Sustained delivery by a cyclodextrin material-based nanocarrier potentiates antiatherosclerotic activity of rapamycin via selectively inhibiting mTORC1 in mice

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Increasing evidence has demonstrated special advantages of the nanomedicinal approach for the management of cardiovascular disease. We hypothesize that sustained delivery of rapamycin (RAP) may provide more desirable therapeutic effects than traditional oral administration by selectively inhibiting mammalian target of rapamycin complex 1 (mTORC1) signaling. To evidence this assumption and develop an effective, safe, and translational nanotherapy for atherosclerosis, this study was designed to examine antiatherosclerotic efficacy of a RAP nanotherapy based on an acetalated (β-cyclodextrin (Ac-bCD) material in apolipoprotein E-deficient (ApoE−/−) mice. First, biodegradable and biocompatible materials of Ac-bCDs were synthesized by kinetically controlled acetalation, giving rise to carrier materials that may not generate acidic byproducts after hydrolysis. Then RAP-loaded nanoparticles based on various Ac-bCDs were prepared by a nanomulsion technique, which can sustain drug release for different periods of time, depending on the composition of Ac-bCDs. For a RAP/Ac-bCD180-derived nanotherapy (RAP-NP) that may continue RAP release for up to 20 days in vitro, it afforded constant drug levels in both the blood and aortic tissue after subcutaneous injection, while orally administered free RAP showed typical peak-valley profiles with remarkably high peak concentrations. Therapeutic studies conducted in an experimental model of atherosclerosis established in ApoE−/− mice revealed that RAP-NP significantly reduced the formation of atherosclerotic lesions and dramatically enhanced the stability of plaques, which was more efficacious than orally delivered free RAP. Moreover, rupture-prone proinflammatory factors in both serum and aortas were significantly decreased after treatment. Whereas oral administration of RAP simultaneously inhibited mTORC1 and mTORC2 in the aorta, sustained delivery by RAP-NP selectively suppressed mTORC1, agreeing with in vitro results in smooth muscle cells. These findings demonstrated that antiatherosclerotic activity of RAP may be considerably improved by sustained release via the Ac-bCD derived nanocarrier, which was achieved through selectively inhibiting mTORC1.

1. Introduction

Cardiovascular disease remains the leading cause of morbidity and mortality in developed countries. Etiologically, the dominant trajectory involves atherosclerosis, a chronic inflammatory process characterized by the formation of atheromatous plaques in the intima of arteries that can cause life-threatening coronary artery disease, carotid artery disease, stroke, and peripheral vascular disease [1–4]. Pharmaceutical intervention is probably the most effective regimen for the prevention and therapy of atherosclerotic diseases [3,5,6]. Besides the frequently prescribed lipid-lowering statins, therapeutics including inhibitors of cholesterol biosynthesis or absorption, antioxidants, and vaccines have been broadly investigated for the management of atherosclerosis [7]. Although certain success has been achieved and risk can be reduced by existing therapies, tremendous challenges remain in the development of more efficient drugs and strategies [3].

Recent insight that atherosclerosis is an inflammatory disease offers new opportunities for discovering new therapeutic agents for prevention and treatment of atherosclerosis and its complications, leading to
the identification of several promising targets [5]. These intriguing findings have been translated into the clinical development of drugs such as selective phospholipase A2 inhibitors and antitumor necrosis factor agents [8]. It has also been recognized that immune responses of vascular cells play significant roles in the etiology of atherosclerosis [9]. Immune cells including subsets of T cells and B cells are generally involved in the pathogenesis of atherosclerosis, and they have been considered to likely play pro-atherogenic roles. Accordingly, treatment with immunosuppressants also represents an attractive strategy. Besides, accumulating evidence has suggested that autophagy occurs in advanced atherosclerotic plaques [10]. Stabilization of vulnerable and rupture-prone lesions can be achieved by selectively activating autophagic death of macrophages [11]. These findings strongly imply that therapeutic agents with multifaceted activities are favorable for the treatment of atherosclerosis. Rapamycin (RAP), a potent immunosuppressant and an inhibitor of the mammalian target of rapamycin (mTOR) pathway, has been clinically used for transplantation. In view of its anti-inflammatory, immunosuppressive, anti-mitogenic, and anti-proliferative properties as well as its capability of activating autophagy, RAP may be a promising antiatherosclerotic agent. Both preclinical and clinical studies revealed that RAP is able to reduce neointima formation, and therefore prevent the development of instent restenosis after percutaneous transluminal coronary angioplasty [12,13], by inhibiting migration and proliferation of abnormal vascular smooth muscle cells (SMCs) [14]. In addition, recent studies suggested that RAP may attenuate inflammation, modulate atherosclerotic plaque, and enhance plaque stability [14–17]. However, current treatment regimens by oral delivery generally results in uncontrolled and fluctuated RAP concentrations in the blood, thereby leading to increased levels of plasma triglyceride (TG) and low-density lipoprotein (LDL) as well as adverse effects to other organs. Furthermore, it has been found that, stimulation by high doses of RAP can inhibit both mTOR complex 1 (mTORC1) and mTORC2 signaling pathways [18,19], while mTORC2 has multiple beneficial functions such as anti-inflammation, promoting endothelial cell survival and migration, and regulating RIT-mediated oxidative stress resistance [19–21]. These issues, together with the fact that chronic feature of atherosclerosis necessitates intervention for a long period of time, strongly suggests sustained delivery of RAP at low doses is highly desirable for the effective treatment of atherosclerosis without appreciable side effects.

On the other hand, the nanomedical strategy has been proposed as a new approach for the management of cardiovascular disease by site-specific cargo delivery for diagnosis and therapy [22–32]. However, the development of efficient targeting nanomedicines for atherosclerosis with clinical significance is still challenging [33,34]. First, due to the rapid clearance of intravenously (i.v.) injected nanoparticles by the mononuclear phagocyte system, their targeting efficiency to atherosclerotic lesions is low, even with delicate structure design and surface functionalization on therapeutic nanoparticles [28,31]. Second, the retention time for nanomedicines accumulated in the plaque is too short from the view point of chronic diseases. To maintain the minimum effective concentration for a drug to achieve desirable efficacy, frequent dosing by i.v. administration is essential for targeted nanomedicines, which may be poorly acceptable by patients over a long period of time [35]. Additionally, frequent administration may bring considerable economic and mental burden on patients’ daily lives. This undesirable risk to benefit ratio makes the bench-to-bedside translation of currently developed nanotherapeutics extremely difficult with respect to antiatherosclerotic therapy. Taking these issues into account, sustained delivery systems that can release payload in a controlled manner over a long period of time are particularly desirable.

Most recently, we have developed nanoparticulate platforms with excellent biocompatibility based on chemically modified cyclodextrins, which may degrade into water soluble and non-acidic parent compounds that have already been widely used in pharmaceutical products for many years [36–38]. Compared with frequently exploited biodegradable materials (such as polyesters and polyanhydrides) with acidic byproducts upon hydrolysis that may lead to local inflammation and therefore further aggravate chronic inflammation in cardiovascular disease [39,40], our new nanovehicles only caused slight tissue inflammatory response [36], thus making them satisfactory for the treatment of chronic inflammatory diseases. The main purpose of this study was to demonstrate our hypothesis that sustained delivery of RAP may provide more significant therapeutic effects over traditional administration by selectively inhibiting mTORC1 signaling in apolipoprotein E-deficient (ApoE−/−) mice. In addition, we attempted to examine safety profile of a new nanovehicle based on an acetalated β-cyclodextrin (Ac-bCD) material for the management of chronic disease. To this end, both in vitro and in vivo studies were performed to substantiate therapeutic advantages and long-term safety of the controlled delivery system of RAP/ Ac-bCD nanoparticles.

2. Materials and methods

2.1. Materials

β-cyclodextrin (β-CD), 2-methoxypropene (MP), and oil-red O (ORO) were purchased from Sigma-Aldrich (St. Louis, USA). Pyridinium p-toluene sulfonate (PTS) and poly(vinyl alcohol) (PVA) (88 mol% hydrolyzed, MW = 25 kDa) were obtained from Acro Organics. RAP was purchased from Beijing Huamai Biotechnology Co, Ltd. Ascomycin was supplied by Apollo Scientific, Ltd. (UK). Cyanine7.5 NHS ester (Cy7.5) was obtained from Lumiprobe, LLC (U.S.A.). All the other reagents are commercially available and used as received.

2.2. Synthesis of acetalated β-CD materials

Acetalated β-CD materials (Ac-bCDs) were synthesized according to our previously established procedures (Fig. 1A) [36]. Specifically, 4 g β-CD and 16 mL of MP were dissolved into 80 mL of anhydrous dimethyl sulfoxide (DMSO), into which 64 mg PTS was introduced. After acetalation for various periods of time, 10 mL of mixture solution was collected and the reaction was terminated by mixing with 0.1 mL triethylamine. The product was precipitated from deionized water, thoroughly washed with deionized water, and finally hophilized to give rise to white powder. The obtained materials were abbreviated according to the reaction time, i.e. Ac-bCD collected after 2.5 min of acetalation is denoted as Ac-bCD2.5. All materials were characterized by 1H NMR spectroscopy on a Varian INOVA-400 spectrometer operating at 400 MHz.

2.3. Fabrication of RAP-loaded Ac-bCD nanoparticles

RAP-loaded Ac-bCD nanoparticles were fabricated by an oil-in-water (o/w) emulsion solvent evaporation technique [37]. Briefly, 50 mg Ac-bCD and 20 mg RAP were dissolved in 1 mL of dichloromethane, which was emulsified into 6 mL of 1.0 wt% PVA aqueous solution by probe sonication at 118 W. The obtained o/w emulsion was then poured into 20 mL of 0.3 wt% PVA solution under magnetic stirring. After magnetic stirring at room temperature for 3 h, nanoparticles were collected by centrifugation at 21,752 g (Sigma 3 K18, Germany) for 10 min, and washed four times with deionized water. Similar procedures were followed to prepare Cy7.5-labeled Ac-bCD nanoparticles. In this case, both 2 mg Cy7.5 and 50 mg Ac-bCD180 were dissolved in dichloromethane to give an oil phase.

2.4. Characterization of RAP/Ac-bCD nanoparticles

The size and size distribution of RAP/Ac-bCD nanoparticles were measured by dynamic light scattering with a Malvern Zetasizer Nano ZS instrument at 25 °C. Scanning electron microscopy (SEM) observation was performed on a S-3400N II electron microscope (Hitachi, Japan). Transmission electron microscopy (TEM) was taken on a

**References**

TECNAI-10 microscope (Philips, Netherland) operating at an acceleration voltage of 80 kV.

2.5. Quantiﬁcation of the RAP content in RAP/Ac-bCD nanoparticles

To quantify the drug content, 5.0 mg freeze-dried RAP-loaded nanoparticles was thoroughly dissolved in 10 mL of DMSO. Then the RAP concentration was determined by UV measurement at 278 nm. The drug loading content and entrapment efﬁciency were calculated according to the following equations.

Drug loading content (%) = \[
\frac{\text{Weight of RAP in nanoparticle}}{\text{Weight of RAP-loaded nanoparticle}} \times 100\%\]

Entrapment efficiency (%) = \[
\frac{\text{RAP content in nanoparticle} (%)}{\text{Theoretical RAP content} (%)} \times 100\%\]

2.6. In vitro release study on RAP/Ac-bCD nanoparticles

For a standard in vitro release test, 5.0 mg RAP-containing nanoparticles in 0.2 mL of deionized water was placed into dialysis tubing (MWCO: 3500 Da), which was immersed into 10 mL of PBS (0.01 M, pH 7.4). At predetermined time intervals, 4.0 mL of release medium was withdrawn, and fresh PBS was supplemented. The RAP concentration in release buffer was quantified by UV at 278 nm.

2.7. Animals

All the animal care and experimental protocols were performed according to the guidelines for the Care and Use of Laboratory Animals of Third Military Medical University. Male C57BL/6 mice (22–25 g) were obtained from the Animal Center of the Third Military Medical University, while male ApoE−/− mice at 8 weeks of age were supplied by the
ApoE knockout mice were acclimatized to the laboratory for at last 7 days for further experimentation.

2.8. In vivo pharmacokinetic study

Forty male C57BL/6 mice were administered with RAP/Ac-bCD180 nanoparticles (RAP-NP; with mean size of 250 nm) via subcutaneous injection at 3.0 mg/kg of RAP. At each time point, the blood samples and the whole aortas were collected from four animals. The accurately weighed aortic tissue was grinded with 500 μL of saline. Previously established methods were then employed to extract RAP from blood and aortic tissues [15]. The RAP concentration was quantified by liquid chromatography-mass spectrometry. Similar procedures were followed to examine the pharmacokinetic profiles of orally administered RAP in C57BL/6 mice at 3.0 mg/kg. In this case, raw RAP was dispersed in saline, and administered by oral gavage.

2.9. Experimental atherosclerosis in ApoE−/− mice

ApoE−/− mice were received normal diet containing 0.25 wt% cholesterol and 2 wt% lard for three months during the whole experiment. After one month of Western diet, forty mice were randomized into five groups (n = 8) and subjected to various treatments for additional two months: one control group treated with saline; one group received free RAP by oral gavage at 3.0 mg/kg every three days; two groups treated with subcutaneous injection of RAP-NP (with mean size of 250 nm) every fifteen days at 1.0 and 3.0 mg/kg of RAP, respectively; and one group of the vehicle control treated with blank Ac-bCD180 nanoparticles. In a separate study, free RAP dispersed in saline was subcutaneously administered at 3.0 mg/kg in ApoE−/− mice every fifteen days. During treatment, changes in the body weight of mice were monitored.

2.10. Examination on biodistribution of subcutaneously injected nanoparticles

Biodistribution of subcutaneously administered nanoparticles was examined by fluorescence imaging. To this end, Cy7.5-labeled Ac-bCD180 nanoparticles containing 0.2 mg Cy7.5 were injected in ApoE−/− mice with established atherosclerosis. At pre-defined time points, the presence of Ac-bCD nanoparticles at the subcutaneous site of interest was detected by a living imaging system (IVIS Spectrum, PerkinElmer, U.S.A.). Subsequently, mice were euthanized. The blood samples and aortic tissues were collected for ex vivo fluorescence imaging.

2.11. Quantification of atherosclerotic plaques

Post different treatments, mice were sacrificed after anesthesia. The degree of pathological changes was determined by measuring the lesion area of aortas from the heart to the iliac bifurcation. Briefly, the aorta was fixed by perfusion with formalin (10% in PBS) for 50 min. After the periadventitial tissue was cleaned, the aorta was opened longitudinally, and then the entire aorta was stained with ORO to quantify the plaque area. To evaluate the extent of atherosclerosis at the aortic origin and brachiocephalic artery, the tissues embedded in Tissue-Tek® OCT™ compound (Sakura Finetek Inc. USA) were cross-sectioned serially at 8-μm intervals and stained by ORO for quantifying the area of atheroma plaques. Analysis of the plaque area was carried out with Nis-Elements BR 3.2 software (Nikon, Japan).

2.12. Histology and immunohistochemistry analyses

The aortic sinus was fixed in 10% formalin for 50 min, and then it was embedded in paraffin and cut into sections. The 6-μm sections were stained with hematoxylin-eosin (H&E) for histopathological analysis. Staining with Masson’s trichrome was also conducted to quantify the content of collagen. For immunohistochemistry, 4-μm sections were deparaffinized, dried at 60 °C. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide and methanol for 20 min, and sections were blocked in PBS containing 1% bovine serum albumin and 0.3% Triton X-100 for 60 min. Subsequently, sections were incubated with antibodies to CD68 (for macrophage staining), matrix metalloproteinase-9 (MMP-9), and α-smooth muscle actin (α-SMA, for SMCs staining) overnight. Histology and immunohistochemistry analyses were carried out with Nis-Elements BR 3.2 software (Nikon, Japan). The main organs including heart, liver, spleen, lung, and kidney were also harvested, fixed in formalin, and then pathological sections were made and stained with H&E.

2.13. Complete blood counts and clinical chemistries

Blood was collected in EDTA spray-coated tubes and immediately analyzed for hematological parameters such as red blood cell (RBC), white blood cell (WBC), platelet (PLT), and hemoglobin (HGB) (Sysmex KX-21, Sysmex Co., Japan). The plasma concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA), and blood urea nitrogen (UREA) were also quantified (Roche Cobas C501, Roche Co., Switzerland).

2.14. Lipid and lipoprotein analysis

The levels of serum lipids including total cholesterol (TCH), triglyceride (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were detected by an AU-2700 automatic biochemical analyzer (Olympus, Japan). The detection kits were purchased from Medical Co. Ltd. (Kang Taike, Beijing), while the standard serum sample was supplied by Randox Laboratories Ltd. (Northern Ireland, UK).

2.15. Determination of serum cytokines

Quantibody® Mouse Inflammation Antibody Array 1 (Raybiotech Inc., USA) was used to determine the levels of various cytokines including tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1), according to the manufacturer’s instructions. Briefly, the arrays were blocked, incubated with 100 μL of working diluent buffer were added into each well, and then with Cy3-labeled streptavidin for 1.5 h. Finally, the chip was exposed to a laser scanner (Axon GenePix). Quantitative array analysis was performed using the GenePix 4000B and Raybio QAM-INF-1 data processing software.

2.16. Quantification of apolipoprotein B and low density lipoprotein receptor

After various treatments, 0.5 g of the liver tissue from each mouse was homogenized with 2 mL of saline using an automatic agitator. After centrifugation at 825 g for 10 min, the supernatant was extracted, and the levels of apolipoprotein B (ApoB) and low density lipoprotein receptor (LDLR) were detected following the instruction of Elisa kits (Shanghai Di Jin Biological Co., Ltd). Briefly, 10 μL of tissue supernatant and 40 μL of working diluent buffer were added into each well, and then incubated with 100 μL of HRP-conjugated reagent for 60 min at 37 °C. Subsequently, the solution was removed. After repeated washing, 50 μL of chromogen solution A and 50 μL of chromogen solution B were added, and gently mixed and incubated for 15 min at 37 °C. Then 50 μL of stop solution was added into each well, and the optical density was quantified by a microtiter plate reader within 15 min.
2.17. Quantification of proteins involved in mTOR signaling

Mouse vascular SMCs (MOVAS-1) were planted in a 6-well plate. After incubation with various doses of RAP (50, 150, 500, and 1500 ng/mL) for predetermined periods of time, cells were harvested, and proteins were extracted. Then, the expression of proteins including p70S6K (Thr389) and AKT (Ser473) and their phosphorylated forms were measured using a specific multiplex kit from Millipore (No. 48.602: AKT/PKB total; M-TOR total; p70s6 total; AKT/PKB pho; M-TOR pho; p70S6 pho; b-Tubulin). Also, the expression of these proteins in the aortas of treated ApoE−/− mice was detected.

2.18. Flow cytometry quantification of T and B cells in the spleen

After different treatments, the spleen was cut into pieces that were thoroughly grinded in the presence of saline. Then cells were collected by centrifugation in the lymphocyte separation medium. After cells were stained with antibodies to CD3 and CD19, flow cytometry analysis was performed to quantify the percentage of T and B cells in the splenic tissues.

2.19. Statistical analysis

Data are presented as mean ± standard error (SE). Statistical analysis was performed by SPSS 12.0 using one-way ANOVA test for experiments consisting of more than two groups, and with a two-tailed, unpaired t-test in experiments with two groups. Statistical significance was assessed at p < 0.05.

3. Results

3.1. Synthesis of Ac-bCDs and fabrication of RAP-loaded nanoparticles

Biodegradable and biocompatible materials of Ac-bCDs were synthesized by acetalation of β-CD according to our previous strategy (Fig. 1A) [36]. The obtained materials were characterized by 1H NMR spectrometry. A typical spectrum of Ac-bCD180 is illustrated in Fig. S1, indicating successful acetalation of β-CD. As demonstrated in our previous studies, the composition of acetalated cyclodextrin materials could be easily regulated by controlling acetalation time [36, 37]. Calculation based on 1H NMR spectra revealed the molar ratio of cyclic acetal (CA) to linear acetal (LA) was 0.27:1, 0.53:1, 0.58:1, 0.62:1, and 0.63:1, for Ac-bCD2.5, Ac-bCD10, Ac-bCD30, Ac-bCD90, Ac-bCD180, and Ac-bCD240, respectively. As a result, the hydrolysis period of Ac-bCDs may be modulated, since hydrolysis of CA is slower than that of LA [41].

Traditionally, other chemical derivatives of cyclodextrins, such as 2-hydroxypropyl-β-CD, randomly methylated β-CD, sulphobutylether β-CD, and 2-hydropropyl-γ-CD are generally used to improve solubility of various hydrophobic drugs by forming water-soluble inclusion complexes [42]. However, these compounds can only be dissolved in highly polar solvents such as DMSO and dimethylformamide, and they cannot realize sustained drug delivery. By contrast, acetalated cyclodextrins are essentially hydrophobic. These compounds can be easily dissolved in common solvents, such as dichloromethane, acetone, ethanol, and tetrahydrofuran. Therefore they can serve as carrier materials, and can be processed into microparticles by different techniques for sustained release of various therapeutics [43,44]. RAP-loaded nanoparticles based on Ac-bCDs were prepared by an o/w nanomulsion technique. Observation by TEM and SEM indicated that these nanoparticles exhibited spherical shape (Fig. 1B and Fig. S2). According to measurements by dynamic light scattering, the average size was 238, 220, 223, 185, 250, and 234 nm for RAP-containing nanoparticles based on Ac-bCD2.5, Ac-bCD10, Ac-bCD30, Ac-bCD90, Ac-bCD180, and Ac-bCD240, respectively (Fig. 1C). Of note, size of resulting RAP/Ac-bCD particles can be conveniently tailored by changing processing parameters during preparation of the o/w emulsion. For example, microparticles with mean size of 533 and 2482 nm could be successfully obtained (Fig. 1D-E), by forming the o/w emulsion via homogenization at 32,000 and 1000 rpm, respectively.

Independent of varied materials, high entrapment efficiency (>90%) was achieved (Table S1). The RAP loading content was 26.4%, 27.4%, 28.3%, 27.6%, 27.2%, and 27.5% for RAP-containing nanoparticles based on Ac-bCD2.5, Ac-bCD10, Ac-bCD30, Ac-bCD90, Ac-bCD180, and Ac-bCD240, respectively. This suggested that the composition of Ac-bCD materials had no significant effect on the loading of RAP into nanoparticles.

3.2. In vitro release of RAP/Ac-bCD nanoparticles

To examine release performances of RAP-loaded nanoparticles based on various Ac-bCDs, in vitro release tests were performed in 0.01 M PBS at pH 7.4. As illustrated in Fig. 2A, release profiles of RAP/ Ac-bCD nanoparticles were closely related to carrier materials. Considerably rapid release was achieved when nanoparticles were formulated using Ac-bCDs with the low content of CA units. Whereas drug release was completed within 2 days in the case of RAP/Ac-bCD2.5 nanoparticles, RAP delivery was sustained for 4.4, 9.0, 19.0, to 21.6 days for nanomedicines based on Ac-bCD10, Ac-bCD30, Ac-bCD90, and Ac-bCD180, respectively. Furthermore, dramatically slowed release could be achieved when Ac-bCD240 was employed as a carrier material. Of note, the release rate was relatively constant during the whole period, independent of varied Ac-bCDs. Consequently, the drug release period of RAP/Ac-bCD nanoparticles can be effectively tailored by using materials with different CA/LA ratios which may be easily attained by modulating acetalation time.

Further, we investigated the effect of particle size on release behaviors of RAP/Ac-bCD microparticles. To this end, RAP/Ac-bCD180 particles with mean size of 533 and 2482 nm were employed. Compared with RAP/Ac-bCD180 nanoparticles with average size of 250 nm, particles of 533 and 2482 nm displayed remarkably slowed release rate (Fig. 2B). Accordingly, the RAP release period can also be efficiently regulated by the particle size.

Our previous studies demonstrated that Ac-bCD nanoparticles exhibited pH-dependent hydrolysis profiles. Whereas hydrolysis of Ac-bCD nanoparticles may be notably accelerated in acidic solutions, they can be hydrolyzed slowly under physiological conditions (Fig. S3). Accordingly, drug molecules were largely released from RAP-loaded nanoparticles by diffusion out of the Ac-bCD nanomatrix. Agreeing with these results, analysis of the release curve of RAP/Ac-bCD180 nanoparticles (RAP-NP) by mathematic modeling revealed a linear release profile for 8 days after burst release within the first 3 h (Fig. S4A), which was followed by a power-type phase (Fig. S4B). According to the modeling equation established by Peppas [45], the first stage of RAP release from RAP-NP displayed a nearly zero-order profile, and this was mainly dominated by molecular diffusion. By contrast, the second stage corresponded to a non-Fickian transport, in which both passive diffusion and disintegration of nanoparticles were responsible for drug release, in line with the hydrolysis of blank Ac-bCD180 nanoparticles during this period (Fig. S3). Nevertheless, it should be noted that in both two release phases, partial inclusion of hydrophobic units of RAP into the cavity of β-CD might slightly delay release of drug molecules. As substantiated by our previous studies, hydrophobic units of RAP may be complexed with β-CD via host-guest interactions, although the molecular size of RAP itself is too large to match the cavity [46].

3.3. In vivo pharmacokinetic studies

Then we performed in vivo pharmacokinetic studies in mice after subcutaneous injection of Ac-bCD180-based RAP-NP (mean size, ~250 nm), which was employed for the following therapeutic studies. Consistent with the in vitro release behavior, in vivo pharmacokinetic
study rendered a sustained profile of RAP concentration in the blood post subcutaneous administration of RAP-NP at 3 mg/kg of RAP (Fig. 2C). After an initial phase with relatively low concentration of RAP, nearly constant RAP levels (around 50 ng/L) were maintained for 13 days. In addition, in vivo release of RAP from RAP-NP was slightly accelerated when compared with in vitro release in PBS at pH 7.4. Previously, we found intramuscularly injected Ac-bCD180 nanoparticles could be absorbed or eliminated from the injection site after about one week in rabbits [36], although much longer time was required for their hydrolysis in vitro at pH 7.4 (Fig. S3). Four days after subcutaneous administration of Cy7.5-labeled Ac-bCD180 nanoparticles, their presence at the injection site could be clearly observed (Fig. S5A). Whereas in-depth studies are necessary to fully elucidate metabolism and elimination profiles of subcutaneously injected Ac-bCD nanoparticles, the current findings suggested that hydrolysis of Ac-bCD nanoparticles might be significantly accelerated under in vivo conditions. In addition, encapsulation of highly hydrophobic RAP molecules may delay in vivo hydrolysis of Ac-bCD nanoparticles. These results suggested that in vivo release of RAP after subcutaneous administration was dominated by both passive diffusion and hydrolysis of nanocarriers. This is different from in vitro release at pH 7.4, in which diffusion largely dominated the whole process. Of note, currently we cannot exclude the possibility that subcutaneously administered Ac-bCD nanoparticles might be translocated to blood circulation through lymphatic transport [47]. Indeed, significant fluorescence could be detected in the blood samples collected at various time points after subcutaneous injection of Cy7.5/ Ac-bCD180 nanoparticles in mice (Fig. S5B), which might be contributed by both free Cy7.5 and entrapped Cy7.5 molecules.

Also, we determined the levels of RAP in the aorta, since atherosclerotic plaques develop at particular sites in the arterial tree. As illustrated in Fig. 2D, relatively constant levels of RAP (about 1.2 ng/g) could be detected in the aorta of mice, which well agrees with RAP concentrations in the blood. Similar to that observed in the blood, fluorescence distribution could also be examined in the aorta of ApoE−/− mice subjected to Western diet for three months (Fig. S5C). This implied that subcutaneously injected nanoparticles might be transported to atherosclerotic plaques in the aorta, which need to be further affirmed in future studies.

By contrast, oral gavage of the same dose of free RAP led to rapid distribution and elimination of RAP in the blood and aorta (Fig. S6), with the peak drug concentration of 438 ng/L and 3.7 ng/g in the blood and aorta, respectively. In this case, relatively high levels of RAP (average 200 ng/L in the blood and 2.0 ng/g in the aorta) were maintained for about 8 h, although RAP was completely eliminated within 72 h.

3.4. Orally administered RAP inhibited the growth of atherosclerotic plaques

To evidence antiatherosclerotic activity of RAP in ApoE−/− mice, oral gavage was conducted at 3.0 mg/kg of RAP once every three days after one month of Western diet. Fig. 3A shows representative en face micrographs of ORO-stained aortas from ApoE−/− mice subjected to various treatments for two months. Significant atherosclerotic lesion formation could be observed for the control mice without therapeutic intervention. Treatment by orally administered RAP dramatically inhibited the plaque development, as indicated by the reduced lesion formation. Quantification based on ORO-stained aortas showed the average plaque area decreased from 27.3% to 17.7% after RAP therapy (Fig. 3B). Similarly, ORO-stained cross-sections of aortic roots also revealed reduced lesions (Fig. 3C). The plaque area was decreased by 21.2% after treatment with free RAP (Fig. 3D). In line with this, the relative lumen area was 31.5% and 43.0% for the control and RAP group (Fig. 3E), respectively. Besides, plaque formation in the brachiocephalic artery was
evidently inhibited (Fig. 4A-B). In this case, the relative lesion area was 75.1% and 38.9%, while the relative lumen area was 27.6% and 44.4% for the control and RAP-treated group (Fig. 4F), respectively. Additional information was provided by H&E sections of aortic roots. Lesions in the control group were mainly consisted of acellular cores and cholesterol clefts that are characteristic of complex lesions, while treatment with orally delivered RAP significantly decreased the area of necrotic cores (Fig. 4G). Quantitative analysis suggested that the relative area of necrotic cores was decreased by 72.2% after RAP treatment (Fig. 4H).

3.5. Sustained delivery of RAP more significantly attenuated the progression of atherosclerosis

In RAP-NP groups, ApoE−/− mice were treated by subcutaneous administration every 15 days at the lower (1.0 mg/kg of RAP) and higher (3.0 mg/kg of RAP) dose, respectively. Whereas treatment by the vehicle of blank Ac-bCD180 nanoparticles almost had no effect on the lesion area, RAP-NP significantly reduced the growth of atherosclerotic lesion (Fig. 3A). The average lesion area was 4.7% and 3.9% at 1.0 and 3.0 mg/
kg of RAP, respectively (Fig. 3B). In sections of aortic roots, the relative plaque area was 69.9%, 13.1%, and 9.7% for the vehicle group, RAP-NP at 1.0 and 3.0 mg/kg, respectively (Fig. 3C-D). There was significant difference between the free RAP group and RAP-NP groups at either lower or higher dose. Nevertheless, no statistical difference was found between the lower and higher dose of RAP-NP. Likewise, treatment with RAP-NP afforded notably increased lumen area (Fig. 3E).

Also, the lesion area in cryosections of brachiocephalic arteries from RAP-NP groups was dramatically decreased when compared with the RAP group (Fig. 4C-E). The relative lesion area was 23.9% and 22.0% for the RAP-NP groups at 1.0 and 3.0 mg/kg, while the corresponding lumen area was 58.9% and 61.6%, respectively (Fig. 4F). Accordingly, atherosclerotic development was strikingly attenuated by sustained delivery of RAP, to a greater extent than that of orally administered RAP. In similar to the free RAP group, treatment by RAP-NP markedly decreased the formation of necrotic cores in plaques, and a reduction of 82.4% and 79.9% could be discerned at lower and higher dose, respectively (Fig. 4G-H). It should be noted that subcutaneous administration of RAP alone at 3 mg/kg twice a month caused insignificant improvement as compared to the model control, with respect to atherosclerotic lesion formation, relative plaque area, necrotic cores in plaques, and relative lumen size. Accordingly, this group was not subjected to further analyses.

3.6. Immunohistochemistry analysis

RAP therapy significantly decreased macrophages and MMP-9 in the plaques, and more remarkable effect could be found for RAP-NP groups as compared to the free RAP group (Fig. 5A). Quantitative analysis showed that the number of macrophage was decreased by 58.1%, 89.1%, and 90.0% after treatment with free RAP, RAP-NP at 1.0 and 3.0 mg/kg of RAP, respectively (Fig. 5B). Free RAP as well as RAP-NP groups at 1.0 and 3.0 mg/kg displayed a reduction in the MMP-9 level of 43.8%, 75.5%, and 77.9%, respectively (Fig. 5C).
Consistent with reduced MMP-9 and macrophage, the content of collagen around plaques was well-retained post therapy. Compared with the control group, an increase of 20.9%, 115.5%, and 104.9% was observed for RAP, RAP-NP at 1.0 mg/kg, and RAP-NP at 3.0 mg/kg, respectively (Fig. 5D). All RAP-treated groups exhibited prominently thicker fibrous caps. Furthermore, remarkably more SMCs were observed after RAP therapy, with 94.8%, 265.7%, and 267.5% more SMCs for RAP, and RAP-NP at 1.0 and 3.0 mg/kg, respectively, as compared to the model group (Fig. 5E). Besides, T cells in the plaques were strikingly reduced after treatment with either RAP and RAP-NP (Fig. S7).

3.7. Serum inflammatory cytokines

Compared with the control, treatment with orally administered RAP remarkably reduced the levels of inflammatory cytokines including TNF-α, INF-γ, IL-1α, IL-1β, IL-6, and MCP-1 (Fig. 6A-F). Intervention with RAP-NP at either lower or higher dose more significantly decreased the serum levels of these cytokines. Furthermore, levels of TNF-α, INF-γ, IL-1α, and IL-1β in the RAP-NP groups were markedly lower than those of the RAP group. For IL-6, significant difference could be found between RAP and RAP-NP at 3.0 mg/kg.

3.8. Levels of proteins involved in mTOR signaling pathways

Treatment with RAP by oral administration significantly decreased the phosphorylated levels of p70S6K at the Thr389 site (p-p70S6K(Thr389)) and AKT at the Ser473 site (p-AKT(Ser473)) in the aortas of ApoE−/− mice (Fig. 6G-H). This indicated that both mTORC1 and mTORC2 signaling pathways were inhibited. By contrast, only p-
3.9. Changes in lipid profiles

No significant changes appeared in the levels of serum lipids including TCH, TG, and HDL in ApoE−/− mice (Fig. 8A). However, orally delivered RAP significantly enhanced the LDL level. Treatment by either RAP or RAP-NP significantly decreased the level of ApoB in the liver (Fig. 8B). Likewise, the production of LDLr in the liver was inhibited by different RAP formulations (Fig. 8C). Nevertheless, the ApoB/IDLr ratio was markedly increased only in the free RAP group as compared to the control, while no significant change was found in RAP-NP groups (Fig. 8D). This result is consistent with the enhanced level of LDL in the serum of free RAP-treated mice.

3.10. Evaluation on the safety profile of the sustained delivery system RAP-NP

During treatment, we did not observe any side effects related to general immunosuppressants such as infection and lymphoma in RAP-NP-treated mice, which are main concerns for the long-term use of RAP. At each time point, no noticeable changes in the body weight were found between various groups (Fig. S8A). Post treatment, main organs were resected, and no evident differences appeared in the organ index of the sustained delivery system RAP-NP-treated mice (Fig. S8B). A complete blood count indicated no significant variation in typical hematological parameters for RAP-NP-treated mice (Fig. S8C–F). Likewise, measurement on biochemical markers relevant to liver and kidney functions revealed no significant difference between each group (Fig. S8G–H). In addition, examination on H&E sections showed that treatment by either vehicle or RAP-NP did not result in discernable injuries for the examined organs (Fig. S8I). Furthermore, examination by immunohistochemistry and flow cytometry revealed that RAP-NP did not cause significant variation in the number of T and B cells in the spleen of ApoE−/− mice (Fig. S9 and Fig. 9), implicating that sustained delivery of RAP had no remarkable negative effects on the adaptive immune system of treated mice.

4. Discussion

Currently, there is still an urgent demand for discovering and developing new effective and safe therapeutics for the prevention and therapy of atherosclerotic diseases. Increasing evidence has indicated the important role of inflammation and immune response in the pathogenesis of atherosclerosis [1,3,4,9,48], in which a number of cellular and molecular events are involved [1–4,9,49], while they have been poorly treated by existing drugs [5,50]. Accordingly, therapeutics with versatile pharmacological activities that can inhibit multiple components of the complex atherosclerotic process are highly desirable. RAP has been found to possess multiple functions, including immunosuppression, anti-inflammation, anti-migration, antiproliferation, and autophagy activation. These properties support its potential as an antiatherosclerotic agent, and recent studies have partly revealed atheroprotective effects of RAP in animal models [15–17,51–54]. Nevertheless, extensive studies remain necessary to convincingly demonstrate the antiatherosclerotic effect of RAP. Herein oral administration of RAP at 3.0 mg/kg every three days clearly showed that RAP may attenuate the progression of atherosclerosis in ApoE−/− mice by reducing lesions in the entire aorta. Also, atheroma formation was dramatically suppressed at the aortic root and brachiocephalic artery. Concomitantly, RAP therapy decreased typical proinflammatory cytokines such as TNF-α, INF-γ, IL-1α, IL-1β, and IL-6.
IL-6 as well as a chemotactic cytokine MCP-1, while all these cytokines may deteriorate atherosclerosis by activating inflammation and/or perpetuating a chronic inflammation [3]. Accordingly, inflammation in atherosclerosis was significantly inhibited by RAP. In addition, RAP prevented the development of atherosclerosis to a more advanced stage, as indicated by the significantly reduced necrotic cores in plaques. RAP therapy also decreased macrophage and MMP-9 in plaques. Since the presence of macrophage and MMP-9 can lead to thin fibrous cap, destabilize atherosclerotic plaques, and eventually cause vulnerable lesions in the late stage of atherosclerosis [55,56], RAP may enhance the plaque stability. This is further verified by increased collagen and thickened fibrous cap in plaques. Increment in collagen well agrees with lowered INF-γ, because secretion of INF-γ by Th1 cells in plaques may reduce collagen synthesis by SMCs [57]. Moreover, orally delivered RAP inhibited both mTORC1 and mTORC2. Accordingly, the underpinnings responsible for the antiatherosclerotic activity of RAP lie in the fact that, by inhibiting mTOR, RAP may suppress the proliferation of SMCs and maintain their viability to attenuate inflammation resulted from proinflammatory cytokines released by apoptotic or necrotic SMCs and macrophages. Additionally, through mTOR inhibition, RAP can suppress proliferation and migration of T cells as well as selectively deplete macrophages to reduce MMP-9.

Consequently, the synergistic effect of multiple activities of RAP may have contributed to its atheroprotective function. However, regular administration of RAP or its analogues, especially at high doses for a long period of time, may lead to hyperlipidemia [17,58,59]. Other side effects such as polyarthralgia, mild stomatitis, diarrhea, and over immunosuppression have also been found in humans [60]. Herein we observed significantly enhanced LDL after therapy with orally administered RAP (Fig. 8A). This may be attributed to the varied degrees of effects on ApoB and LDLr that is responsible for production and elimination of LDL, respectively. After long-term treatment by orally administered RAP at 3 mg/kg, the ratio of ApoB/LDLr significantly increased (Fig. 8D). Besides, RAP efficacy was compromised by notably inhibiting mTORC2 that has been confirmed to have antiatherosclerotic effects by anti-inflammation, promoting endothelial cell survival and migration, and regulating Rit-mediated oxidative stress resistance [19–21].

Recently, different nanovehicles have been developed to realize spatiotemporally controlled delivery of various therapeutics against atherosclerosis [27–31,35,61,62]. However, the development of efficient nanomedicines for atherosclerosis with clinical significance remains challenging [33], largely due to rapid therapeutic clearance, short retention time, and limited targeting efficiency. To address these issues and develop effective, safe, and translational nanomedicines, we constructed RAP-loaded Ac-bCD nanoparticles based on biocompatible nanoplatforms derived from acetalated cyclodextrin materials of Ac-bCDs (Fig. 1). Under physiological conditions, Ac-bCDs may degrade into a water-soluble and non-acidic parent compound that has already been clinically used in formulations for many years [63,64]. Compared with frequently employed nanoparticles based on other biodegradable materials with acidic byproducts upon hydrolysis that may cause local inflammation and therefore further aggravate chronic inflammation in cardiovascular disease [39,65], nanocarriers derived from Ac-bCDs only induces minimal tissue inflammatory response, making them satisfactory for treating chronic inflammatory disease. Notably, the release period of RAP-loaded Ac-bCD microparticles can be easily tailored by

**Fig. 8.** The levels of serum lipids and lipid-related proteins in the liver of ApoE−/− mice after various treatments. A, Typical lipid levels. TCH, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; and LDL, low-density lipoprotein. B–C, The relative levels of ApoB (B) and LDLr (C) in the liver. D, The ratio of ApoB to LDLr level in the liver. Data are mean ± SE (n = 6); * p < 0.05, ** p < 0.01, *** p < 0.001.
their size or by using Ac-bCDs with different compositions (Fig. 2). The release period of RAP from Ac-bCD nanoparticles can be modulated from 2 days to months by using materials with varied CA/LA ratios. As a proof-of-concept study, RAP/Ac-bCD180-derived RAP-NP with a moderate release period was employed for detailed in vivo evaluations. After subcutaneous administration, RAP-NP can continuously release RAP at a constant rate for nearly 20 days.

In the current dosing regimen, oral administration needs ten times of dosing with a total dose of 30 mg/kg in each month, while RAP-NP only requires two times of administration with a total dose of 2 or 6 mg/kg at lower or higher dose, respectively. Despite its lower dose, RAP-NP showed much better therapeutic outcome, as manifested by the more significantly reduced lesion area and notably increased lumen size, as compared to RAP (Figs. 3 and 4). Compared with RAP, RAP-NP more efficaciously reduced necrotic cores, and therefore effectively delayed the progression of atherosclerosis to a more advanced and vulnerable stage. This is further affirmed by prominently reduced macrophages and MMP-9 as well as increased collagen in the plaques of RAP-NP groups (Fig. 5). In line with the better efficacy, RAP-NP more markedly decreased inflammatory cytokines (Fig. 6), which is favorable for attenuating atherosclerosis progress. Since low doses of RAP specifically inhibited mTORC1 (Figs. 6 and 7), the potentiated anti-inflammatory activity by RAP-NP may be largely resulted from selective inhibition of mTORC1, while maintaining the anti-inflammation capability of the mTORC2 signaling pathway. On the other hand, whereas proliferation of SMCs was inhibited by RAP, their apoptosis was also notably suppressed due to reduced macrophages and lowered inflammatory cytokines after RAP therapy. These collective effects should account for the significantly higher level of SMCs in RAP-NP treated groups as compared to that in the free RAP group, because RAP-NP attenuated inflammation to a much more significant degree than free RAP (Fig. 6). Furthermore, recent studies suggested that SMCs are protective in advanced lesions, by preventing rupture of fibrous caps and promoting plaque repair [66]. Together, these results demonstrated that the antiatherosclerotic activity of RAP may be dramatically enhanced by delivery in a sustained manner.

Of note, RAP-NP did not significantly increase LDL in the serum, primarily due to inhibition of both ApoB and LDLr to the same degree (Fig. 8). Furthermore, sustained release of RAP may not cause significant adverse effects (Fig. 9 and Fig. S8–9). This may be due to the fact that only lower concentrations of RAP was constantly released from RAP-NP, which is lower than that capable of causing perceptible side effects. Also, our results implied that nanoparticles based on Ac-bCD180 are safe for the treatment of chronic diseases.

5. Conclusions

In summary, by packaging RAP into a biodegradable and biocompatible nanocarrier based on an acetalated cyclodextrin material, we successfully constructed an effective, safe, and translational nanotherapy RAP-NP that can sustain RAP release in a well-controlled manner both in vitro and in vivo. In an atherosclerosis model established in ApoE<sup>−/−</sup> mice, RAP-NP more effectively delayed the progression of

![Fig. 9. Quantification of T and B cells in the spleen of ApoE<sup>−/−</sup> mice post various treatments by flow cytometry. A–C, Typical flow cytometric profiles of control (A), RAP-NP at 1.0 mg/kg (B), and RAP-NP at 3.0 mg/kg (C). D, Quantitative results. Data are mean ± SE (n = 5).](image-url)
atherosclerosis, suppressed local inflammation in the lesions, and stabilized atherosclerotic plaques, when compared with free RAP, mainly through selectively inhibiting mTORC1. To the best of our knowledge, this is the first study that demonstrates the beneficial efficacy of sustained delivery of RAP for atherosclerosis therapy. Whereas more comprehensive experiments are necessary to advance this sustained RAP therapy into clinic, the current study unambiguously substantiated that controlled delivery systems hold great potential to realize delicate balance of preserved safety and improved efficacy for drugs against atherosclerotic diseases. The insight obtained herein may also facilitate the discovery of novel therapeutics.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2016.05.049.

References


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