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Oral Absorption Enhancement of Probucol by PEGylated G5 PAMAM Dendrimer Modified Nanoliposomes

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Abstract

Probucol (PB), an antioxidant drug, is commonly used as a lipid concentration lowering drug to reduce blood plasma cholesterol levels in the clinic. However, the therapeutic effects of this drug are negatively impacted by its poor water solubility and low oral absorption efficiency. In this study, a PEGylated G5 PAMAM dendrimer (G5-PEG) modified nanoliposome was employed to increase water solubility, transepithelial transport, and oral absorption of PB. The uptake mechanism was explored *in vitro* in Caco-2 cells with the results suggesting that the absorption improvement of G5-PEG modified PB-liposome (PB-liposome/G5-PEG) was related to P-glycoprotein (P-gp) efflux pump, but was independent of caveolae endocytosis pathways. Additionally, plasma lipid concentration lowering effects of PB-liposome/G5-PEG were evaluated *in vivo* in a LDLR−/− hyperlipidemia mouse model. Compared with saline treated group, treatment with PB-liposome/G5-PEG significantly inhibited the increase of plasma total cholesterol (TC) and triglyceride (TG) of mice induced by a high fat diet. Moreover, its lipid concentration lowering effects and plasma drug concentration were greater than PB alone or commercial PB tablets. Our results demonstrated that PB-liposome/G5-PEG significantly increased the oral absorption of PB and therefore, significantly improved its pharmacodynamic effects.

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Supporting Information

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Keywords

probucol; G5-PEG PAMAM dendrimer; nanoliposome; transepithelial absorption and mechanism; *in vivo* pharmacodynamic effects

1. Introduction

In recent research, probucol (PB), originally an antioxidant drug, has been found to decrease the concentrations of plasma total cholesterol and very low density lipoprotein cholesterol (VLDL-c), while also reducing atherosclerosis (AS) plaques and improving coronary restenosis by enhancing reverse cholesterol transport (RCT)[1–3]. Animal studies have also demonstrated the therapeutic effects of PB on cardiovascular diseases[1, 4, 5]. In addition to a lipid concentration lowering effect, PB also remarkably reduced lipid oxidation, delaying the progression of plaque formation. These effects make it a more promising drug than statins for prevention and cure of hyperlipidemia and AS in clinic.

Unfortunately, the therapeutic effects of this drug are significantly reduced by its poor water solubility (only 5 ng/mL in water) and low oral absorption efficiency (bioavailability only 2–8%)[6]. To achieve satisfactory lipid concentration lowering effects, patients have to increase PB dose at the expense of aggravating side effects including lowering high density lipoprotein cholesterol (HDL-c) and increasing the electrocardiogram (ECG) Q-T interval[7]. Although combining PB with statins can reduce the dose and negative side effects of PB, this strategy does not ameliorate the decreased concentration of HDL-c[8–10]. Therefore, improved strategies to increase the oral absorption efficiency, and thereby reduce side effects from high doses, are greatly needed for the clinic.

Preparation of nano-drug delivery systems is a general strategy to overcome problems of solubility and absorption of hydrophobic drugs, thereby improving their oral bioavailability. There are several kinds of PB nanoparticles reported in literature based on PB/polyvinylpyrrolidone/sodium dodecyl sulfate[11, 12] or PB/sodium dodecyl sulfate/methacrylic acid co-grinding systems[13, 14], self-microemulsion[15–17] and nano-suspension[18]. Chitosan was also used as a carrier and trimeric sodium phosphate as a cross-linking agent to prepare PB containing nanoparticles[19]. Although all of these PB nano-delivery systems made improvements to some extent, for example improving the solubility of PB, there are still many issues remaining unresolved with these strategies including residues of organic solvent from preparation, surfactant related toxicity, and storage stability of liquid pharmaceuticals. Perhaps the largest challenge stems from the fact that the therapeutic dose of PB is about 500 mg per day for an adult with a normal body weight, but drug loading percentages of the present PB nano-delivery systems are usually low (5 to 10 wt.%), making it difficult to achieve the dose demands in clinic[10–17]. Therefore, further pharmaceutical studies on nano-delivery systems for PB are in great demand.

Liposomes are vesicular structures formed by lecithin bilayers with cholesterol insertion to increase membrane rigidity[20]. Hydrophobic drugs can be easily encapsulated in liposome bilayers with a high loading percentage[21]. Encapsulation of hydrophobic drugs in liposomes not only significantly increases solubility of the drug, but also releases the drug at a

sustained and controlled rate[22]. In addition, components of liposome bilayer are very similar to that of the cell membrane, and therefore drugs encapsulated in liposomes can be easily delivered into the cells through confluence effects of the liposome with the cell membranes[23]. Although liposomes possess specific advantages in encapsulation and delivery of hydrophobic drugs, to the best of our knowledge there is no research related to PB liposomes to date.

Poly (amidoamine) (PAMAM) dendrimers, a class of highly branched polymers, have been demonstrated potential as drug delivery carriers due to their low polydispersity and nanoscopic size. Hydrophobic drugs can be attached on surface of dendrimer molecules electrostatically or be encapsulated in their non-polar interiors, resulting in increased water solubility and a sustained release of the encapsulated drugs[24]. In addition, PAMAM dendrimer can improve transepithelial permeation of the encapsulated drugs in the intestinal tract, although the mechanism remains unclear[24–28]. Despite these potential advantages, simple mixing of PB and dendrimers still results in a limited drug loading percentage and low storage stability[29].

A combined drug delivery system composed of dendrimer and liposome was designed to overcome drawbacks of traditional single drug delivery system and offer advantages to the system. This combined drug delivery system can increase encapsulation efficacy[30, 31] and modify the release rates of the loaded drugs[32, 33], improve bioactivities of the therapeutics[34], and promote the stability of the liposome membrane[35].

Our previous studies demonstrate that modification with 8% polyethylene glycol (PEG, MW 5000) not only reduced the cytotoxicity and hemolysis toxicity of generation 5 (G5) PAMAM dendrimer, but also significantly increased gene delivery efficiency of this material[36, 37]. In this study, PEG 5000 conjugated G5 PAMAM dendrimer (G5-PEG) was utilized to modify liposome as a combined formulation for the improvement of solubility, encapsulation, release, transepithelial transport and therapeutic effects of PB. Studies probing the mechanism of permeability enhancement of liposomal formulation of G5-PEG modified PB-liposome (PB-liposome/G5-PEG) were explored in Caco-2 cell monolayers (an intestinal absorption model) and plasma lipid lowering effects of PB-liposome/G5-PEG were studied *in vivo* in low density lipoprotein receptor deficient (LDLR−/−) mice.

2. Materials and methods

2.1. Materials

PAMAM dendrimers of generation 5 with $–NH_2$, –OH, and –COOH termination were purchased from Dendritech Inc (Midland, MI, USA). Cyclosporin A (CsA) and all other reagents at analytical grade were purchased from Sigma-Aldrich Chemical Company (Saint Louis, MO, USA). Probucol was purchased from Japanese Otsuka Corporation (Chiyodakum, Tokyo, Japan). Caco-2 cell line was bought from American Type Culture Collection (ATCC, Manassas, VA, USA). FITC tagged cholera toxin B subunit (CTB-FITC) was from Invitrogen (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and other cell culture related reagents were all purchased from GIBCO (New York, NY, USA). Rabbit-anti-caveolin-1 polyclonal antibody and goat-anti-

rabbit IgG-HRP were from Santa Cruz Biotechnology (Dallas, TX, USA). Ad-CAV-1 and vector Ad-Null were from Vector Biolabs (Philadelphia, PA, USA). Polystyrene 24-well Transwell® with filters of 3.0 μm mean pore size was from COSTAR (Acton, MA, USA).

2.2. PEGylated G5 PAMAM dendrimer

G5 PAMAM dendrimers were purified by dialyzing (cut off: 10 KDa) against distilled water (8 media changes), then centrifuged by Millipore tube (Amicon Ultra, cut off: 5 KDa). Purified G5 was collected by lyophilization.

8% of the terminal amine groups of G5-NH_2 PAMAM dendrimer were PEGylated by PEG 5000 to generate PEGylated G5 PAMAM dendrimer (G5-PEG) according to a previously published procedure[36].

2.3. Water solubility of PB in the presence of G5

To investigate the influence of different charges and terminal groups of dendrimers on solubility of PB, saturation concentration of PB in water was measured in the presence of different G5 PAMAM with different end-group terminations (G5-PEG, G5-NH_2, G5-COOH, and G5-OH). Briefly, excess PB (around 5 mg) was added to 2 mL water, which ensured a saturated condition. G5 dendrimers were added into the PB suspensions with equal molar concentrations of 174 nM dendrimer. Each suspension was mechanically shaken at 37 °C for 48 h and then centrifuged at 10000 rpm. PB concentrations in the supernatant were measured by HPLC (λ = 242 nm). Three repeats were conducted for each experiment.

2.4. Preparation and characterization of PB-liposome and G5-PEG modified PB-liposome

PB-liposomes were prepared by using a thin film dispersion method[38]. To prepare the lipid films, soybean lecithin (225 mg), cholesterol (25 mg) and PB (25 mg) were mixed in a 9:1:1 ratio and dissolved in 3 mL chloroform. Solvent was slowly removed by evaporation at 37 °C for 30 min in a rotary evaporator. The lipid films were then dried under vacuum overnight at room temperature. Multilamellar vesicles (MLVs) were prepared by hydrating the lipid film with 6 mL phosphate buffered saline (PBS, pH 7.5) and the resultant suspensions were sonicated for 30 min using a probe sonicator (at 25 HZôUP 50H, Hiescher, Germany) to achieve small unilamellar vesicles (SUVs) of PB-liposome.

To prepare G5-PEG modified PB liposome (PB-liposome/G5-PEG), the lipid film with PB was formed by the same method described above; however, 174 nM or 696 nM (4 × 174 nM) of G5-PEG in PBS was used to hydrate the lipid film to prepare PB-liposome/G5-PEG or PB-liposome/4(G5-PEG), respectively.

An Amicon® Ultra-15 3K ultrafiltration centrifugal tube (Millipore, USA) was used to remove unloaded free PB from the liposomes. Briefly, 6 mL PB-liposome or G5-PEG modified PB-liposome was added into the internal vial of the tube. Then 6 mL PBS (pH 7.5) was added in the liposomes followed by centrifugation (Eppendorf 5810R, USA) at 1672 g for 15 min. Free PB filtered out in PBS was discarded, and the PBS washing step was repeated for 5 to 6 times to remove all free PB from the liposomes.

The size and ζ-potential of PB-liposome and G5-PEG modified PB-liposome were measured using a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd, Worcestershire, UK), and the encapsulated percentage of PB in the liposomes was measured by HPLC.

2.5. Stability of the formulations in artificially gastrointestinal juice

To evaluate the stability of the PB/4(G5-PEG), PB-liposome and PB-liposome/4(G5-PEG) in the gastrointestinal environment of human body, artificial gastric juice (pH 1.89) and intestinal juice (pH 6.8) were prepared according to the China Pharmacopoeia, and *in vitro* stability of the three formulations in the above two juices were investigated. Briefly, 1 mL of each formulation was added into 9 mL of the artificial gastric or intestinal juice, and the mixture was stirred at 37 °C using a magnetic stirrer (MS-H280-Pro, China). At the time points of 2 h in the artificial gastric juice or 24 h in the artificial intestinal juice, 200 μL of samples were taken out and then demulsified with 800 μL methanol. The suspension was centrifuged (Eppendorf 5810R, USA) at 10450 g for 25 min, and the concentrations of PB in the supernatant were analyzed by HPLC. The stability of the three formulations were evaluated using a ratio of M/M_0 (M was the mass of PB at 2 h in the artificial gastric juice or 24 h in the artificial intestinal juice, and M_0 was the initial mass of PB in the three formulations before the experiment.

2.6. In vitro PB release

Three formulations of PB/4(G5-PEG), PB-liposome and PB-liposome/4(G5-PEG) were adjusted into an equal PB concentration of 1 mg/mL, and were aliquot into three dialysis bags (10 KD) respectively. The dialysis bags with 1 mL of the formulations were placed in glass vials containing 10 mL of artificial intestinal juice (pH 6.8 PBS) and maintained at 37°C in a shaker bath. At predetermined time intervals, 1 mL samples were taken out from the release medium and 1 mL fresh PBS (pH 6.8) were replaced in the vials. The concentrations of PB in the samples were determined by HPLC, and the accumulative release percentages of PB from the formulations were calculated and plotted.

2.7. Caco-2 Cell Culture

Caco-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) at 37 °C with an atmosphere of 95% oxygen and 5% CO_2. To make a complete DMEM, 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1% glutamine, 10,000 units/mL penicillin and 10,000 μg/mL streptomycin were supplemented in the cell culture medium. The cells were passaged at 80–90% confluence by using a 0.25% trypsin/ethylenediamine tetraacetic acid (EDTA) solution. Cell passages from 35 to 45 were used for the following experiments.

2.8. Cytotoxicity Assay

Cytotoxicity of PB, PB/G5-PEG, PB/4(G5-PEG), PB-liposome and PB-liposome/4(G5-PEG) was evaluated by MTT assay. Briefly, Caco-2 cells were seeded on a 96-well plate with a cell density of $2\times l0^4$ per well and grew to 80–90% confluence in the complete DMEM. The cell culture medium was changed to Hank's balanced salt solution (HBSS). PB (dissolved in dimethyl sulfoxide (DMSO)), PB/G5-PEG, PB/4(G5-PEG), PB-liposome and PB-liposome/4(G5-PEG) were added respectively in HBSS with a PB concentration of 20

mM and 174 nM of G5-PEG or 696 nM of 4(G5-PEG). After a 72 h incubation, the incubation medium was removed and 10 μL of MTT stock solution (5 mg/mL) in 200 μL fresh HBSS was added in each well, followed by a four-hour-incubation. Then, supernatant in each well was removed and 200 μL of DMSO was added into the well. The plate was shaken for 10 min at 37 °C to dissolve MTT crystals. Absorbance at 590 nm was measured by utilizing a SpectraMax M2 microplate reader (Bio-rad550, USA). Cell viability with each treatment was expressed as a percentage compared to cells grown in HBSS only, which was considered as 100% cell viability. Six repeats were conducted for each sample.

2.9. Transepithelial Transport

Caco-2 cell monolayers were used as an *in vitro* intestinal absorption model to study the transepithelial permeability of PB, PB/G5-PEG, PB-liposome, and PB-liposome/G5-PEG. Caco-2 cells were seeded onto a 24-well Transwell® with a density of 5×10^5 cells/mL. The monolayer integrity, or ability of the cells to provide their barrier function, was monitored by transepithelial electrical resistance (TEER). After the cells had been cultured for 21 days, monolayers with a TEER of 350–450 $\Omega\bullet cm^2$ were used for the transport studies.

Prior to transport experiments, the complete DMEM was changed to 500 and 1,500 μL HBSS buffer (with Mg^{2+} and Ca^{2+}) in the apical (AP) and basolateral (BL) compartments of the Transwell, respectively. Samples to be tested were added to the AP side of compartment and 200 μL samples were collected from the BL side of compartment at 0, 30, 60, 90, 120, 180 and 240 min, followed by supplying 200 μL of fresh HBSS. PB concentrations in the collected samples were determined by HPLC. The apparent permeability coefficients (Papp) were calculated according to following formula:

$$\mathrm{Papp} = (\mathrm{dQ}/\mathrm{dt}) \,/\, (\mathrm{A} \times \mathrm{C}_0) \;\; \mathrm{cm} \cdot \mathrm{s}^{-1}$$

dQ/dt : amount of drug transported across the monolayers per unit of time (mol • s^{-1}); A: surface area of polycarbonate film of the Transwell, in this model equivalent to an area of support film of 1.13 cm^2; C0: the initial drug concentration in the AP or BL side of the monolayers.

2.10. Mechanism of G5-PEG and liposome in improving the transport of PB across epithelium

The mechanism of G5-PEG and liposomal formulation to enhance the permeability of PB was explored using Caco-2 cells with a focus on the role of the P-glycoprotein (P-gp) efflux pump and caveolae endocytosis pathway.

2.10.1. P-glycoprotein (P-gp) efflux pump—Cyclosporin A (CsA), a P-gp inhibitor, was used to investigate the role of the P-gp efflux pump on transepithelial transport of PB and the liposome formulation across the Caco-2 cells.

Caco-2 cells were seeded onto a 24-well Transwell® and grew to be monolayers according to the procedure described above. Prior to experiments, 20 μM CsA was added in both the AP and BL side of the Transwell for 0.5 h. The medium was then replaced with fresh HBSS.

Samples to be tested were added in the AP or BL side of the Transwells in order to study their transepithelial transport from AP to BL side (AP→BL) or BL to AP side (BP→AL), respectively. Samples (100 μL) from the opposite side of the addition compartment of the Transwell were collected at 60, 120, 180 and 240 min, followed by supplying 100 μL fresh HBSS in the well. PB concentration in the collected samples was determined by HPLC. Efflux ratio (ER, P ratio) was calculated using the following formula: P ratio = Papp (BL→AP) / Papp (AP→BL).

2.10.2. Caveolae—To study the possibility of transepithelial transport of PB and PB-liposome/G5-PEG by the caveolae endocytosis pathway, Caveolin-1 (Cav-1), the most crucial protein to maintain the structure and function of caveolae, was upregulated by adenovirus (Ad-Cav-1) infection of Caco-2 cells.

Briefly, Caco-2 cells were seeded on 6-well plates at a cell density of 5×10^4 per well. After 24h cell culture, the cells were infected by Ad-CAV-1 (titer: 1×10^{10} pfu/mL) at multiplicity of infection (MOI) of 10 or 30. The infected cells were then incubated for 48 h to express Cav-1 protein. To evaluate the influence of adenovirus vector alone on the cells, parallel experiments were conducted by using an empty adenovirus vector (Ad-null, titer: 1×10^{10} pfu/mL) to infect the cells at an equal dose of MOI 30.

After 48 h infection, the cells were collected and western blot (WB) was employed to detect expression of Cav-1 in cell lysates by using a primary antibody of rabbit-anti-caveolin-1 polyclonal antibody (1:1500 dilution) and a second antibody of goat-anti-rabbit IgG-HRP (1:2000 dilution).

CTB, a marker of the caveolae endocytosis pathway, was used to confirm normal endocytosis functions of caveolae regulated by Ad-Cav-1 on the Caco-2 cells. The effects of upregulation of caveolae pathway on uptake of PB or PB-liposome/G5-PEG were measured by flow cytometry.

Transwell experiments were done under the same conditions as described above to analyze the role of Cav-1 and the caveolae pathway in the process of transepithelial transport of PB/G5-PEG and PB-liposome/G5-PEG across the Caco-2 cell monolayers.

2.11. In vivo studies

Male LDLR−/− mice with an age of 7 to 8 weeks were obtained from the animal department of Peking University Health Science Center (Beijing, China). The Laboratory Animal Care Principles (NIH publication no. 85–23, revised 1996) were followed, and the experimental protocol was approved by Animal Care Committee, Peking University Health Science Center. All mice were raised under a 12-hour light/dark cycle with free access to food and water.

LDLR−/− mice were randomly divided into 4 groups with 6 mice in each group. To develop a hyperlipidemia disease model, all of mice were fed for 4 weeks with a western-type diet (TD 96125, Harlan-Teklad; 42% of calories from fat, 43% from carbohydrates, 15% from protein). From day 0, the 4 groups of mice were intragastric administrated twice a day with

saline (NS), PB, commercial PB tablets, or PB-liposome/4(G5-PEG), respectively. All groups of mice receiving PB were treated at an equal PB dose of 133 mg/kg body weight per day. At the end of 4 weeks, blood samples of mice from each group were collected and plasma total cholesterol (TC) and triglyceride (TG) were determined. Plasma PB concentrations in the blood samples were measured by HPLC.

2.12. Statistical analysis

All data are presented as mean values ± standard error of mean (SEM) and analyzed by Student's t-tests. One-way analysis of variance (ANOVA) with Bonferroni correction was used for comparison among multiple groups. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Solubilization of PB by G5 dendrimers

Characterization data for G5-PEG are shown in Fig. S1. A molecular structure of PB (Fig. 1A) and a cartoon of a PB/G5 dendrimer complex (Fig. 1B) are shown in Fig. 1. After forming complexes with G5 dendrimers, the water solubility of PB was improved to different extents depending upon the surface functionalities. Fig. S2 shows the effects of surface groups and charges of the G5 PAMAM dendrimers on the aqueous solubility of PB. G5-PEG and G5-NH_2 showed a five to six folds improvement of aqueous solubility of PB as compared to G5-OH and G4.5-COOH. Fig. 1C illustrates that solubilization of PB by G5-PEG was highly concentration dependent. When the molar concentrations of G5-PEG increased to four times (696 nM) of the initial one (174 nM), the aqueous solubility of PB had a significant increase (3.7 fold), and reached to 1.5 μg/mL (a 300 fold increase compared to PB alone).

3.2. PB-liposome with or without G5-PEG modification

The cartoon in Fig. 2A illustrates the preparation process of G5-PEG modified PB-liposome. The average size of the PB-liposome and G5-PEG modified PB-liposome was determined as 115 ± 4.2 nm and 119 ± 3.7 nm, respectively. PB encapsulation efficiency in the PB-liposome and G5-PEG modified PB-liposome was 85 ± 1.45% and 84.3 ± 1.53%, respectively.

3.3. Stability of the formulations in artificially gastrointestinal juice

As shown in Table 1, the M/M_0 ratios of the three formulations are all above 85%, which indicates they are relatively stable in the gastrointestinal fluid. After 2 h stirring in the artificial gastric juice (pH 1.89), PB-liposome had relatively lower M/M_0 ratio (87.5±0.04% *vs* 94.7±0.01%, P=0.0545) than G5-PEG modified PB-liposome. In the artificial intestinal juice for 24 h, the M/M_0 ratios remained above 90% for all the three formulations, indicating their great stability in the intestinal tract.

3.3. *In vitro* PB release

Fig. 2B demonstrates that the accumulative PB release rate from PB/4(G5-PEG) in 24 h was 20.9 ± 1.1%, while PB-liposome and G5-PEG modified PB-liposome significantly increased

PB release rate, and reached to 49.7 ± 1.2% ($P<0.005$) and 52.8 ± 3.2% ($P<0.005$), respectively. There was no difference between PB-liposome and PB-liposome/4(G5-PEG) in PB release rate.

3.4. Effects of G5 and liposomal formulation on transepithelial transport of PB

An MTT assay (Fig. 3A) shows that viabilities of Caco-2 cells were all above 85% after incubating the cells with 20 mM PB or the PB formulations (PB concentration in the formulations was 20 mM). PB forming complexes with 174 nM or 696 nM G5-PEG (described as PB/G5-PEG or PB/4(G5-PEG), respectively) did not obviously affect the activity of the Caco-2 cells. Modification of the PB-liposome with 696 nM of G5-PEG (described as PB-liposome/4(G5-PEG)) did not result in acute toxicity to the Caco-2 cells. The results demonstrate that the formulations containing high concentration (696 nM) of G5-PEG, such as PB/4(G5-PEG) and PB-liposome/4(G5-PEG), employed in the cellular experiments were all safe to Caco-2 cells.

Fig. 3B illustrates that the transepithelial transport of PB across Caco-2 cell monolayers was significantly increased when 4(G5-PEG) (696 nM) was applied to form complexes with PB. Although it significantly increased water solubility of PB (Fig. S2), G5-NH_2 (174 nM) failed to improve transepithelial transport of PB, even when higher molar concentration of 4(G5-NH_2) (696 nM) was applied (Fig. S3). Comparing Fig. 3C with Fig. 3B, transepithelial transport of PB was increased about 2 folds by liposomal formulation, and modification with 4(G5-PEG) (696 nM) more significantly enhanced the transepithelial transport of PB-liposome (Fig. 3C). These enhancement effects not only depended on the concentrations of G5-PEG, but were also related to the incubation time. With the presence of 4(G5-PEG), the transepithelial transport of PB (Fig. 3B) and its liposome (Fig. 3C) was greater increased within the first than the second 2h of incubation (data of PB-liposome are only shown the first 2h of incubation).

3.5. Mechanism of G5-PEG and liposomal formulation in improving the transport of PB across the epithelium

3.5.1. P-gp efflux pump—Fig. 4A shows that 20 μM CsA significantly improved Papp (AP→BL) of PB-liposome and PB-liposome/4(G5-PEG). In addition, Efflux P ratios of PB/4(G5-PEG) (Fig. 4B) and PB-liposome (Fig. 4C) were significantly reduced by addition of 20 μM CsA, which suggested increased PB transport from AP to BL side, and/or a decreased PB transport from BL to AP side of Caco-2 monolayers. The sum of these results demonstrates that the transepithelial transport of PB/4(G5-PEG), PB-liposome, and PB-liposome/4(G5-PEG) were all related to the P-gp efflux pump on Caco-2 cells.

3.5.2. Cav-1 protein and caveolae endocytosis pathway—WB results in Fig. 5A indicate that Cav-1 expression was successfully upregulated by Ad-Cav-1 at a dose of MOI 30, while adenovirus vector itself (Ad-null) almost had no influence on the expression of Cav-1 at an equal dose. Endocytosis functions of caveolae were confirmed through a significant increase of CTB-FITC uptake in Caco-2 cells facilitated by the adenovirus expressed Cav-1 (Fig. 5B). By way of comparison, Transwell experiments indicated that transepithelial transport of PB/4(G5-PEG) (Fig. 6A), and PB-liposome/4(G5-PEG) (Fig. 6C)

were not influenced by upregulation of Cav-1 expression on Caco-2 cells. Even the transport of PB-liposome had a decreasing trend after Cav-1 expression was upregulated, there was no significant difference (Fig. 6B). The results demonstrate that the permeability enhancement of G5-PEG and liposomal formulation to PB was not related to Cav-1 and the caveolae endocytosis pathway in Caco-2 cells.

3.6. In vivo lipid concentration lowering effects of G5-PEG modified PB-liposome

Fig. 7B&C demonstrate that plasma TC (B) and TG (C) levels of LDLR−/− mice were significantly decreased in the PB-liposome/4(G5-PEG) treated group compared with that of the saline treated group, but both TC and TG levels did not change in the groups treated with either PB or commercial PB tablets. Plasma PB concentrations from the PB-liposome/4(G5-PEG) treated group were 5 ($P<0.005$) and 2 ($P<0.01$) times higher than those from the PB and the commercial PB tablets treated groups, respectively (Fig. 7A). The results demonstrate that G5-PEG modified liposome significantly increased the oral absorption of PB, and therefore, significantly improved its lipid concentration lowering effects.

4. Discussion

In this research, we investigated and reported the effects of G5-PEG modified PB-liposome by *in vitro* transepithelial transport and *in vivo* oral absorption of PB. First, we found that all four surface functionalities of G5 dendrimers investigated improved the solubility of PB in water. In all cases, the hydrophobic drug could be encapsulated in the internal hydrophobic cavities of the G5 dendrimer molecules. Positively charged G5-NH_2 and G5-PEG improved the water solubility of PB substantially more than negatively charged G4.5-COOH or neutral G5-OH did. This agrees with our previous results, which indicate that positively charged dendrimers expand in water whereas the neutral dendrimers collapse on themselves[39, 40], that might make the internal spaces of positively charged dendrimers more available than neutral dendrimers. Therefore, PB is much more soluble in G5-NH_2 or G5-PEG than in G4.5-COOH or G5-OH.

Although it was less effective than G5-NH_2, PEG 5000 could also increase PB solubility in water because of its hydrophilic effects (Fig. S2). Therefore, modification of G5-NH_2 with PEG 5000 resulted in the material with the best PB solubilization capability (Fig. S2).

The solubilization of PB by G5-PEG was concentration dependent, since more dendrimer molecules would be available to encapsulate more drugs at higher concentrations. It was seen that when molar concentrations of G5-PEG were elevated to four times higher than the initial one, its solubilization effect increased rapidly (Fig. 1C).

Although G5-NH_2 and G5-PEG solubilized PB to a similar degree (Fig S2), G5-NH_2 failed to improve transepithelial transport of PB on Caco-2 cell monolayers, even when a high concentration of 696 nM G5-NH_2 was employed to form PB/4(G5-NH_2) complex (Fig S3). By comparison, G5-PEG not only significantly improved solubility of PB, but it also significantly increased transepithelial transport of PB (Fig 3B). The results suggest that absorption enhancement of G5-PEG to PB also related to mechanisms other than

solubilization. For example, inhibition of P-gp on the Caco-2 cell membranes, which was investigated in this work.

Liposomes are an effective approach solving the problems of poor water solubility and low oral absorption for hydrophobic drugs[20]. Our results demonstrate that liposomal formulation significantly increased encapsulation, release rate (Fig. 2B) and transepithelial transport (Fig. 3C) of PB. G5-PEG was chosen to modify PB-liposome, since it gave the best improvement to the solubility (Fig. S2) and permeability (Fig. S3) of PB as compared to the other three investigated G5 dendrimers. Although modification of PB-liposome with G5-PEG did not significantly change encapsulation and release rate (Fig. 2B) of PB, it protected PB-liposome from being degraded in the gastric fluid (Table 1). Besides, the hydrophilic characteristic of G5-PEG might enable it to attach to the external aqueous phase and also enter the internal water phase of the liposome (Fig. 2A), which stabilized the liposomal formulation[36]. On the other hand, PAMAM dendrimer has good permeability across cell membranes, which might help to promote transmembrane transport of liposomes[41, 42]. Our results show modification of PB-liposome with 696 nM G5-PEG significantly increased the transepithelial transport of PB (Fig. 3C).

Strategies for PAMAM dendrimer platforms promoting drug absorption have been mainly focused on two mechanisms: 1) intracellular endocytosis into cells *via* proteins or receptors on the cell membranes[43–45], and 2) intercellular transport by disturbing and opening tight junctions of the cells[46]. Liposomes provide a third mechanism, namely amalgamation or fusion of the liposome bilayers with the cell membrane. However, specific mechanisms of how G5-PEG modified liposome promote drug transmembrane transport has remained unknown. The roles of the P-gp efflux pump and caveolae pathway in endocytosis were investigated in this paper.

P-gp is a 170 kD transmembrane glycoprotein belonging to the ATP binding cassette (ABC) transporter carrier protein family. It inhibits absorption of drugs by an energy dependent discharge of drug from the intracellular side to extracellular side of cells[47]. Correlation of drug transport with P-gp can be determined by testing Papp and P ratio of the drug. If the Papp value remains constant over the entire concentration range, passive diffusion is considered to be the main transport mechanism. In this study, the Papp value of PB increased with concentration, suggesting that the permeability enhancement of PB/G5-PEG did not follow a simple passive diffusion mechanism. Moreover, with 20 μM CsA, Papp (AP→BL) significantly increased for both PB-liposome and PB-liposome/4(G5-PEG). Besides, P ratios of PB/4(G5-PEG) and PB-liposome decreased. The results demonstrate that absorption of PB promoted by G5-PEG, liposome, or G5-PEG modified liposome was all modulated by P-gp efflux pump. Inhibition of P-gp by CsA contributed to increase the transepthelial transport of PB/G5-PEG, PB-liposome and PB-liposome/G5-PEG.

Caveolae is a concave structure of the cytoplasm membrane with a variety of forms and can be found in many types of cells[48]. Caveolin-1 (Cav-1) is the most important protein to maintain the structure and functions of caveolae. Therefore, in order to study the relationship of the transmembrane transport process of G5-PEG modified PB-liposome and the caveolae endocytic pathway, Cav-1 expression was upregulated in Caco-2 cells by adenovirus

infection. The uptake of CTB, a caveolae endocytosis pathway marker, significantly increased after Ad-CAV-1 infection in Caco-2 cells (Fig. 5B), which indicates that upregulation of Cav-1 by the adenovirus can facilitate the normal endocytosis function of caveolae. By way of comparison, transwell studies (Fig. 6) indicate that the transport of PB-liposome/4(G5-PEG) was not proceeded by the caveloae pathway. Additional mechanistic studies are needed to further clarify the other possible mechanism for transepithelial transport of G5-PEG modified PB-liposome.

As PB is commonly used as a lipid-lowering drug to reduce plasma cholesterol and triglyceride concentrations in clinic, we studied oral pharmacodynamics of PB-liposome/4(G5-PEG) *in vivo* in LDLR−/− mice with PB and commercial PB tablets as two controls. LDLR−/− mice can be induced to hyperlipidemia after being fed with a high fat diet for one month. The plasma TC level from the saline treated mice group increased to 600–900 mg/dL after four weeks, which meant severe hypercholesterolemia was successfully developed in LDLR−/− mice. By way of comparison, treatment with PB-liposome/4(G5-PEG) significantly inhibited the increase of plasma TC (Fig. 7B) and TG (Fig. 7C) levels of mice, while treatment with either PB or commercial tablets resulted in no significant difference in plasma TC and TG levels (Fig. 7). The results demonstrate that PB-liposome/4(G5-PEG) had better lipid concentration lowering effects than PB or commercial PB tablets. Additionally, mice body weights from each group showed no difference (Fig. S4), which meant food intake in each group was equal. Therefore, the greater lipid concentration lowering effects of PB-liposome/4(G5-PEG) than PB or commercial PB tablets were not from weight loss of the mice, but from the greater solubilization, encapsulation, stability, PB release, and absorption of PB improved by G5-PEG modified liposome. Plasma drug concentration also confirmed this point, which showed that plasma PB concentrations from PB-liposome/4(G5-PEG) treated mice group were nearly five times higher than those from the PB treated group (Fig. 7A).

5. Conclusions

G5-PEG modified liposome formulation significantly increased the water solubility and transepithelial transport of PB. Enhancement mechanisms were related to P-gp efflux pump, but were independent of caveolae endocytosis pathways. Compared with PB alone and commercial PB tablets, PB-liposome/4(G5-PEG) significantly improved plasma concentration of PB in LDLR−/− mice after oral administration, which resulted in a dramatic inhibition of the elevation of plasma TC and TG levels induced by high fat diet.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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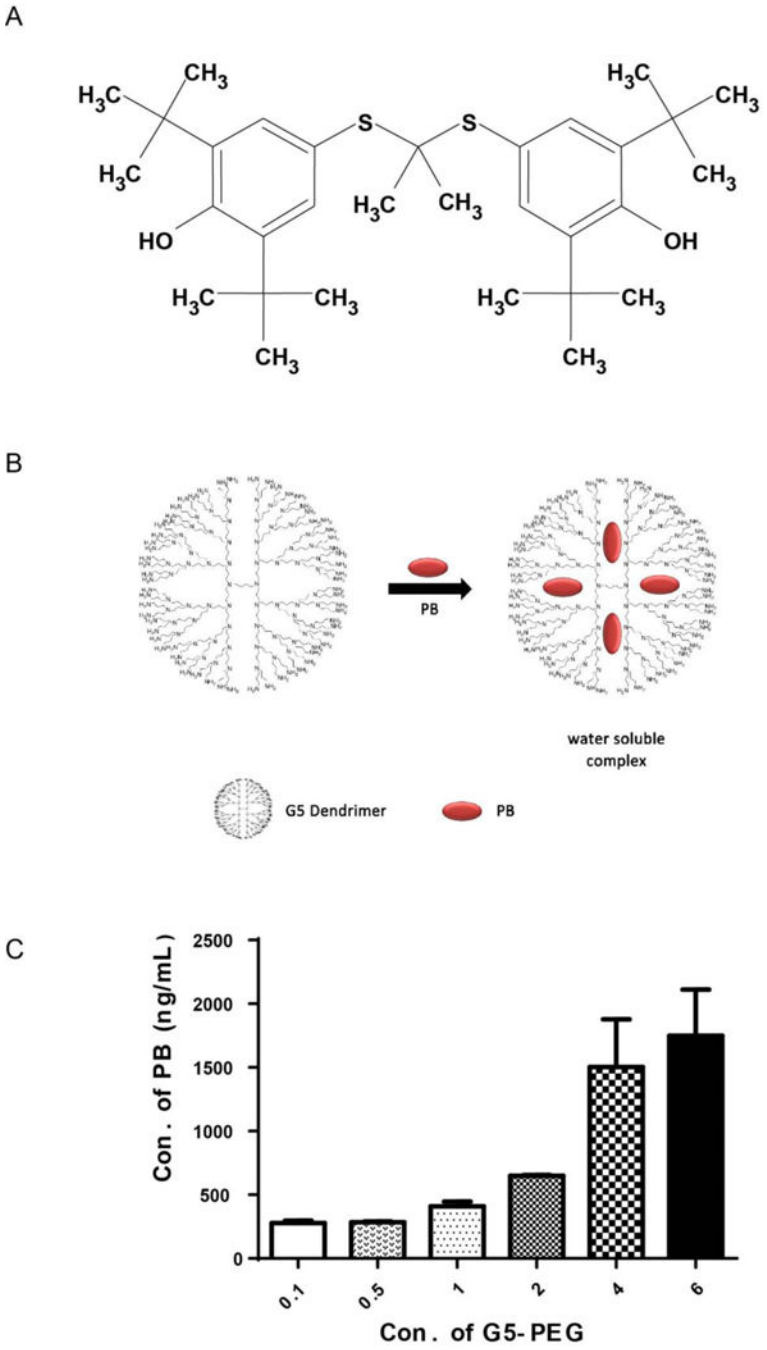

FIG. 1. Chemical structure of PB (A), cartoon of PB/G5 complex (B) and concentration-dependent solubilization of G5-PEG to PB (C)

In cartoon B, red dots represent PB, blue scaffold is schematic dendrimer molecule. In Fig (C), the horizontal axis is the fold of molar concentration of G5-PEG. For example: 1 fold equals to 174 nM, 4 folds equal to 696 nM, and so on.

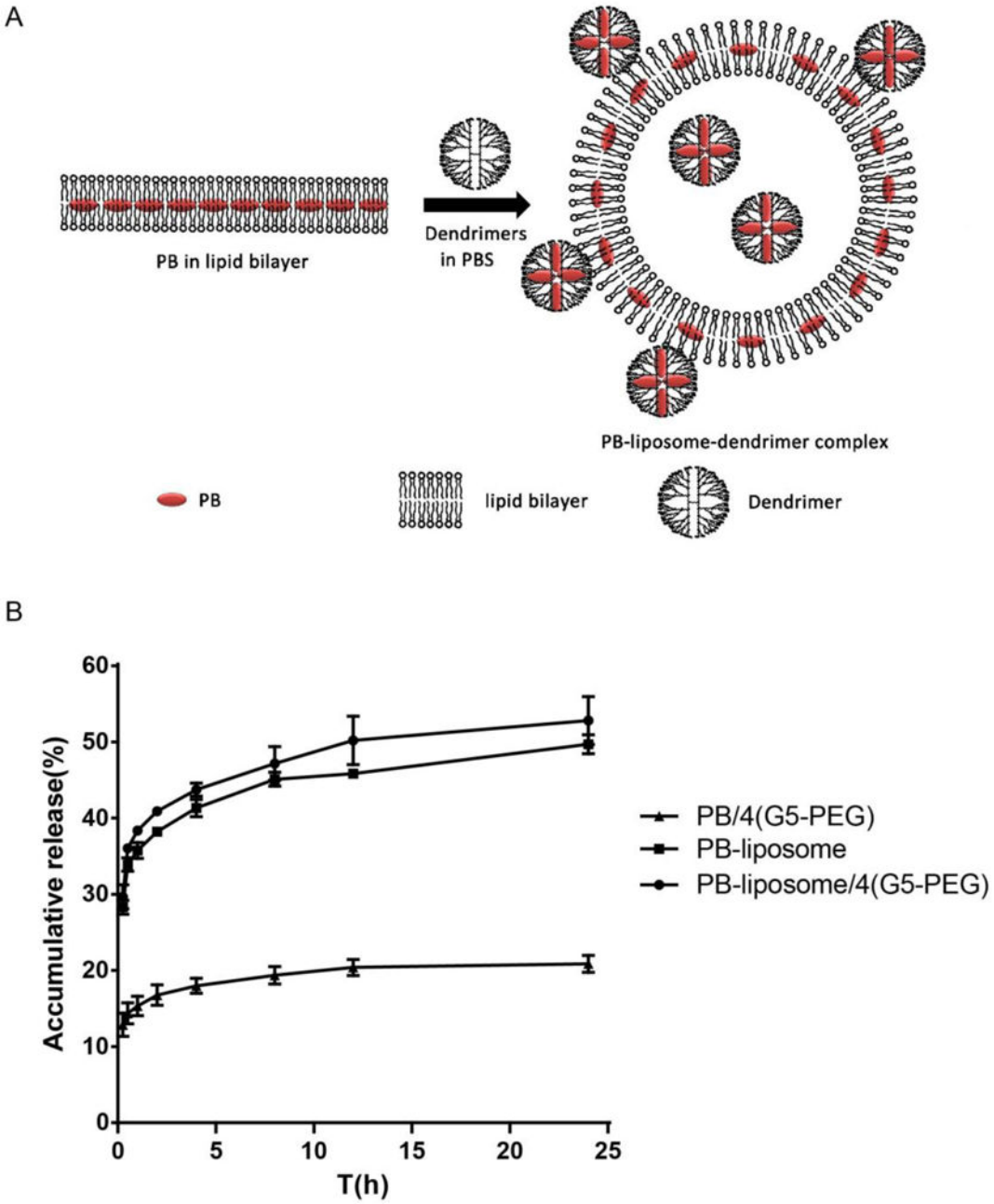

FIG. 2. *In vitro* PB release profiles

(A) Cartoon of preparation process of G5-PEG modified PB-liposome. Red dots represent PB. (B). *In vitro* PB release profiles of PB/4(G5-PEG), PB-liposome, and PB-liposome/4(G5-PEG) in an artificial intestine juice (pH 6.8 PBS). ***p<0.005 PB-liposome/4(G5-PEG) or PB-liposome *vs.* PB/4(G5-PEG) at all the investigated time points. n=3.

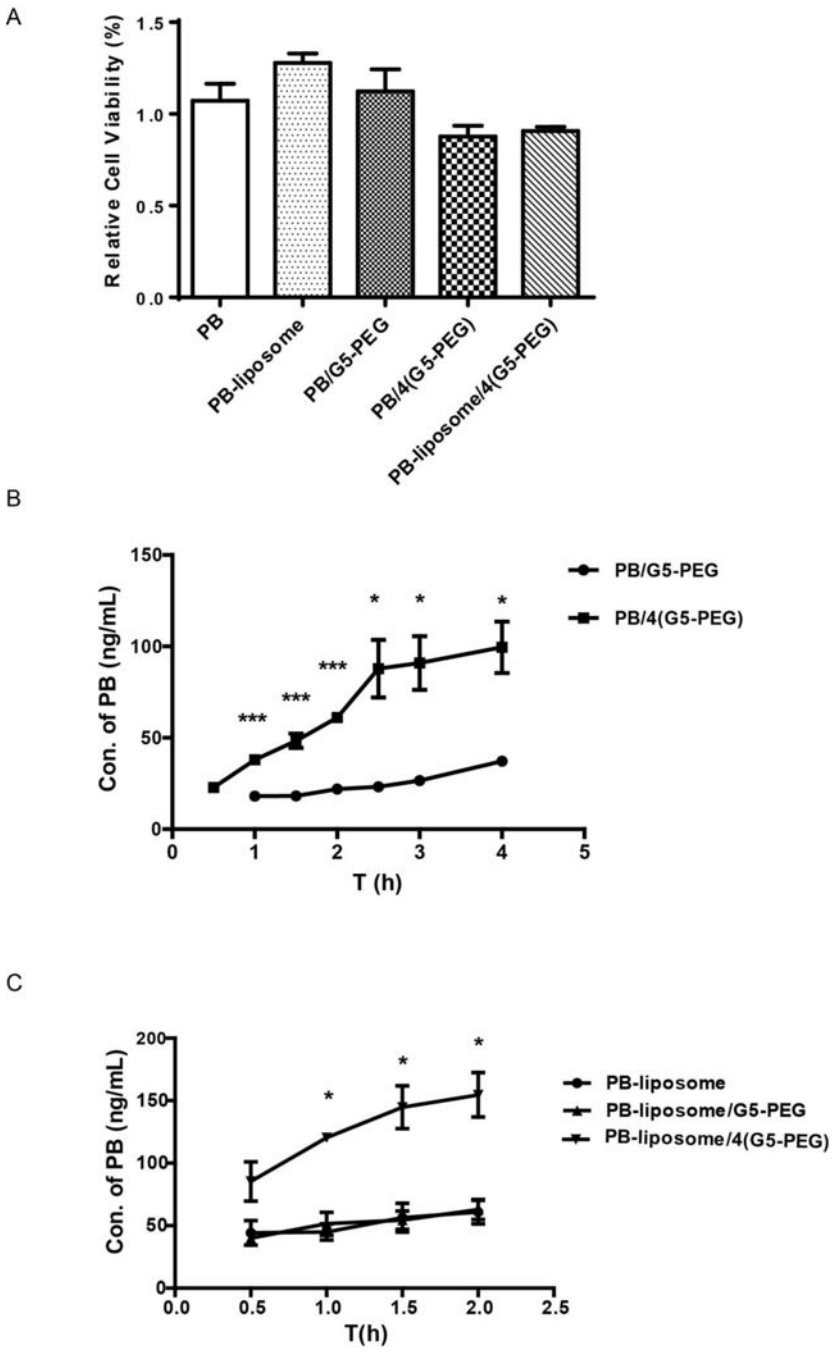

FIG. 3. Effects of G5-PEG and liposomal formulation on the transepithelial transport of PB across Caco-2 cell monolayers

(A) Caco-2 cell viabilities in the presence of PB, PB-liposome, PB/G5-PEG, PB/4(G5-PEG) and PB-liposome/4(G5-PEG). n=6. (B) Effect of G5-PEG concentrations on the transport of PB across Caco-2 cell monolayers over a period of 4 h. *p<0.05, ***p<0.001 *vs.* PB/G5-PEG. n=3. (C) Effects of G5-PEG concentrations on transport of PB-liposome across Caco-2 cell monolayers over a period of 2 h. *p<0.05 vs. PB-liposome. n=3.

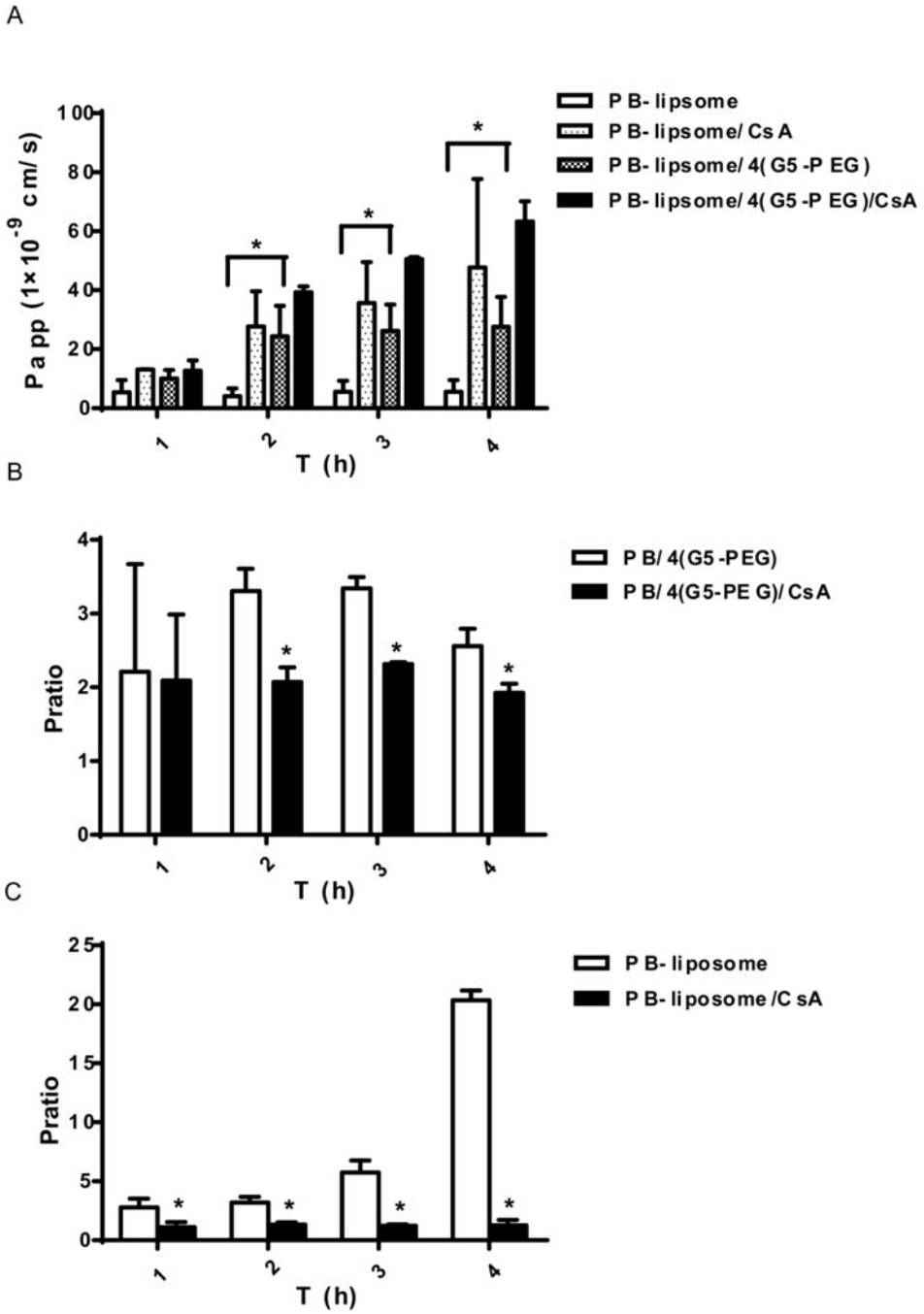

FIG. 4. Effects of CsA on transepithelial transport of PB/4(G5-PEG), PB-liposome and PB-liposome/4(G5-PEG) in Caco-2 cell monolayers

(A) Papps of PB-liposome and PB-liposome/4(G5-PEG) before and after 20 μM CsA being applied. (B & C) Efflux P ratios of PB/4(G5-PEG) (B) and PB-liposome (C) before and after 20 μM CsA being applied. P ratio=Papp (BL-AP)/Papp (AP-BL). *$p<0.05$ *vs.* data without CsA. n=3.

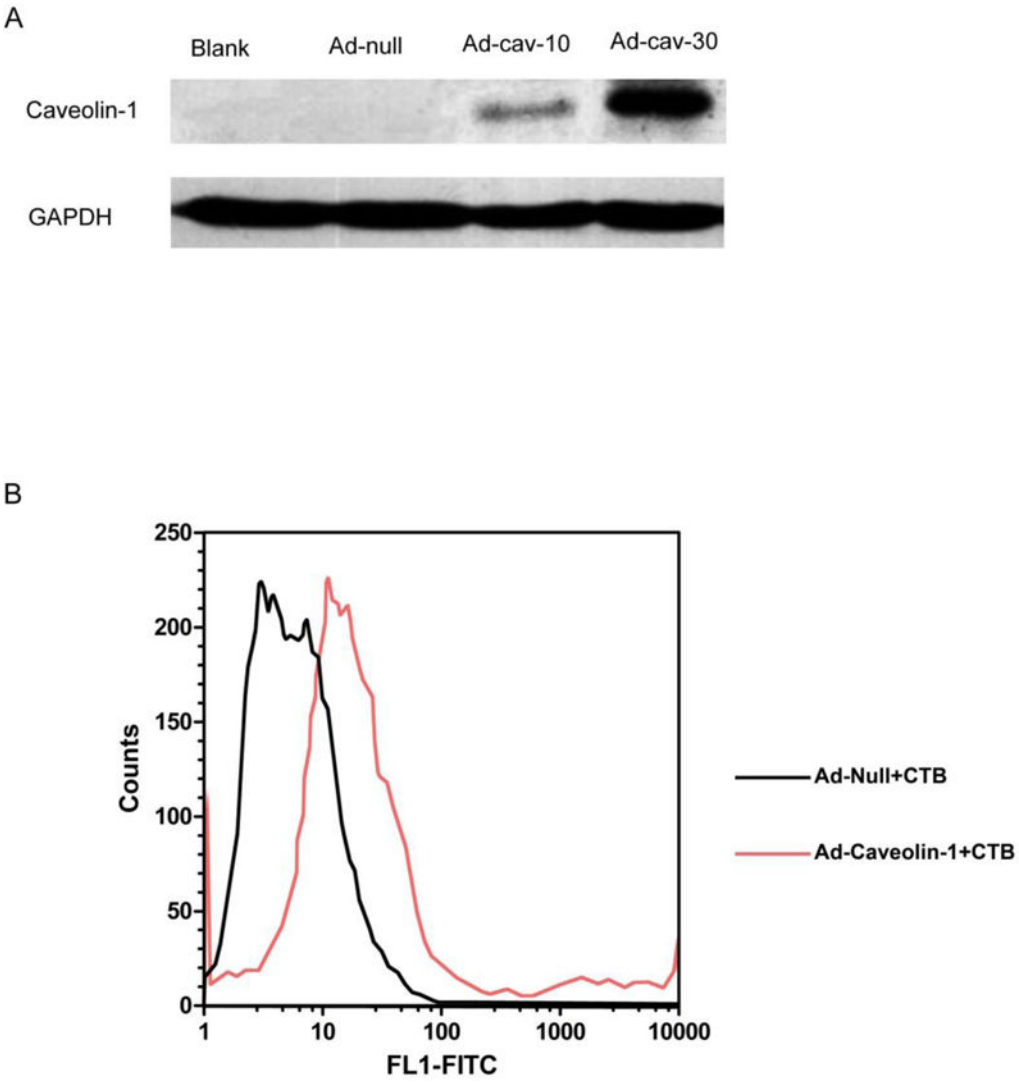

FIG. 5. Effects of upregulation of Cav-1 on uptakes of CTB in Caco-2 cells

(A) Cav-1 expression detected by western blot before and after the cells being infected by Ad-Cav-1 and with GAPDH as an internal control. (B) Uptakes of CTB-FITC before and after Cav-1 being upregulated in Caco-2 cells.

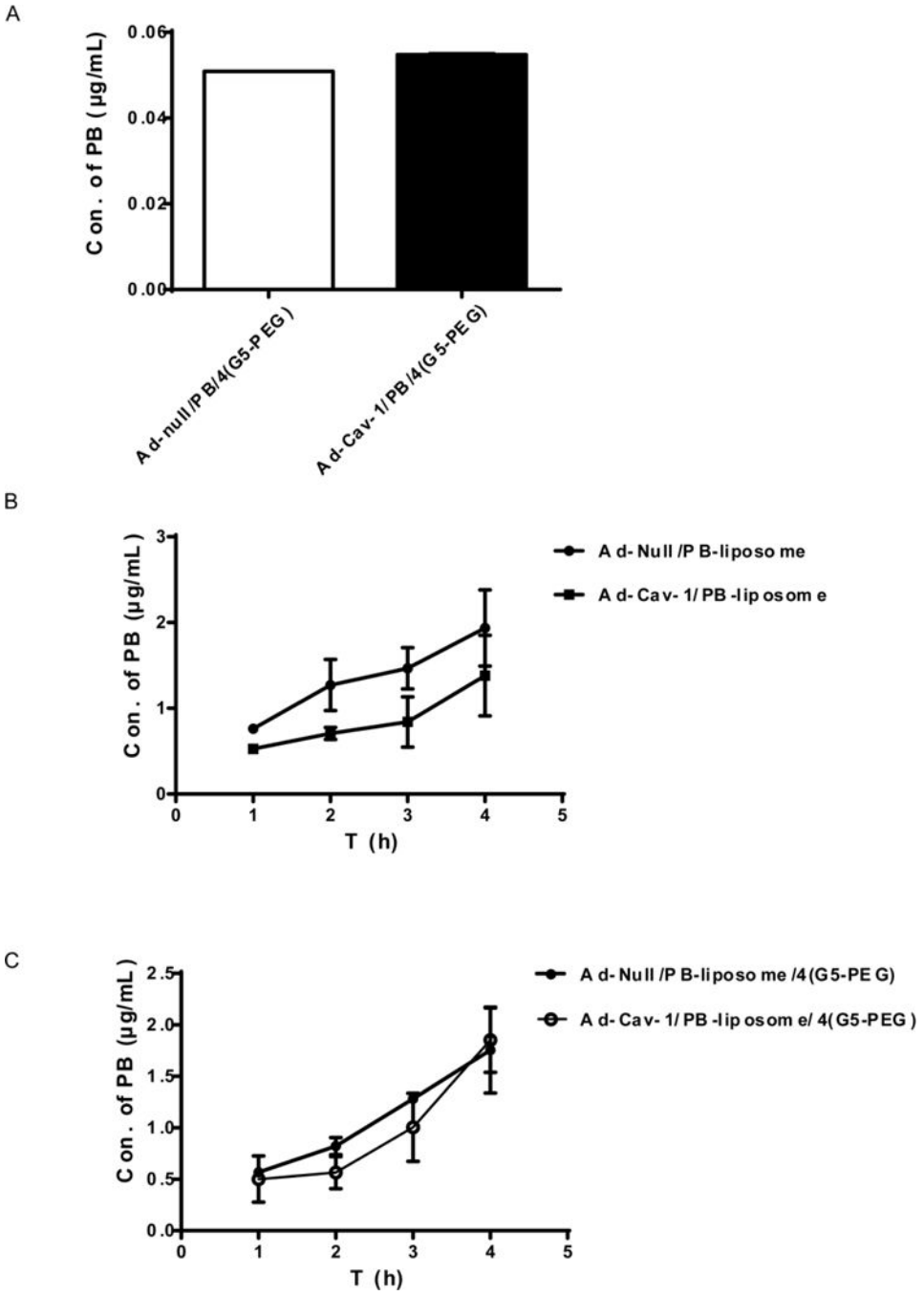

FIG. 6. Effects of upregulation of Cav-1 on transepithelial transport of PB/4(G5-PEG) (A), PB-liposome (B), and PB-liposome/4(G5-PEG) (C) in Caco-2 cell monolayers

Fig (A) was a sum of 4h transport.

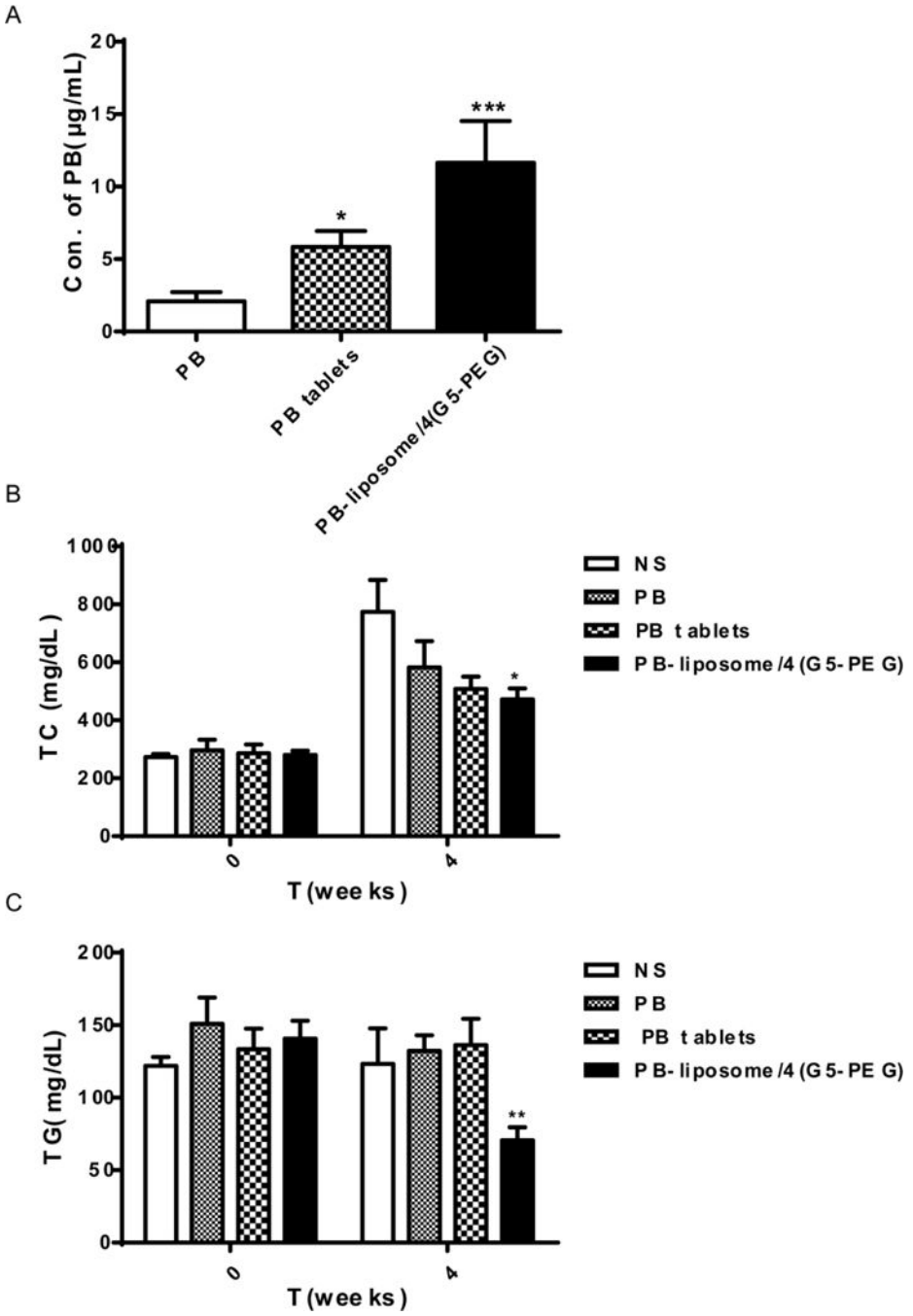

FIG. 7. Plasma PB concentrations and lipid concentration lowering effects of G5-PEG modified PB-liposome in LDLR −/− mice. (A) Plasma PB concentrations of mice from each group determined by HPLC. *p<0.05, ***p<0.001 *vs.* PB. (B) Plasma total cholesterol level of mice from each group. *p<0.05 *vs.* NS. (C) Plasma TG level of mice from each group. **p<0.01 vs. NS. NS means saline-treated group. n=6. All data were measured after the mice having been treated with the formulations in company with high fat diet for 4 weeks.

Table 1

The stability of the three formulations in the artificially digestive tract fluid. (n=3, mean±SD)

Formulations	M/M_0 ratios of the formulations (%)	
	Artificial gastric juice (pH1.89, 2 h)	Artificial intestinal juice (pH6.8, 24 h)
PB/4(G5-PEG)	91.9±0.14	91.1±0.002
PB-liposome	87.5±0.04	95.4±0.07
PB-liposome/4(G5-PEG)	94.7±0.01	93.4±0.10