assay. Data (mean ± standard deviation) are representative of three independent experiments. CCK, Cell Counting Kit; HUVECs, human umbilical vein endothelial cells; HemECs, hemangioma endothelial cells; R-PLNPs, rapamycin-encapsulated lipid polymer nanoparticles; R-PLNPs-V, R-PLNPs coupled with anti-VEGR2 antibody.

Table II. IC_{50} of various treatments in HUVECs and HemECs.

	72 h		
IC_{50}, ng/ml	Rapamycin	R-PLNPs	R-PLNPs-V
HUVECs	101.3±18.3	39.1±7.5	21.3±7.3
HemECs	75.6±7.2	38.5±7.1	19.8±8.7

Data are expressed as mean ± standard deviation (n=3). IC_{50}, half-maximal inhibitory concentration; HUVECs, human umbilical vein endothelial cells; HemECs, hemangioma endothelial cells; R-PLNPs, rapamycin-encapsulated lipid polymer nanoparticles; R-PLNPs-V, R-PLNPs coupled with anti-VEGR2 antibody.

The cytotoxic effects of rapamycin, R-PLNPs, and R-PLNPs-V were then evaluated in HUVECs and HemECs, and all of them displayed a dose-dependent cytotoxicity in both cell lines (Fig. 6). As presented in Table II, the IC_{50} of rapamycin, R-PLNPs, and R-PLNPs-V was 101.3, 39.1, and 21.3 ng/ml in HUVECs, respectively. These results suggest that R-PLNPs-V were 2.6- and 4.8-fold more effective than R-PLNPs and rapamycin, respectively. In HemECs, similar results were obtained (Table II). R-PLNPs-V were 2- and 3.8-fold more effective than R-PLNPs and rapamycin, respectively. Thus, R-PLNPs-V displayed significantly increased cytotoxic effects towards the endothelial cells compared with rapamycin and R-PLNPs.

Effect of nanoparticles in cytokine production by ELISA. The production of VEGF-A and bFGF is dependent on mTOR activation, and rapamycin could decrease their production by mTOR inhibition (20). As illustrated in Fig. 7, levels of secreted VEGF-A and bFGF were examined following various treatments in the endothelial cells. The relative protein levels of the treated groups are presented as the % of the levels of the untreated group. Although the blank nanoparticles PLNPs-V barely affected VEGF-A and bFGF production, R-PLNPs-V, R-PLNPs and rapamycin treatments inhibited VEGF-A and bFGF production in HUVECs and HemECs, in a dose-dependent manner (Fig. 7). At 50 ng/ml, R-PLNPs-V inhibited the production of VEGF-A in HUVECs more effectively than rapamycin ($P<0.01$) and R-PLNPs ($P<0.05$), and R-PLNPs-V at 10 ng/ml were also more effective than rapamycin ($P<0.01$) and R-PLNPs ($P<0.05$). In the case of bFGF in HUVECs, similar results were obtained. R-PLNPs-V were more effective in inhibiting the production of bFGF in HUVECs compared with rapamycin ($P<0.001$) and R-PLNPs ($P<0.01$), and R-PLNPs-V was also more effective than rapamycin ($P<0.001$) and R-PLNPs ($P<0.01$) at 10 ng/ml. In the case of HemECs, similar results were obtained. Taken together, these results demonstrated that R-PLNPs-V were more effective in inhibiting VEGF-A and bFGF production in the endothelial cells, compared with rapamycin and R-PLNPs.

Effect of nanoparticles in anti-hemangioma activity in vivo. The therapeutic efficacy of the various treatments was evaluated in hemangioma-bearing mice (Fig. 8). The blank nanoparticle, PLNPs-V, did not exhibit any activity against hemangiomas, as reflected by the progressive growth of tumors following treatment with PLNPs-V (Fig. 8A). By contrast, rapamycin, R-PLNPs and R-PLNPs-V inhibited the growth of hemangiomas at different degrees. At the end time point, R-PLNPs-V treatment resulted in a striking 86% decrease in the volume of hemangiomas, whereas R-PLNPs and rapamycin alone had induced a 68 and 50% decrease in the volume of hemangiomas, respectively. Compared with other groups, the R-PLNPs-V-treated group had a significantly smaller hemangioma volume ($P<0.001$, compared with PLNPs-V; $P<0.01$, compared with R-PLNPs; Fig. 8B). In addition, compared with other groups, the R-PLNPs-V-treated group had a significantly lower hemangioma weight ($P<0.001$, compared with PLNPs-V; $P<0.01$, compared with R-PLNPs; Fig. 8C). The overall body weight of the mice did not change significantly with any of the treatments (Fig. 8D).

Furthermore, histology staining and microvessel density (MVD) analysis were performed on the excised hemangiomas

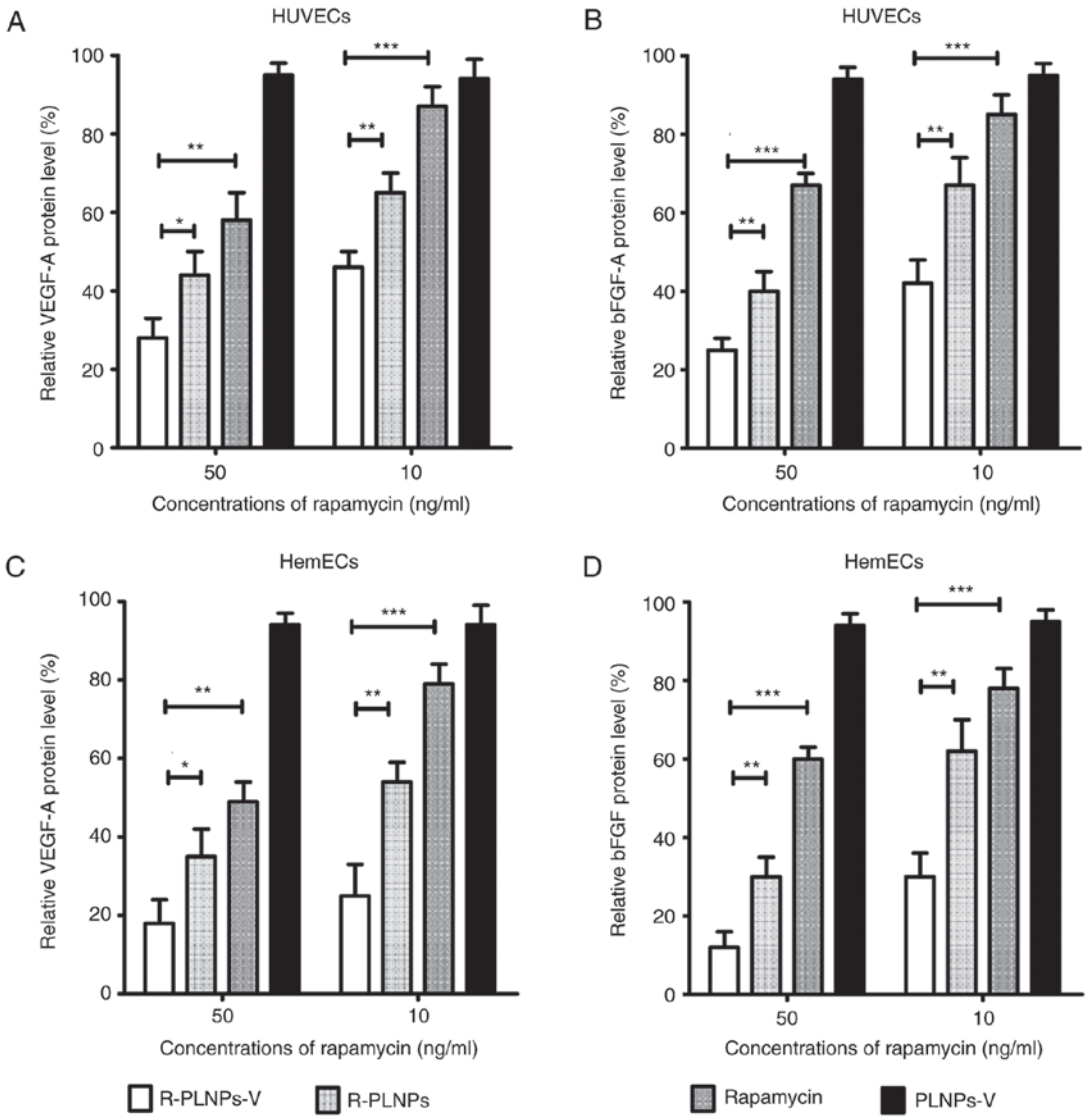

Figure 7. VEGF-A and bFGF production in (A and B) HUVECs and (C and D) HemECs analyzed by ELISA. The relative protein levels in each treatment group were expressed as a % relative to the untreated group. Data are expressed as mean ± standard deviation (n=3). *P<0.05, **P<0.01 and ***P<0.001, with comparisons indicated by brackets. VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; HUVECs, human umbilical vein endothelial cells; HemECs, hemangioma endothelial cells; PLNPs-V, lipid polymer nanoparticles coupled with anti-VEGR2 antibody; R-PLNPs, rapamycin-encapsulated lipid polymer nanoparticles; R-PLNPs-V, R-PLNPs coupled with anti-VEGR2 antibody.

(Fig. 9). Compared with other groups, the R-PLNPs-V-treated group had the lowest MVD (P<0.001, compared with saline, PLNPs-V, rapamycin or R-PLNPs; Fig. 9B). Thus, these results demonstrated that R-PLNPs-V inhibited the microvessel density of hemangiomas and displayed the best efficiency against hemangiomas among the tested treatments.

Discussion

Hemangeol is the only FDA-approved drug for infantile hemangiomas, but has several disadvantages and adverse effects. Based on the previously developed R-PLNPs, anti-VEGFR2 antibody was coupled to the nanoparticles in the present study, in order to enhance the targeting of R-PLNPs to infantile hemangiomas. The results demonstrated that R-PLNPs-V displayed sustained rapamycin release lastingly and superior efficiency in reducing hemangioma activity *in vitro* and *in vivo*.

The superior safety profile of nanoparticles could facilitate their clinic use due to their reduced damage to humans (21). The components of the R-PLNPs-V generated in the present study include rapamycin, lecithin, PEGylated lipid, PLGA, and VEGFR2 antibodies (Fig. 1). Lecithin, PEGylated lipid, and PLGA are biocompatible, and all of them are FDA-approved pharmaceutical materials. The use of these FDA-approved pharmaceutical materials may facilitate a potential transition of the presented nanoparticles in the clinic. Rapamycin is an FDA-approved drug for renal transplantation. Due to the numerous antibodies approved by FDA to treat various diseases, the safety of a large amount of monoclonal antibodies has been well-demonstrated (22). Although the anti-VEGFR2 antibody has not been approved by FDA, its safety is expected to be sufficient, although this will need to be demonstrated in future studies. In the present study, the results from cytotoxicity assays demonstrated the good biocompatibility of the blank nanoparticles coupled with anti-VEGFR2 antibody

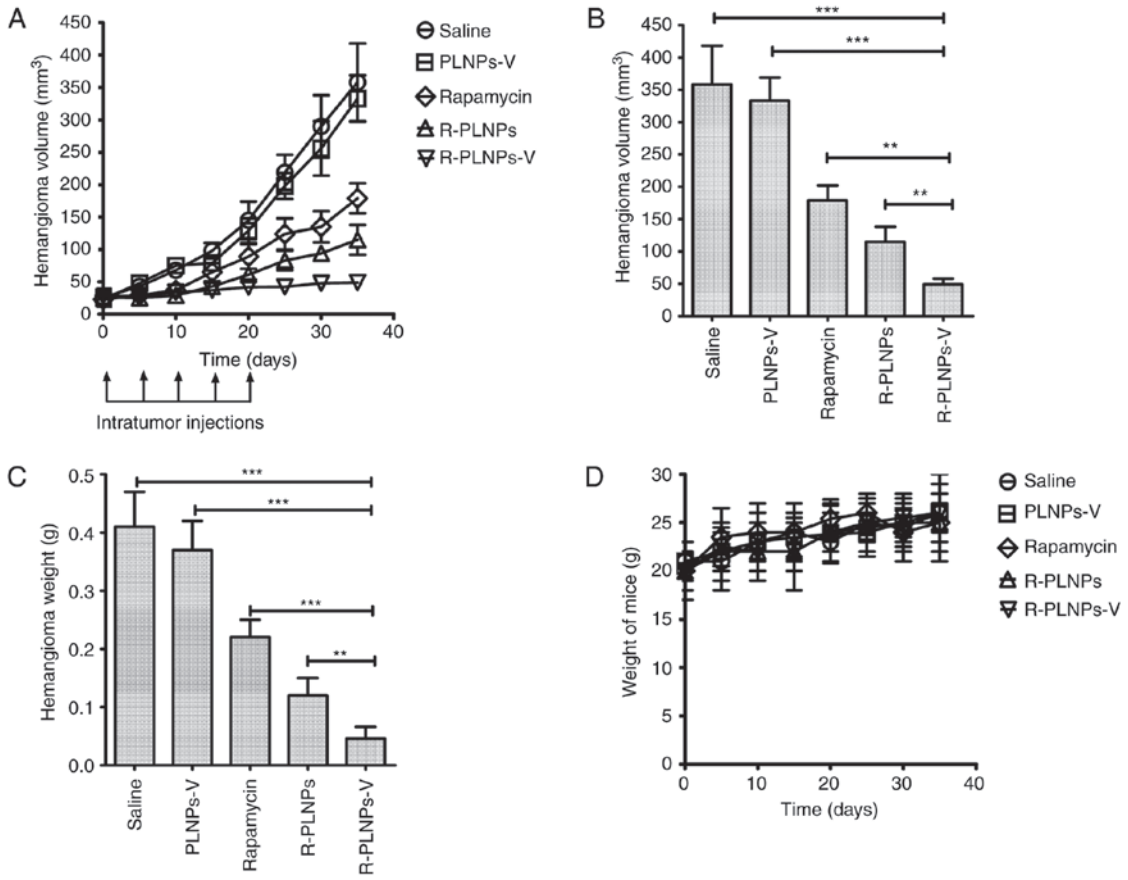

Figure 8. Anti-hemangioma activity of nanoparticles *in vivo*. After hemangioma was ~25 mm^3 in size (day 0), mice received single intratumoral injections of saline, R-PLNPs (2 mg rapamycin/kg), R-PLNPs-V (2 mg rapamycin/kg), rapamycin (2 mg rapamycin/kg) or PLNPs-V (40 mg/kg). Additional intratumoral injections were performed on days 5, 10, 15 and 20 (indicated by black arrows in the schematic). (A) Growth curve of hemangiomas during the course of treatments. (B) Tumor volume of each experimental group analyzed at the end point. (C) Weight of excised hemangiomas analyzed at the end point. (D) Measurements of the total body weight of the mice during the treatment. Data are expressed as mean ± standard deviation (n=8). *P<0.05, **P<0.01 and ***P<0.001, with comparisons indicated by brackets. PLNPs-V, lipid polymer nanoparticles coupled with anti-VEGR2 antibody; R-PLNPs, rapamycin-encapsulated lipid polymer nanoparticles; R-PLNPs-V, R-PLNPs coupled with anti-VEGR2 antibody.

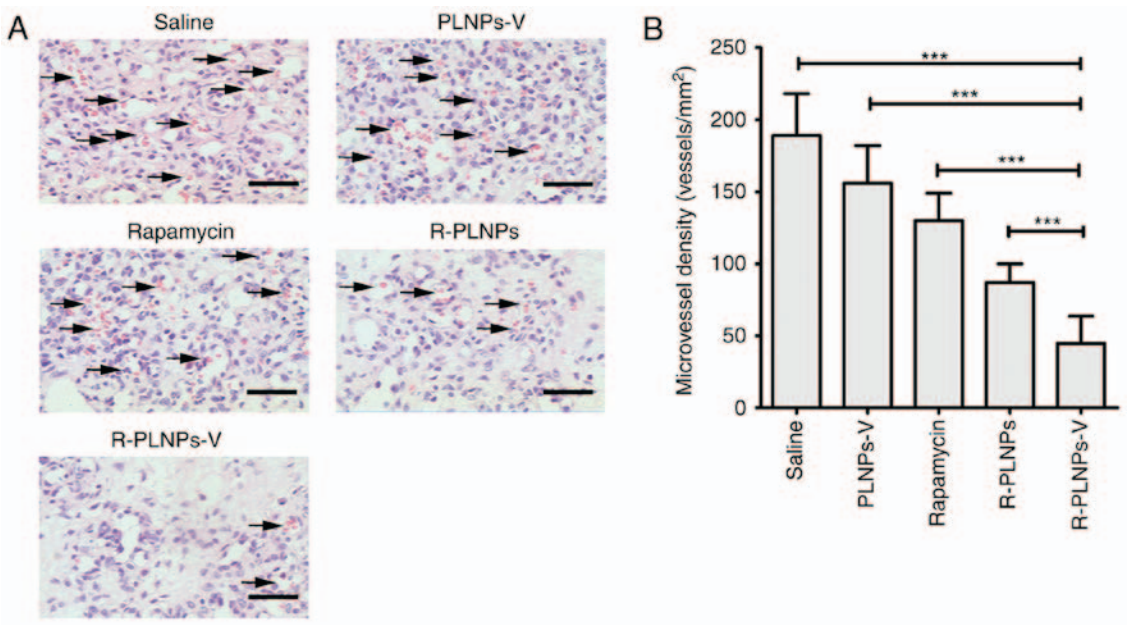

Figure 9. Histology and microvessel density analysis of the excised hemangiomas. (A) Representative images from H&E staining of the hemangioma tissues in each treatment group. Black arrows indicate lumens with red blood cells. Scale bar, 50 μm. (B) Microvessel density quantification of hemangiomas, by counting the numbers of lumens with red blood cells per mm^2 of tissue. Data are expressed as mean ± standard deviation (n=16). *P<0.05, **P<0.01 and ***P<0.001, with comparisons indicated by brackets. PLNPs-V, lipid polymer nanoparticles coupled with anti-VEGR2 antibody; R-PLNPs, rapamycin-encapsulated lipid polymer nanoparticles; R-PLNPs-V, R-PLNPs coupled with anti-VEGR2 antibody.

(PLNPs-V). Furthermore, the present results from the mice study suggested that the prepared R-PLNPs-V did not cause significant weight changes in the mice. Therefore, R-PLNPs-V is anticipated to possess good safety properties which could facilitate the clinical translation of R-PLNPs-V.

A series of targeted nanoparticles have been developed to target various cancers (14,15). The most prominent example is the doxorubicin-loaded immunoliposomes that exhibited enhanced cytotoxic effects towards human epidermal growth factor receptor 2 (HER2)-expressing breast cancers (23). However, there has been few targeted nanoparticles that have been developed for infantile hemangiomas. To the best of our knowledge, the only nanoparticle developed for infantile hemangiomas to date is the urea-immunoliposomes coupled with the anti-VEGFR antibody (19). The R-PLNPs-V developed in the present study have three advantages over the urea-immunoliposomes. First, rapamycin is a FDA-approved drug, whereas urea has not been approved. Second, liposomes possess a structure of soft-membrane, making its drug release rather quick. By contrast, the PLGA core of R-PLNPs-V will be more rigid, making its drug release last for a longer period. The drug release assay demonstrated that R-PLNPs-V released rapamycin gradually in 6 days, thus having the possibility to maintain a high level of the drug in infantile hemangiomas. Third, the therapeutic efficacy of the urea-immunoliposomes was only tested in hemangioma vascular endothelial cells *in vitro*, and not *in vivo* (19). In the present study, R-PLNPs-V have not only been demonstrated to inhibit HUVECs and HemECs *in vitro*, but also significantly impede hemangioma growth *in vivo*, using a patient-derived xenograft.

The present results have confirmed that the anti-VEGFR2 antibody was pivotal for the special targeting of R-PLNPs-V to HUVECs and HemECs. Confirmed by flow cytometry, the anti-VEGFR2 antibody promoted the nanoparticle uptake in both the endothelial cells. The quantitative HPLC assay demonstrated that 40-50% of rapamycin could be taken by R-PLNPs-V, in contrast to the only ~5% of rapamycin uptake for the treatment with rapamycin alone, suggesting that both the anti-VEGFR2 antibody conjugation and the nanoparticle formulation significantly facilitated the rapamycin uptake. The enhanced rapamycin uptake dramatically increased the cytotoxic effects of R-PLNPs-V. R-PLNPs-V was 2.6- and 4.8-fold more effective than R-PLNPs and rapamycin in HUVECs, respectively, and 2- and 3.8-fold more effective than R-PLNPs and rapamycin in HemECs, respectively. Notably, the *in vivo* results demonstrated that the therapeutic efficacy of R-PLNPs-V was superior to rapamycin alone or R-PLNPs, as reflected by the fact that the R-PLNPs-V treatment resulted in the lowest hemangioma volume, weight and MVD. These results suggested that the therapeutic efficacy of rapamycin was promoted by R-PLNPs-V, and the interaction of VEGFR/anti-VEGFR antibody could facilitate effective rapamycin delivery to endothelial cells. Furthermore, the local administration of rapamycin by R-PLNPs-V could target endothelial cells directly and efficiently, resulting in minimal unpredictable absorption and side effects of rapamycin. The lack of anti-hemangioma activity for PLNPs-V, the blank nanoparticles with anti-VEGFR2 antibody, may be attributed to the low amount of anti-VEGFR2 antibody on PLNPs-V, which may not be sufficient to induce obvious cytotoxic effects towards endothelial cells. In summary, the superior activity of R-PLNPs-V is attributed to its targeted delivery and sustained release of rapamycin.

The present data aid in clarifying the mechanism underlying the anti-hemangioma activity of R-PLNPs-V. In brief, following local administration, due the interaction of VEGFR with the anti-VEGFR2 antibody, R-PLNPs-V bound to hemangioma endothelial cells and inhibited the growth of hemangioma endothelial cells. Additionally, R-PLNPs-V were also able to reduce VEGF-A and bFGF production in hemangioma endothelial cells, while hemangioma growth was inhibited *in vivo* to a great extent.

In conclusion, R-PLNPs-V released rapamycin lastingly, and achieved superior therapeutic efficacy in inhibiting hemangiomas *in vitro* and *in vivo*, compared with rapamycin. Taken together, the present results suggest that R-PLNPs-V may represent a promising and safe treatment for infantile hemangiomas.

Competing interests

The authors declare that they have no competing interests.

References

1. Kilcline C and Frieden IJ: Infantile hemangiomas: How common are they? A systematic review of the medical literature. Pediatr Dermatol 25: 168-173, 2008.
2. Chang LC, Haggstrom AN, Drolet BA, Baselga E, Chamlin SL, Garzon MC, Horii KA, Lucky AW, Mancini AJ, Metry DW, *et al*: Growth characteristics of infantile hemangiomas: Implications for management. Pediatrics 122: 360-367, 2008.
3. Chen TS, Eichenfield LF and Friedlander SF: Infantile hemangiomas: An update on pathogenesis and therapy. Pediatrics 131: 99-108, 2013.
4. Sethuraman G, Yenamandra VK and Gupta V: Management of infantile hemangiomas: Current trends. J Cutan Aesthet Surg 7: 75-85, 2014.
5. Léaute-Labrèze C, Boccara O, Degrugillier-Chopinet C, Mazereeuw-Hautier J, Prey S, Lebbé G, Gautier S, Ortis V, Lafon M, Montagne A, *et al*: Safety of oral rapamycin for the treatment of infantile hemangioma: A Systematic Review. Pediatrics 138: pii: e20160353, 2016.
6. Kaur A and Sharma S: Mammalian target of rapamycin (mTOR) as a potential therapeutic target in various diseases. Inflammopharmacology 25: 293-312, 2017.
7. Del Bufalo D, Ciuffreda L, Trisciuoglio D, Desideri M, Cognetti F, Zupi G and Milella M: Antiangiogenic potential of the Mammalian target of rapamycin inhibitor temsirolimus. Cancer Res 66: 5549-5554, 2006.
8. Medici D and Olsen BR: Rapamycin inhibits proliferation of hemangioma endothelial cells by reducing HIF-1-dependent expression of VEGF. PLoS One 7: e42913, 2012.
9. Zheng N, Ding X and Jahan R: Low concentration of rapamycin inhibits hemangioma endothelial cell proliferation, migration, and vascular tumor formation in mice. Curr Ther Res Clin Exp 76: 99-103, 2014.
10. Johnson RW: Sirolimus (Rapamune) in renal transplantation. Curr Opin Nephrol Hypertens 11: 603-607, 2002.
11. Stallone G, Infante B, Grandaliano G and Gesualdo L: Management of side effects of sirolimus therapy. Transplantation 87 (8 Suppl): S23-S26, 2009.
12. Almeida H, Amaral MH, Lobao P, Frigerio C and Sousa Lobo JM: Nanoparticles in ocular drug delivery systems for topical administration: Promises and challenges. Curr Pharm Des 21: 5212-5224, 2015.
13. Li H, Teng Y, Sun J and Liu J: Inhibition of hemangioma growth using lipid polymer nanoparticles for delivery of rapamycin. Biomed Pharmacother 95: 875-884, 2017.
14. Gao J, Chen H, Song H, Su X, Niu F, Li W, Li B, Dai J, Wang H and Guo Y: Antibody-targeted immunoliposomes for cancer treatment. Mini Rev Med Chem 13: 2026-2035, 2013.

15. Gao J, Feng SS and Guo Y: Antibody engineering promotes nanomedicine for cancer treatment. Nanomedicine (Lond) 5: 1141-1145, 2010.
16. Li W, Li J, Gao J, Li B, Xia Y, Meng Y, Yu Y, Chen H, Dai J, Wang H and Guo Y: The fine-tuning of thermosensitive and degradable polymer micelles for enhancing intracellular uptake and drug release in tumors. Biomaterials 32: 3832-3844, 2011.
17. Eskens FA and Verweij J: The clinical toxicity profile of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR) targeting angiogenesis inhibitors; a review. Eur J Cancer 42: 3127-3139, 2006.
18. Tugues S, Koch S, Gualandi L, Li X and Claesson-Welsh L: Vascular endothelial growth factors and receptors: Antiangiogenic therapy in the treatment of cancer. Mol Aspects Med 32: 88-111, 2011.
19. Wang Z, Li J, Xu X, Duan X and Cao G: Urea immunoliposome inhibits human vascular endothelial cell proliferation for hemangioma treatment. World J Surg Oncol 11: 300, 2013.
20. Humar R, Kiefer FN, Berns H, Resink TJ and Battegay EJ: Hypoxia enhances vascular cell proliferation and angiogenesis in vitro via rapamycin (mTOR)-dependent signaling. FASEB J 16: 771-780, 2002.
21. Nyström AM and Fadeel B: Safety assessment of nanomaterials: Implications for nanomedicine. J Control Release 161: 403-408, 2012.
22. Nelson AL, Dhimolea E and Reichert JM: Development trends for human monoclonal antibody therapeutics. Nat Rev Drug Discov 9: 767-774, 2010.
23. Park JW, Hong K, Kirpotin DB, Colbern G, Shalaby R, Baselga J, Shao Y, Nielsen UB, Marks JD, Moore D, *et al*: Anti-HER2 immunoliposomes: Enhanced efficacy attributable to targeted delivery. Clin Cancer Res 8: 1172-1181, 2002.