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Improved intervention of atherosclerosis and cardiac hypertrophy through biodegradable polymer-encapsulated delivery of glycosphingolipid inhibitor

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Abstract

D-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), a glycosphingolipid synthesis inhibitor, holds promise for the treatment of atherosclerosis and cardiac hypertrophy but rapid *in vivo* clearance has severely hindered translation to the clinic. To overcome this impediment, we used a materials-based delivery strategy wherein D-PDMP was encapsulated within a biodegradable polymer composed of poly ethylene glycol (PEG) and sebacic acid (SA). PEG-SA was formulated into nanoparticles that were doped with ¹²⁵I-labeled PEG to allow *in vivo* bio-distribution and release kinetics of D-PDMP to be determined by using γ -scintigraphy and subsequently, by mass spectrometry. Polymer-encapsulation increased the residence time of D-PDMP in the body of a treated mouse from less than one hour to at least four hours (and up to 48 h or longer). This substantially increased *in vivo* longevity provided by polymer encapsulation resulted in an order of magnitude gain in efficacy for interfering with atherosclerosis and cardiac hypertrophy in apoE^{-/-} mice fed a high fat and high cholesterol (HFHC) diet. These results establish that D-PDMP encapsulated in a biodegradable polymer provides a superior mode of delivery compared to unconjugated D-PDMP by way of increased gastrointestinal absorption and increased residence time thus providing this otherwise rapidly cleared compound with therapeutic relevance in interfering with atherosclerosis, cardiac hypertrophy, and probably other diseases associated with the deleterious effects of abnormally high glycosphingolipid biosynthesis or deficient catabolism.

Keywords

Atherosclerosis; Cardiac hypertrophy; Biomaterials-based drug delivery; Glycosphingolipids

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1. Introduction

Atherosclerosis and cardiac hypertrophy are the leading causes of death worldwide. To date, methods to treat these diseases – such targeting cholesterol using statins and cholesterol absorption inhibitors (ezetimibe) – have not substantially improved outcomes. Lowering glycosphingolipid (GSL) load offers an attractive alternative method for preventing and interfering with atherosclerosis and cardiac hypertrophy, since accumulation of cholesterol, triglycerides and GSLs contribute to the plaque formation that leads to these conditions [1–3]. The promise of the widely used inhibitor of GSL synthesis, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), is evident in our recent study where we showed that this compound could *slow* and in some cases actually *prevent* the progression of atherosclerosis in a rodent model [4]. Unfortunately, D-PDMP is rapidly metabolized by the cytochrome P450 system and cleared from the body in less than an hour severely compromising the *in vivo* effectiveness of D-PDMP. As a result, the efficacy of this potential drug to the more stringent task of *reversing* already present atherosclerotic tissue or extending this treatment approach to additional endpoints such as cardiac hypertrophy is severely hindered. The current report describes a polymer-based approach that overcomes the poor pharmacological properties of D-PDMP and thereby improves this drug candidate's *in vivo* activity.

Although the concept of encapsulating drugs in biomaterials to improve their *in vivo* delivery is a decades-old concept [5], many challenges inherent in implementing this strategy have slowed its widespread adoption. For example, non-immunogenic materials that could be reproducibly molded into particles or wafers of defined sizes and shapes – while at the same time accommodating a robust “payload” of drug – had to be developed. Over the past decade, many pertinent technical issues have been overcome (as reviewed elsewhere, [6,7]), setting the stage for a materials-based strategies for treating prevalent human ailments such as cardiovascular disease [8,9]. In particular, in this study we extended biodegradable and biocompatible sebacic acid – poly(ethylene glycol) (SA-PEG) copolymers as drug delivery vehicles [10–12] by (1) successfully encapsulating D-PDMP for controlled release, (2) controlling the size of biomaterial particles to approximately 100 nm for release over a multi-hour time scale, and (3) incorporating radioactive tracers into the material for *in vivo* tracking. The success of this strategy in increasing the *in vivo* longevity of encapsulated D-PDMP and thereby dramatically enhancing this compound's efficacy in reversing atherosclerosis and cardiac hypertrophy was demonstrated using an animal model consisting of apoE^{−/−} mice fed a high fat and high cholesterol (HFHC) diet [13] and an extensive set of biomarkers indicative of cardiovascular disease.

2. Materials and methods

D-PDMP was purchased from Matreya LLC (Pleasant Gap, PA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless mentioned otherwise.

2.1. Preparation of polymer-encapsulated D-PDMP

Poly(ethylene glycol) sebacic acid co-polymer (PEG-SA) was prepared following the procedure published by Fu and coworkers [10]. Briefly, sebacic acid prepolymer was made by refluxing sebacic acid (SA) in acetic anhydride followed by drying under high vacuum (evaporation), crystallized from dry toluene, washed with 1:1 anhydrous ethyl ether-petroleum ether and finally air dried. PEG prepolymer was made by refluxing of polyoxyethylene dicarboxylic acid in acetic anhydride, volatile solvents were removed under vacuum. The solid mass was extracted with anhydrous ether and air dried. The poly(PEG-SA) co-block polymer was then synthesized by the melt polycondensation method and characterized by proton NMR [10]. Note that this copolymer was used before in our laboratory and have been extensively characterized for the composition and structural identity [14].

Encapsulation of D-PDMP in poly(PEG-SA) (to prepare polymer-encapsulated drug subsequently referred to as BP-D-PDMP, with the “BP” standing for biodegradable polymer) followed by the melt polycondensation method described above for SA and PEG prepolymers but with the inclusion of D-PDMP at starting ratios of poly(PEG-SA) to D-PDMP of 70:30 by weight. Subsequently, microparticles were prepared using a single emulsion solvent evaporation method [10]. Briefly, D-PDMP and poly(PEG-SA) were dissolved in chloroform (50 mg/mL) and emulsified into a 1.0% w/w poly(vinyl alcohol) aqueous solution under sonication condition keeping the temperature below 25 °C. Particles were hardened by allowing chloroform to evaporate at room temperature while stirring for 12 h. Particles were collected and washed three times with double distilled water via centrifugation at 2600× g (30 min) and lyophilized for 48 h before use.

2.2. Preparation of [¹²⁵I]-BP-D-PDMP and imaging and metabolic experiments

2.2.1. Radiolabeling the SA-PEG co-polymer—L-Tyrosine (20 mg, 0.14 mmol) (Sigma Aldrich, St. Louis, MO) was introduced to 45 mCi (810 kBq) of [¹²⁵I]NaTyrosine in 100 mL of PBS in a glass vial containing plated Iodogen (Pierce, Rockford IL USA). The radioiodinated reaction proceeded at room temperature for 12 min before withdrawing the supernatant. The supernatant was then added to 100 mg of *O,O'*bis[2-(*N*-succinimidylsuccinylamino) ethyl]polyethylene glycol (Sigma Aldrich, St. Louis MO USA) and this mixture was allowed to sit at room temperature for one hour. After one hour, the reaction mixture was then loaded onto a PBS-conditioned G25 Sephadex size-exclusion column (Pierce, Rockford, USA) to remove any unreacted iodide and free tyrosine. The absence of free radioiodine and tyrosine in the elute was confirmed using ITLC (Gelman strips, Vernon Hills IL USA) in ACD buffer (Sigma–Aldrich, St. Louis MO USA). Finally, the labeled PEG was incorporated into poly (SA-PEG) polymer.

2.2.2. In vivo γ -scintigraphy of ¹²⁵I labeled polymer—Drug-loaded polymer [45 mCi (810 kBq) of the ¹²⁵I-labeled samples] was orally administered by gavage to each of three wild type, C57bl/6 adult male mice. The mice were anesthetized using 2.5% isoflurane gas in oxygen delivered via tent. The mice were lined up side-by-side directly over a high-resolution parallel hole collimator in an X-SPECT SPECT-CT scanner (GammaMedica Ideas, North Ridge CA USA). Scans consisted of several 10 min acquisitions through two

hours post-tracer administration with a CT scan followed by additional acquisitions as indicated with accompanying CT scans (512 slice, 50 keV beam). The data were reconstructed using the manufacturer's software and co-registered using AMIDE (<http://www.sourceforge.net>). All scintigraphy images are displayed on the same scale.

2.3. Animals and treatments

Apolipoprotein E-deficient ApoE^{-/-}, male mice aged 12 weeks (Jackson Labs, Bar Harbor, ME) were used. At the age of 12 weeks, the ApoE^{-/-} mice were placed on a high fat and high cholesterol diet (HFHC) of 4.5 kcal/g, 20% fat and 1.25% cholesterol (D12108C, Research Diet Inc., New Brunswick, NJ) until 20 weeks of age. This time point was chosen so as to allow for adequate atherogenesis prior to therapeutic intervention. A control group of mice on normal diet consisting only of chow food was used for comparison.

Starting at 20 weeks, mice on the HFHC diet were treated with unconjugated D-PDMP at 10 mg/kg (mpk) and with 1 and 10 mpk of polymer-encapsulation drug and compared to controls fed normal chow diet or placebo fed with HFHC + Vehicle (100 µL of 5% Tween 80 in PBS) for another 16 weeks. A sample size of n = 5–6 subjects per group was designated.

During D-PDMP treatment, vehicle control, unconjugated, and polymer-encapsulated drug was delivered daily by oral gavage. During treatment, the mice were supplied with a known quantity of food on a weekly basis allowing food intake to be estimated; the growth rate of the animals was also monitored on a weekly basis. Physiological studies and tissue harvest were performed around 12 and 36 weeks for molecular and histopathological studies. The 20 week time point was designed for the start of treatment because we have found that mice show significant plaque accumulation, significant increase in ascending aortic intima media thickness (IMT_{AsAo}) and vascular stiffness using histopathology, ultrasound and pulse wave velocity (PWV), respectively at this time point (unpublished studies).

2.4. Ultrasound imaging

Vascular ultrasound was performed in conscious mice using the 2100 VisualSonics ultrasound imaging system equipped with MS400: 38 MHz MicroScan transducer (Toronto, Ontario, Canada) as previously described [15,16]. The aorta was first viewed in 2 dimensional (2D) mode and the parasternal long axis view. The ascending aorta external and internal dimensions and intima media thickness were measured at end systolic phase and in blinded fashion so that the genotype and treatments were not revealed. The intima media thickness (IMT) was measured from the ascending aortic wall and derived according to the following equation:

$$IMT (AsAo) (mm) = \frac{\text{External Ascending aorta diameter (mm)} - \text{Internal Ascending aortic diameter (mm)}}{2}, \text{ where AsAo refers to ascending aorta.}$$

All measurements were performed according to the guidelines set by the American Society of Echocardiography. For each mouse, three to five values for each measurement were obtained and averaged for evaluation.

2.5. Histopathology

Masson trichrome staining of the ascending aorta and left ventricle was used to quantify hypertrophy, wall thickening and assess for the elastin fibers structure and morphology, plaque and Ca^{2+} accumulation, 16 weeks after treatment and 36 weeks of HFHC diet and aging time. Nikon 80I Eclipse equipped with Nikon DS-EI1 camera and NIS-Elements software (Nikon, Japan) were used for image analysis.

2.6. Measurement of glycosphingolipids

A MALDI-TOF/TOF (AB SCIEX TOF/TOF 5800, Applied Biosystems, Framingham, MA) was used in this study for both MS and MS/MS analyses of glycosphingolipids. Extracted lipids were reconstituted in 100 μL acetonitrile–methanol (9:1, v/v) and approximately 0.5 μL was spotted on an AB SCIEX Opti-TOF MALDI plate. Sample spots were overlaid with 0.5 μL of the MALDI matrix 2,5-dihydroxybenzoic acid (DHB) in an acetonitrile–methanol solution (5 mg/mL DHB in 9:1 ACN-MeOH, v/v). A 355 nm laser at a repetition rate of 200 Hz was employed for ionization. For MS analysis, mass spectra were the sum of 4000 laser shots and acquired in reflector positive mode. For MS/MS analysis, mass spectra were the sum of 1000 laser shots with collision energy of 2 keV and pressurized air was utilized as the collision gas to induce fragmentation. The following standards were used to calibrate the mass spectrometer: polypeptide hormones (ACTH I-III, Sigma–Aldrich, St. Louis, MO) and a peptide (Fibrinopeptide B, human, Sigma–Aldrich, St. Louis, MO). External GSL standards were spotted separately and used for the comparative analysis and quantification.

2.7. Measurement of cholesterol, triglycerides, and oxidized LDL

The serum level of oxidized LDL (oxLDL), was measured using an ELISA assay and monoclonal antibody [17] against human oxLDL according to instructions given by the supplier (Avanti Polar Lipids, Alabaster, AL). Plasma levels of cholesterol and triglycerides were taken from microtiter readings following Wako kit assays. The serum level of triglycerides and cholesterol were measured using commercially available kits from Wako (Wako Diagnostics, Richmond, VA). After plating with two colorimetric reagents, 4 μL of the apoE $^{-/-}$ serum samples were measured at 600 nm with a 700 nm reference wavelength.

2.8. Quantitative real-time PCR

Liver and heart tissues (50 mg of each) were homogenized from each subject and total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen). Two micrograms of RNA was reverse-transcribed with Super-Script II (Invitrogen, USA) using random primers. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, USA) in an Applied Biosystems StepOne™ Real-Time PCR System with the following thermal cycling conditions: 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min for denaturation, annealing and elongation. Relative mRNA levels were calculated by the method of $2^{-\Delta\Delta\text{Ct}}$. Data were normalized to GAPDH mRNA level. To determine the specificity of amplification, melting curve analysis was applied to all final PCR products. All samples were performed in triplicate. Primers were synthesized by Integrated DNA Technologies (Coralville, USA). Expression suite software (Applied Biosystems) was used to analyze the data.

3. Results

3.1. Design and characterization of polymer-encapsulated D-PDMP

Sebacic acid poly ethylene glycol polymers (poly(SA-PEG)) were synthesized based on the strategy outlined in Fig. 1A and were then formulated into particles designed to have an average diameter of ~100 nm (Fig. 1B). This size was selected to be sufficiently large to avoid nonspecific cellular uptake of nanoparticles, which occurs in the 20–60 nm size range, but small enough to ensure fairly rapid release of drug. Because the payload of poly(SA-PEG) formulated particles is released through surface erosion [18], release rates do not depend on the chemical properties of the encapsulated drug but are primarily determined by the size of the particle. Therefore, based on our previous experience using poly(SA-PEG) constructs for small molecule delivery [14], *in vivo* release was tuned to take place over a multi-hour time period (which was experimentally confirmed *in vivo*, as described below).

3.2. Polymer encapsulation facilitates the controlled release of D-PDMP in mice

We first investigated the bio-distribution kinetics of the PEG₃₀₀ constituent formulated with sebacic acid by terminally conjugating the PEG with [¹²⁵I] tyrosine (Fig. 1C) and doping this radiolabeled material into the nanoparticles described above with D-PDMP also encapsulated within this biodegradable polymer. Following feeding by oral gavage, γ -scintigraphic imaging was conducted and recorded at the indicated time points from 0.5 h through 48 h post-gavage (Fig. 2). Scintigraphic analysis showed that the radiolabeled nanoparticles passed through the stomach and the duodenum within 24 h, after which no radioactivity was detected in the mouse tissues. Based on the bio-distribution studies of intact particles (Fig. 2), in which biopolymer encapsulation enabled availability in the stomach and duodenum in a 24 h period, we next independently determined the tissue bio-distribution kinetics of the PEG vehicle and the D-PDMP drug payload. The bio-distribution of D-PDMP was determined and compared as encapsulated in the PEG₃₀₀ + sebacic acid vehicle versus when administered as free drug. In these experiments, D-PDMP was administered to female C57bl/6 mice either as 10 mpk of free D-PDMP or as polymer-encapsulated doses of 1 or 10 mpk. Mice were then sacrificed at 0.5, 1.0, 2.0, 4.0, 6.0, 24 and 48 h post-gavage and the distribution of D-PDMP was determined by collecting whole blood and serum and in addition, various tissues were excised. Total lipids were extracted and analyzed quantitatively by tandem mass spectrometry to determine the levels of D-PDMP.

These experiments showed that when unconjugated D-PDMP was delivered by oral gavage, very little drug was detected in the stomach at any time point and levels observed in the duodenum 0.5 h after administration rapidly decreased to undetectable levels (i.e., to zero; Fig. 3A). Similarly, levels observed in the kidney at 4 h became undetectable by 24 h; these results are consistent with the known fast clearance of D-PDMP from circulation [19]. In contrast to unconjugated D-PDMP, very little polymer-encapsulated D-PDMP (BP-DPDMP) delivered at a dose of 1 mpk (Fig. 3B) or 10 mpk of D-PDMP (Fig. 3C) was associated with the kidney until many hours after administration; at 24 h and beyond, a marked increase in unconjugated D-PDMP was found in the kidneys. This result – indicating a final step in the elimination of drug from the body – confirms a dramatically

increased half-life for the drug in the body when delivered via our strategy to encapsulate it within a biodegradable polymer. Overall these experiments show that unconjugated D-PDMP is rapidly absorbed and metabolized with renal clearance within an hour of oral gavage wherein D-PDMP encapsulated within a biopolymer is rapidly (<30 min) absorbed in murine GI tissues and thereafter displays a steady and increasing renal excretion profile through 48 h post-gavage. Thus, biopolymer encapsulation both increased GI absorption and the residence time of D-PDMP in the mouse.

3.3. Polymer-encapsulated D-PDMP is more effective than unconjugated D-PDMP in reducing aortic intima media thickening

Masson Trichrome-stained thin aortic tissue sections revealed extensive atherosclerosis plaque buildup, cholesterol ester crystal deposits, and extensive fibrosis in placebo-treated apoE^{-/-} mice fed a HFHC diet compared to controls fed normal chow (Fig. 4A). These endpoints were noticeably reduced by treatment with 1.0 mpk of biodegradable polymer-encapsulated D-PDMP (BP-DPDMP) or 10 mpk of unconjugated D-PDMP from week 24 to week 36. The ability of 1.0 mpk of encapsulated D-PDMP (BP-DPDMP) to be equally effective in returning the aortic intima-media thickness of the ascending aorta towards control levels was confirmed by two dimensional mode images of the aortic artery under the same dietary and treatment conditions (Fig. 4B and C).

3.4. Impact of polymer-encapsulated D-PDMP on glycosphingolipids, cholesterol, triglycerides, and oxidized LDL levels

The finding that 1.0 mpk of polymer-delivered D-PDMP could be as effective as a 10-fold higher level of unencapsulated drug was further examined by measuring the levels of various lipids and lipoproteins associated with atherosclerosis and cardiac hypertrophy in the serum and liver tissues of treated animals. In these experiments, mass spectrometric analysis of triglycerides (Fig. 5A) and cholesterol (Fig. 5B) showed that treatment with 1.0 mpk of polymer-encapsulated D-PDMP interfered with the rise of deleterious levels of these lipids in liver tissue as compared to placebo mice fed a HFHC diet. Measurement of oxidized LDL levels using an ELISA assay revealed a marked increase in the serum of placebo-treated mice fed a HFHC diet while treatment with 1.0 mpk of polymer-encapsulated D-PDMP interfered/reduced the oxLDL level to close to the basal level (Fig. 5C). Finally treatment with either unconjugated D-PDMP at 10 mpk or 1.0 or 10 mpk of polymer-encapsulated D-PDMP reduced the levels of glucosylceramide in the liver, the major target of drug action, to the levels found in animals fed a normal chow diet (Fig. 5D); similarly the levels of lactosylceramide were dramatically decreased in animals treated with the various dosing regimens of D-PDMP (Fig. 5E) while levels of the other precursor lipids were not changed significantly.

3.5. Polymer-encapsulated D-PDMP interferes with cardiac hypertrophy in apoE^{-/-} mice fed a HFHC diet

One of the salient features of feeding a HFHC diet to apoE^{-/-} mice is marked atherosclerosis and increase in blood levels of lipids and lipoproteins, vascular stiffness and an increase in aortic intima media thickening. M mode echocardiography revealed extensive cardiac hypertrophy in mice fed a HFHC diet as compared to mice fed chow only.

Treatment with 1.0 or 10 mpk of polymer-encapsulated D-PDMP or 10 mpk of free D-PDMP reversed cardiac hypertrophy in these mice (Fig. 6A–E). Collectively, these changes impact the heart by way of an increase in left ventricular mass (Fig. 6F) and a decrease in fractional shortening (Fig. 6G). Such changes were reversed with the use of either 10 mpk of D-PDMP or 1.0 mpk of polymer-encapsulated D-PDMP, suggesting that optimal interference in left ventricular mass and fractional shortening was effectively achieved by an order of magnitude less polymer-encapsulated D-PDMP compared to unconjugated drug. The salient features of feeding a HFHC diet to apoE^{−/−} mice is marked atherosclerosis and increase in blood levels of lipids and lipoproteins, vascular stiffness and an increase in aortic intima media thickening. Collectively, these changes impact the heart by way of an increase in left ventricular mass (Fig. 6F) and a decrease in fractional shortening (Fig. 6G). Such changes were reversed with the use of either 10 mpk of D-PDMP or 1.0 mpk of polymer-encapsulated D-PDMP, suggesting that optimal interference in left ventricular mass and fractional shortening was effectively achieved by an order of magnitude less polymer-encapsulated D-PDMP compared to unconjugated drug.

3.6. Polymer-encapsulated D-PDMP efficiently alters the expression of genes implicated in cholesterol homeostasis and cardiac hypertrophy

The relative efficacy of polymer-encapsulated and unconjugated D-PDMP was next compared by measuring the transcription of genes implicated in the regulation of cholesterol synthesis (Fig. 7A) and efflux (Fig. 7B), triglyceride metabolism (Fig. 7C) and bile acid formation (Fig. 7D) in the livers of the test animals. In general, the genes involved in these processes that contribute to the pathology of atherosclerosis are down-regulated in the diseased state, which is reproduced by a HFHC in apoE^{−/−} mice; therefore a desired response in the data shown in Fig. 7A–D is increased gene expression in D-PDMP-treated animals compared to placebo-treated controls.

To first briefly discuss cholesterol synthesis (Fig. 7A), expression of HMG-CoA reductase (Hmgcr), the key enzyme involved in the regulation of cholesterol biosynthesis is rescued most effectively by treatment with 1.0 mpk of polymer-encapsulated D-PDMP compared to either 10 mpk of polymer-encapsulated or unconjugated drug. Similarly Ldlr and Srebf2 most effectively responded to treatment with 1.0 mpk of polymer-encapsulated D-PDMP. The mouse Ldlr gene codes for LDL receptors and the Srebf2 gene codes for the sterol regulatory element binding factor 2, which both are important for maintaining cholesterol homeostasis. These results are consistent with previous endpoints presented above where 1.0 mpk of D-PDMP subject to controlled release can be 10 times more efficacious than unconjugated D-PDMP in interfering with atherosclerosis, in this case by raising the expression of genes relevant to cholesterol metabolism.

Moving to cholesterol efflux (Fig. 7B), expression of scavenger receptor class B member 1 (Scarb1) and ApoA1 are depressed under atherosclerosis. These genes, however, were increased in ApoE^{−/−} mice treated with any of the D-PDMP dosing regimens to approximately the same level, again indicating the efficacy of the lower 1.0 mpk dose of polymer-delivered drug. Scarb1 is an integral membrane protein found in many tissues including the liver and adrenal that facilitates the uptake of cholesteryl esters from high-

density lipoproteins in a process drives the movement of cholesterol from peripheral tissues towards the liver for excretion. ApoA1 has an amino acid motif which binds to cholesterol and carries it out of cells and transports to the liver where it is converted to bile acids [20]. Together, the increased expression of these two genes in D-PDMP-treated animals has important therapeutic potential.

To gain a broader sense of the impact of D-PDMP on triglyceride metabolism, Lpl and Vldlr transcription was monitored (Fig. 7C). Lipoprotein lipase (Lpl) serves as a conduit to bind to circulating triglyceride rich lipoproteins such as VLDL to the VLDL receptor (Vldlr) thus facilitating the delivery of VLDL to the liver [21]. We found that 1.0 mpk of polymer-encapsulated D-PDMP increased mRNA levels of LPL and Vldlr approximately 2–3-fold similar to 10 mpk of either unconjugated or polymer-encapsulated D-PDMP. Once cholesterol has experienced egress from outlying tissues and been trafficked to the liver, subsequent catabolism was monitored by RT-PCR quantification of Abca1 and Cyp7A1 (Fig. 7D). Abca1 gene expression was of interest because this protein regulates the egress of cholesterol from peripheral tissues back to the liver. Once cholesterol reaches the liver, Cyp7a1 is one of the genes required for its conversion to bile acids. We again observed equal or superior efficacy of 1.0 mpk of polymer-delivered D-PDMP for increasing the transcription of these genes.

Finally, we tested the expression of genes in the heart tissues of these mice that are established biomarkers of cardiac hypertrophy (Fig. 7E) to gain a sense of how D-PDMP and its mode of delivery affects underlying biochemistry and molecular biology to achieve the beneficial effects on left ventricular mass and fractional shortening (as shown in Fig. 6). In this case, the diseased state – recapitulated in this study by feeding the ApoE^{-/-} mice a HFHC diet – is most often characterized by increased transcription and a desired therapeutic endpoint is the modulation of genes that contribute to or are linked to pathogenesis. Altered transcription of these genes was achieved as efficiently with 1.0 mpk of polymer-encapsulated D-PDMP as 10 mpk of unencapsulated drug for Nppa, Nppb, Myh7, and Myh6. Nppa and Nppb are the natriuretic peptides type A and type B that function as cardiac hormones that regulate blood pressure as well as play roles in ventricular remodeling and in humans, these genes are associated with congestive heart failure and their efficient knockdown by 1.0 mpk of polymer-encapsulated D-PDMP is consistent with a beneficial therapeutic effect. Myh7 is the gene that encodes myosin heavy chain beta (MHC- β) and Myh6 encodes myosin heavy chain alpha (MHC- α); MHC- β is typically associated with failing hearts while MHC- α is predominant in healthy tissue; the impact of D-PDMP is to reduce disease associated Myh7 transcription and concomitantly enhance Myh6. It might be noted that increased Myh6 expression is one of the only endpoints monitored in this study where 1.0 mpk of polymer-encapsulated D-PDMP was not as effective as 10 mpk of either polymer-delivered or unencapsulated drug. Nevertheless, the overwhelming trend through the gene expression studies (as well as the preceding physiological measurements) was that treatment with 1.0 mpk of polymer-encapsulated D-PDMP was superior to treatment with 10 mpk of unconjugated D-PDMP, thereby strongly establishing the benefits of the materials-based delivery strategy. Furthermore, the results in Fig. 7E show that D-PDMP not only interferes with atherosclerosis in apoE^{-/-} mice fed a HFHC diet (as shown in panels A–D) but also is also proactively cardio-protective.

4. Discussion

Glycosphingolipids such as LacCer may play multiple causative roles in cardiovascular disease [22–31], as depicted in Fig. 8. This figure also indicates how using D-PDMP can interfere with biochemical pathways and the several endpoints investigated in this report that contributes to atherosclerosis and cardiac hypertrophy. We recently showed that feeding a minimum of 10 mpk D-PDMP to apoE^{−/−} mice fed a HFHC diet prevented atherosclerosis over a six month period [4]. Unfortunately, the poor pharmacological properties, in particular the extremely rapid serum clearance, of this otherwise promising drug candidate hinder investigation towards related disease endpoints (e.g., cardiac hypertrophy) and slows the development of clinically-relevant therapeutic approaches (e.g., reversal of pre-existing cardiovascular damage). To overcome these impediments, in this study we examined whether using an order of magnitude lower dose of polymer-encapsulated D-PDMP interferes with GSL biosynthesis with sufficient efficacy to reverse pre-existing atherosclerosis and whether this approach could be extended to treat cardiac hypertrophy *in vivo*. To accomplish these goals we successfully exploited the versatile ability of SA-PEG polymers, which have been used for the controlled release of the cancer drug etoposide [32], carbohydrate-based drug candidates [14], and growth factors [33] to encapsulate the glycosyltransferase inhibitor D-PDMP (Fig. 1).

To carefully monitor polymer-mediated delivery *in vivo*, we employed γ -scintigraphy using a X-SPECT-SPECT-CT scanning to quantitatively compare the kinetics of release and bio-distribution of D-PDMP with and without polymer encapsulation in mice (Fig. 2). A major finding of this study was that encapsulation of D-PDMP within a SA-PEG polymer increased the gastro-intestinal clearance and residence time of the drug to ~48 h in the body of the mice compared to one hour or less for unconjugated D-PDMP. Importantly, the encapsulated material was absorbed from the gastrointestinal tract, suggesting that it retained bioavailability upon the preferred oral route of administration. These promising features – dramatically increased residence time for polymer-encapsulated D-PDMP in the body combined with retention of oral bioavailability – provided a foundation for detailed evaluation of endpoints known to be associated with atherosclerosis (as described in detail in part in our recent publication [4]) and, cardiac hypertrophy. Accordingly, we used ultrasound imaging, MALDI-MS-MS and other established biomolecular methods to compare the efficacy of unconjugated and polymer-encapsulated D-PDMP to interfere these endpoints in apoE^{−/−} mice fed a high fat and cholesterol (HFHC) diet.

At macroscopic level, we showed that feeding a HFHC diet to apoE^{−/−} mice for 20 weeks markedly increased left ventricular hypertrophy and decreased fractional shortening, an index of the contraction of the heart, measured by Doppler. The effectiveness of polymer-encapsulated D-PDMP was evident by a marked decrease in lipid load and increased lumen volume in the aorta in animals treated with 1.0 mpk of polymer-encapsulated drug due to reduced levels of glycosphingolipids and bulk lipids such as cholesterol and triglycerides (Fig. 5). Importantly, these beneficial effects were often exhibited to a greater extent at 1.0 mpk of polymer-encapsulated D-PDMP than in animals treated with a 10-fold higher level of unconjugated D-PDMP; in most cases a higher dose of polymer-encapsulated drug showed no added benefit either. Most importantly, fractional shortening – an indicator of the

contraction of heart – as well as left ventricular mass-a marker of cardiac hypertrophy – returned to normal levels in treated apoE^{-/-} mice compared to mice fed a HFHC diet (Fig. 6F,G). These observations were replicated by echocardiographic studies as well (Fig. 6A–E).

At the molecular and genetic level, several biochemical endpoints (as measured experimentally in Fig. 7 and outlined mechanistically in Fig. 8 were monitored and found to be consistent with the known activity of D-PDMP. In particular, genes related to several aspects of cholesterol metabolism are shown in panels A through D of Fig. 7 and in almost every case not only is protective, increased activity observed but further this activity is achieved with an order of magnitude lower dose of polymer-encapsulated D-PDMP than with unencapsulated drug which our previous studies showed was only effective at 10 mpk [4]. In summary, feeding a HFHC diet to apoE^{-/-} mice adversely down regulates the expression of key genes involved in cholesterol, triglyceride and bile acid metabolism as well as genes implicated in cholesterol efflux. Encapsulating a glycosphingolipid glycosyltransferase inhibitor within a biopolymer markedly interferes with atherosclerosis by way of increasing the expression of genes critical in the metabolism of lipids and lipoproteins. Finally, by providing a protective effect, biomarkers of hypertrophy such as Nppa, Nppb, and Myh7 experienced increased expression in the left ventricle in mice fed the HFHC diet (Fig. 7E).

5. Conclusions

This study shows many disease markers of atherosclerosis and cardiac hypertrophy can be ameliorated as effectively with 1.0 mpk of polymer-encapsulated D-PDMP as with 10 mpk of unconjugated compound. Moreover, both polymer components (polyethylene glycol and sebacic acid) are FDA approved, which promises to facilitate human clinical trials of polymer-encapsulated D-PDMP to treat these manifestations of cardiovascular disease. In addition to the wide use of D-PDMP to study and manipulate glycosphingolipids, this compound offers new treatment options for a spectrum of diseases characterized by excessive GSL biosynthesis or deficient catabolism that in addition to cardiovascular disorders includes other prevalent disease states such as cancer [31,34] and various glycosphingolipidoses. Therefore, we expect that the methodology we describe in this work to encapsulate D-PDMP within a biocompatible and biodegradable polymer and achieve controlled release constitutes a major step in overcoming the poor pharmacological properties of this promising drug candidate and will foster accelerated research and clinical translation towards multiple disease endpoints.

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Fig. 1. Schematic for polymer preparation and nanoparticle formulation. (A) Sebacic acid – poly(ethylene glycol) (SA-PEG) polymers were prepared and (B) formulated into nanoparticles in the presence of D-PDMP. (C) Radioisotopically-labeled PEG ([¹²⁵I]Y-PEG) was synthesized and doped into the particles for *in vivo* imaging experiments.

Fig. 2. [^{125}I]Y-PEG planar gamma scintigraphy with CT. Three healthy C57bl/6 mice received 810 kBq of [^{125}I]Y-PEG-spiked D-PDMP-loaded polymer via oral gavage and were scanned at the indicated times. Radiotracer uptake, representing the path of the nanocarrier vehicle, is entirely biliary and is largely dispersed by 24 h post-administration in two of three mice and completely dispersed by 48 h post-administration. White arrow indicates the stomach and the red arrow the kidney. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

MSMS of drug dispersion in stomach, duodenum, and kidney. C57/bl6 mice ($n = 3$) were treated with unencapsulated D-PDMP at 10 mpk (A) or with 1.0 mpk (B) or 10 mpk (C) of polymer-encapsulated D-PDMP via oral gavage for the indicated time periods (e.g., 0.5, 1.0, 4.0, 24, and 48 h). In all cases, the tissues were homogenized (chloroform-methanol 2:1, v/v) and lipids extracted from the stomach, duodenum, liver and kidney and analyzed by mass spectrometry to determine the abundance of D-PDMP.

(A) Masson trichrome staining **(B) 2-D Mode Images** **(C) Quantification** **Fig. 4.**

Polymer-encapsulated D-PDMP was more effective in ameliorating atherosclerosis in ApoE $-/-$ mice compared to unencapsulated D-PDMP. (A) Masson trichrome staining of representative tubular ascending aorta revealed a significant amount of fibrosis, plaque, Ca^{2+} accumulation, wall thickening and defragmentation of elastin fibers, in the apoE $-/-$ placebo-treated mice fed a high fat high cholesterol (HFHC) diet compared to control animals fed normal chow. Treatment of animals fed a HFHC diet and with either 10 mpk D-PDMP or 1.0 mpk of polymer-encapsulated D-PDMP for 16 weeks after treatment intervention and 36 weeks of aging time reduced these endpoints. Nikon 80I Eclipse equipped with Nikon DS-EI1 camera and NIS-Elements software (Nikon, Japan) were used for image analysis. (B) Representative two dimensional (2-D) mode images of aortic artery from the control (normal chow) and HFHC-red animals subject to treatments in Panel (A). Again, the placebo treated animals showed an increase in aortic wall thickening (indicated

by the arrows) compared to the control mice and this effect was prevented by feeding 10 mpk D-PDMP or 1.0 mpk polymer-encapsulated D-PDMP. (C) These results are in agreement with the significant increase in the intima media thickness (IMT_AsAo) in the ascending aorta; this quantification confirms that the polymer-encapsulated D-PDMP was an order of magnitude more effective in ameliorating IMT_AsAo in ApoE^{-/-} mice compared to D-PDMP.

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Fig. 5.

Encapsulated D-PDMP is more effective in mitigating lipid accumulation than unconjugated D-PDMP. Levels of triglycerides (A), cholesterol (B), and oxidized LDL (C) were measured by ELISA. Glucosylceramide (D) and lactosylceramide (E) were measured by MSMS. In all cases, placebo-treated animals fed a HFHC diet experienced dramatic increases in each type of lipid and lipid accumulation was significantly reduced following treatment with D-PDMP (each condition is given in the Legend). Furthermore polymer-encapsulated D-PDMP was always equally or more effective than unencapsulated drug even at one tenth the dose (e.g., 1.0 mpk compared to 10 mpk) $n = 3$ (** $p < 0.0001$, ** $p < 0.001$, * $p < 0.01$).

Fig. 6.

Treatment with polymer-encapsulated D-PDMP (PE D-PDMP) prevents the onset of cardiac hypertrophy. Representative M-mode echocardiography of heart obtained from the left ventricle in the short axis view 36 weeks after the start of the intervention study showing results from control animals fed a normal chow diet (A) or a high fat high cholesterol (HFHC) diet (B–E). Animals on the HFHC diet included placebo-treated controls (B), animals treated with polymer-encapsulated D-PDMP at 1.0 mpk (C) or 10 mpk (D), or treated with unconjugated D-PDMP at 10 mpk (E). Panel (F) shows left ventricle mass (LV mass) and Panel (G) shows fractional shortening (FS) from control ApoE^{-/-} mice fed normal chow compared to mice fed a HFHC diet without D-PDMP treatment (placebo-treated), treated with 1.0 or 10 mpk of polymer-encapsulated D-PDMP, or 10 mpk unencapsulated D-PDMP 36 weeks after treatment intervention. The placebo mice showed a significant increase in LV mass with significant decline in cardiac contractility (FS) indicating maladaptive left ventricular hypertrophy. FS was not significantly different between the D-PDMP treated animal fed the HFHC diet and the control group fed normal chow indicating that the drug restored normal cardiac contractility. The LV mass changes were not significantly different between treatments but significantly reduced as compared to normal control suggesting that 1) 1.0 mpk D-PDMP is as effective as 10 mpk of unconjugated D-PDMP and 2) that these treatments reduce the aging effects of thickening and fibrosis and preserve cardiac contractility. $n = 5$ (** $p < 0.0001$, ** $p < 0.001$, * $p < 0.01$).

(A) Cholesterol synthesis and homeostasis **(B) Cholesterol efflux** **(C) Triglyceride metabolism** **(D) Bile acid formation** **(E) Hypertrophic genes** **Fig. 7.**

Effect of D-PDMP on the expression of hepatic genes that play roles in cholesterol and lipid metabolism and cardiac genes implicated in hypertrophy. Quantitative real-time polymerase chain reaction (RT-PCR) was used to determine the expression of hepatic genes involved in (A) cholesterol synthesis and homeostasis (**Hmgcr**, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; **Ldlr**, low density lipoprotein receptor; and **Srebf2**, sterol regulatory element binding transcription factor 2), (B) cholesterol efflux (**Scarb1**, scavenger receptor class B; and **Apoa1**, apolipoprotein A-I), (C) triglyceride metabolism (**Lpl**, lipoprotein lipase; and **Vldlr**, very low density lipoprotein receptor), (D) bile acid formation (**Abca1**, ATP-binding cassette sub-family ABCA1; and **Cyp7a1**, cholesterol 7 α -hydroxylase). (E) RT-PCR was also used to measure biomarkers of cardiac hypertrophy: **Nppa** (natriuretic peptide precursor A), **Nppb** (natriuretic peptide precursor B), **Myh7** (myosin heavy chain β), and **Myh6** (myosin heavy chain α). Values are means $\pm$ SEM. $n = 3$. (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

Fig. 8.

Proposed mechanism by which D-PDMP mitigates atherosclerosis and cardiac hypertrophy in mice. D-PDMP, which as this paper has shown can be delivered with an order of magnitude increased potency using a materials-based delivery approach, is a potent inhibitor of LacCer synthase (LCS). By inhibiting LCS and blocking conversion of GlcCer to LacCer, this drug candidate initiates a complex series of downstream effects that are discussed in detail elsewhere [22–31]. This report focus on measuring the effects of therapeutic intervention on lipids shown such as oxLDL, GlcCer, and LacCer (Fig. 5) as well as downstream effects on the transcription of the genes listed that are linked to atherosclerosis and cardiac hypertrophy.