

SUPPLEMENTAL MATERIAL

Paclitaxel Drug-Coated Balloon Angioplasty Suppresses Progression and Inflammation of Experimental Atherosclerosis in Rabbits

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METHODS

Inflammatory aortic lesions model suitable for endovascular PTA

Lesions were induced in the aortas of male and female New Zealand White rabbits (aortic diameter 3-3.5mm, weight 3-4 kg; Charles River Laboratories; Wilmington, MA, USA) by local balloon endothelial injury and high-cholesterol diet (HCD). After a 2-week lead-in atherogenic diet (1% cholesterol, 5% peanut oil; Research Diets; New Brunswick, NJ, USA), the infrarenal abdominal aorta of rabbits underwent balloon injury at week 2 as demonstrated previously (1).

Week 2: Anesthesia was induced with intramuscular ketamine (35mg/kg) and xylazine (5mg/kg), with a small dose of buprenorphine (0.01mg/kg). Anesthesia was continued using inhaled isoflurane (1-5% v/v, Novaplus, UK) and supplemental oxygen. The rabbit skin was shaved and cleaned and draped in a sterile manner. A small groin incision was made (approximately 1.5cm) overlying the natural lie of the common femoral artery. The artery was dissected and slung, with the distal end ligated. A small arteriotomy was made and a 4F (French) sheath introduced into the vessel. Blood was aspirated and collected for analysis. An

angiogram was taken using 50% saline and 50% iopamidol contrast media (McKesson Corporation, San Francisco, CA, USA). Next, a 3F Fogarty arterial embolectomy catheter (Edwards Lifesciences, Irvine, CA) was advanced through a 4F sheath into the infrarenal aorta, approximately 15 mm below the lowest renal artery. The aorta was injured by three sequential manual pullbacks of a nominally inflated 3F Fogarty under x-ray angiographic guidance (ARCADIS Varic C-arm; Siemens; Erlangen, Germany) over a distance of approximately 60 mm. The balloon was filled with a 0.9% saline/contrast 50:50 mix to a volume of 0.3 mL. The artery was then ligated, and muscular and skin closure was achieved by 3-0 vicryl. Rabbits were then recovered and placed in an E-collar for one week to prevent any manual disruption of the wound. Rabbits continued on a 1% high-cholesterol diet until week six, whereby this was replaced with a normal chow diet for the remainder of the study (four additional weeks; Figure 1).

Week 6 (PTA/balloon overdistension and DCB-PTA): To image arterial inflammation (cathepsin protease activity) in vivo, rabbits were injected with ProSense VM110 24 hours prior to imaging (400nmol/kg, via ear vein intravenous injection, a quenched sensor engineered to generate fluorescence following protease activation by cathepsins B, L, or S; PerkinElmer, MA, USA) (2, 3). At the baseline time-point of six weeks (Figure S1), rabbits underwent the same preparation and anesthetic protocol as at week two, and femoral and carotid artery access was secured using open surgical cutdown as above. Next using NIRF-OCT, IVUS and angiographic guidance to localize arterial inflammation and aortic lesions, a single paclitaxel-coated angioplasty (DCB-PTA) balloon of size 4.0x40mm (IN.PACT Admiral Medtronic, 3.5ug/mm² of drug coated concentration) or a single plain angioplasty (PTA) balloon 4.0x40mm (INVATEC Admiral Xtreme Medtronic) was inflated to a maximal pressure of 8 atmospheres (atm) for a period of 120 seconds, as per the manufacturer's instructions for use

(IFU). For the control sham-PTA group, a plain PTA balloon with the same 4.0x40mm dimension was placed in the aorta and was inflated to 0 atm for a period of 120 seconds. The average aortic diameter in the rabbit was 3.25mm correlating with a balloon overdilatation of 1.2:1 (4). Only the approximate distal 40 mm of the balloon injured area was subject to angioplasty. Care was taken not to disrupt the IN.PACT balloon once out of packaging (no direct handling) and to work as swiftly as possible. Based on the instructions for use, the DCB-PTA region was pre-dilated with a plain balloon before IN.PACT use. The area subject to angioplasty was determined using a combination of the angiogram, IVUS and OCT to ensure the NIRF co-registration was accurate. This involved a combination of distance from renal arteries, side branches and fiducial markers. This was analyzed across two separate timepoints to ensure both readings were comparable.

Week 10 (sacrifice): Rabbits were injected with ProSense VM110 24 hours prior to imaging as above (400nmol/kg). At the week 10 time-point (Figure 1), rabbits underwent the same experimental protocol as in week 2, with femoral and contralateral carotid access secured with surgical cut down. Angiograms, NIRF-OCT pullback and IVUS pullback were performed followed by sacrifice.

Molecular analysis

RNA extraction and cDNA synthesis: RNA extraction was achieved by homogenization of the tissue sample in question using a rotor-stator homogenizer. The RNeasy mini-kit (Qiagen, Waltham, MA) was used to extract RNA from the tissue. The RNAlater-stabilized tissue was cut into small pieces and placed in 600µL of RLT buffer and homogenized until the solution was uniformly homogenous. This was then centrifuged at full speed for 3mins. The resultant supernatant was extracted into a fresh Eppendorf and 600µL of 70% ethanol was added and

pipetted multiple times to mix the solution. 700 μ L of this solution was added to the RNeasy spin column and centrifuged for 15s at 8000rcf (relative centrifugal force). The flow through was discarded and 700 μ L of Buffer RW1 was added to the spin column and centrifuged for 15s at 8000rcf. The flow through was again discarded and 500 μ L of Buffer RPE added to the column and centrifuged for 15 seconds, 8000rcf. This step was repeated but the column was centrifuged for 2 minutes at 8000rcf. The collection tube was changed and a further centrifugation at max speed for 1 minute to dry the membrane. The spin column was then placed in a 1.5mL collection microcentrifuge tube and 30 μ L of RNase-free water was directly added to the center of the column and spun down for 1min at 8000rcf. Samples were then immediately placed on ice and RNA concentration (ng/ μ L) were obtained using the Nanodrop.

Complementary DNA (cDNA) was synthesized from 1 μ g of total RNA using a reaction mix with a final volume of 20 μ L: 2 μ L of 10x reverse buffer, 0.8 μ L of dNTP (10nM), 2 μ L of 10x random buffer 1 μ L Multiscribe and 14.2 μ L of RNA and water mix (VENDOR). The reaction was performed for 10mins at 25 °C, 120mins at 37 °C, 5 mins at 85 °C and held at 4 °C to produce cDNA. The cDNA was then diluted to 25ng/ μ L and stored in -20 °C, until further use.

qPCR: Assessment of Cathepsin B, Cathepsin L, Cathepsin S, TGF-beta, and IL-1beta RNA expression, was performed using SYBR Green real-time PCR. PCR primer sequences were designed using design software (Primer Express™, ver. 3.0.1, Thermo Fisher Scientific). BLAST (Basic Local Alignment Search Tool) searches were performed for all primer sequences to confirm gene specificity. The q-RT-PCR assays were performed using the QuantStudio 3 Real-Time PCR System (ThermoFisher Scientific) in a 96-well plate format. Real-time data were analyzed using the QuantStudio 3 Sequence Detection System 2.2.1 software (v20040907-2, 2004, ThermoFisher Scientific) with the detection threshold set manually at 0.05 for all the assays. All transcripts were standardized to GAPDH (housekeeping

gene). Quantification of relative gene expression was calculated by the comparative Ct method ($2^{-\Delta\Delta C_t}$).

In-vitro studies: primary human vascular aortic smooth muscle cells and PTX experimental set-up

Primary human smooth muscle cells (HSMC) from healthy donors were purchased from Cell Applications Inc., California, USA (Cat. # 354K-05a). Smooth muscle cell identity was assessed by immunofluorescence staining of contractile markers including SM22 α , calponin, smoothelin and vinculin. In order to preserve cell identity all experiments were carried out at passages 1-8. Human SMCs were grown with smooth muscle cell growth medium from Cell Applications Inc. (Cat.# 311-500). During the experiments, cells were kept in a standard air-incubator (95% air – 5% carbon dioxide; Haereus, Osterode, Germany) in a saturated atmosphere at 37°C. Cells were grown to passage 6-8 for experimental analysis, on 10cm² dishes. Prior to experiments, cells were plated in a 9.6cm² 6-well plate. On day 0, culture medium was changed to a fresh medium and all plates were treated with 100ng/mL of lipopolysaccharide (O26:B6 Escherichia coli, Sigma-Aldrich, USA) for a period of 24 hours. Following this, media was changed to an FBS-free media and cells were treated with graded concentrations of paclitaxel (Taxus yannamensis, T1912, Lot number: SLBS9204, Sigma-Aldrich, USA). For each plate (n=4), the following conditions were constructed; well 1: no LPS, only plated cells (control), well 2: LPS only, no drug, well 3: 1x10⁻⁵M paclitaxel in DMSO (total of 1% solution), well 4: 1x10⁻⁶M paclitaxel, well 5: 1x10⁻⁷M paclitaxel, and well 6: 1x10⁻⁸M paclitaxel. Duplicate plates were run as standard controls. Following paclitaxel application for 90 minutes (single dose), cells were then extracted for RNA and qPCR was performed (5, 6).

Immunohistochemistry

Serial sections were stained with RAM-11 (OCT-embedded) macrophage marker (conc: 1:500, Dako), with primary incubation overnight at 4°C, or SMA (conc: 1:1000, Abcam). This was then labelled with a biotinylated link antibody directed against mouse antigen with the use of an alkaline phosphatase enzyme and visualized with a Vulcan Fast Red Chromogen substrate kit (BioCare Medical). Sections were counterstained with Mayer's hematoxylin (Sigma-Aldrich). Omission of a primary antibody served as a negative control (data not presented). Computer-assisted color image analysis segmentation (Fiji) with background correction was used to quantify IHC staining of positive macrophages and SMCs.

IVUS Formulas:

$$\text{Lesion}_{\text{CSA}} (\text{mm}^2) = \text{EEM}_{\text{CSA}} - \text{Lumen}_{\text{CSA}}$$

$$\text{Plaque Burden (PB) (\%)} = (\text{Lesion}_{\text{CSA}} \div \text{EEM}_{\text{CSA}}) \times 100\%$$

$$\Delta \text{PB (\%)} = \text{PB}_{\text{follow-up}} - \text{PB}_{\text{baseline}}$$

$$\text{Percent atheroma volume (PAV) (\%)} = (\sum \text{Lesion}_{\text{CSA}} \div \sum \text{EEM}_{\text{CSA}}) \times 100\%$$

$$\text{Percentage Maximum Stenosis (\%)} = (1 - (\text{MLA} / \text{reference luminal area})) \times 100\%$$

$$\Delta \text{NIRF (nM)} = \text{NIRF}_{\text{follow-up}} - \text{NIRF}_{\text{baseline}}$$

NIRF Ratio = integrated NIRF signal at 10 weeks divided by the integrated NIRF signal at baseline 6 weeks (a measure of whole plaque inflammation change over time).

The minimal lumen area (MLA) and maximum PB were defined as the smallest lumen CSA and greatest PB measured within the plaque at a given time point.

Abbreviations: CSA: cross-sectional area; EEM: external elastic membrane; PB: plaque burden; PAV: percent atheroma volume; NIRF: near-infrared fluorescence; IVUS: intravascular ultrasound; OCT: optical coherence tomography.

Role of the funder

The funding sources had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. All authors had access to all the data in the study and had final responsibility for the decision to submit for publication.

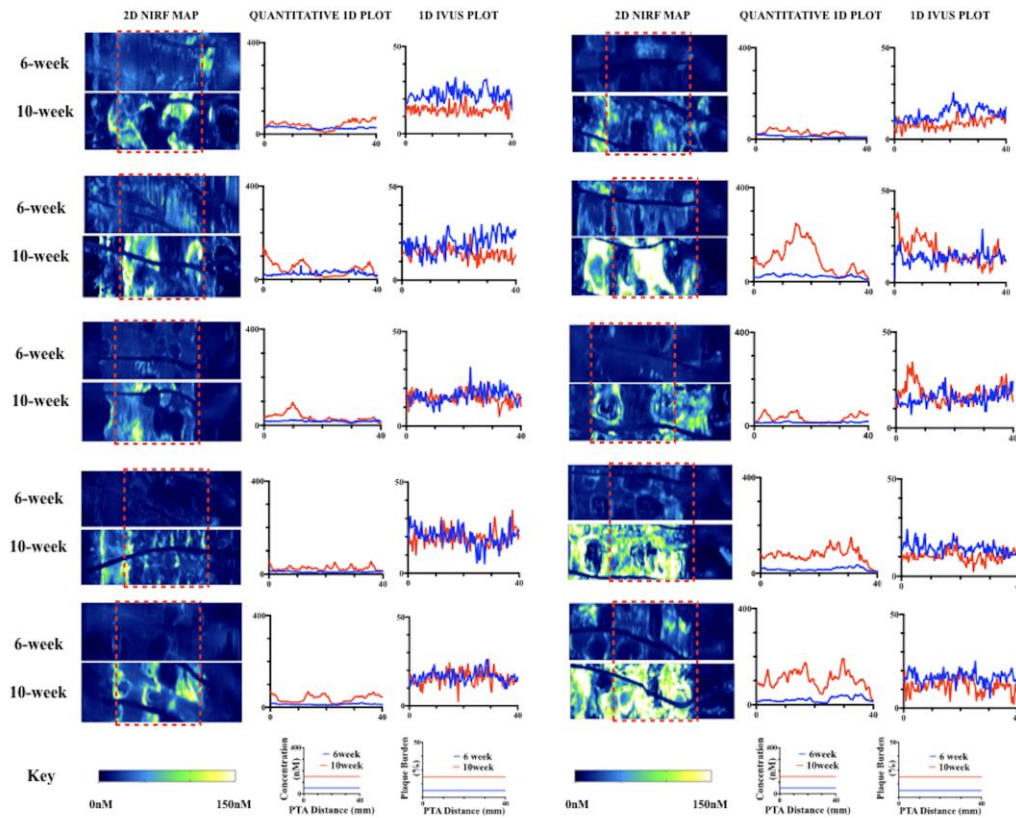
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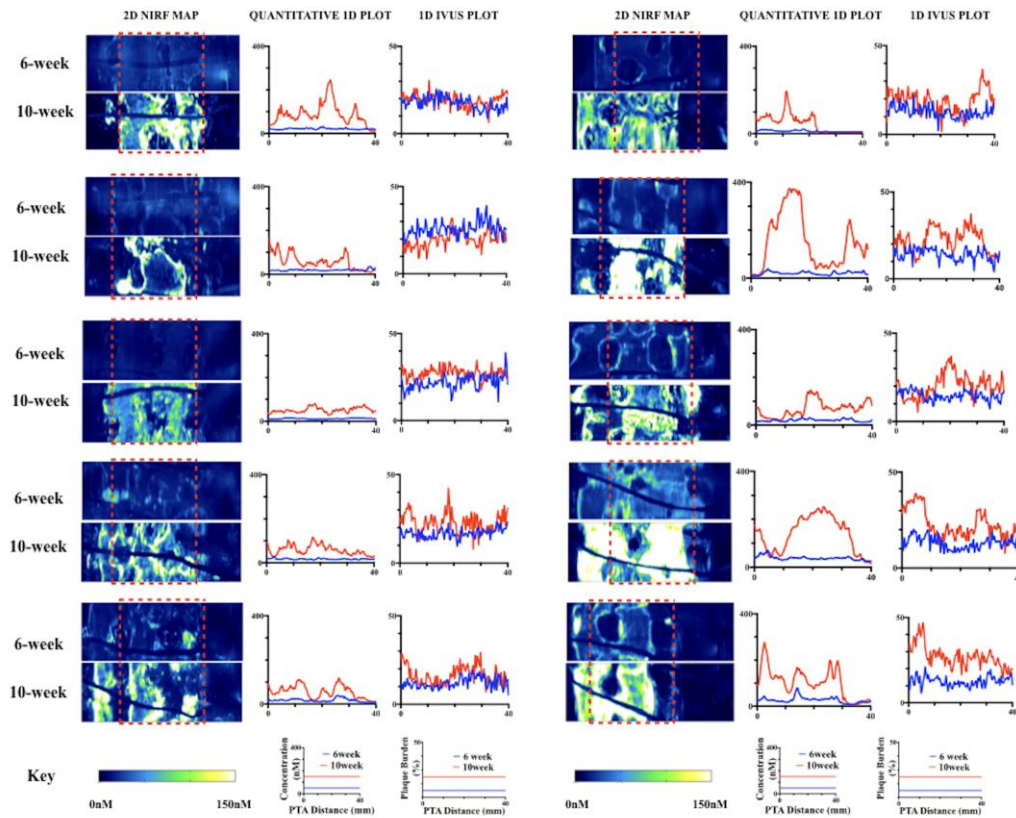
Supplemental Table 1

Parameter	PTA (n=10) Median [Q1, Q3]	DCB-PTA (n=10) Median [Q1, Q3]	P value
Cholesterol (mg/dL)			
6 weeks	2153 [1863, 2730]	1654 [1349, 2040]	0.151
10 weeks	572.5 [371, 1005]	439.5 [309, 619]	0.364
Δ Cholesterol	-1620 [-1708, -949]	-1082 [-1396, -1014]	0.257
CRP (ng/mL)			
6 weeks	26.8 [15.9, 28.5]	19.4 [18.4, 26.4]	0.706
10 weeks	24.6 [20.1, 72.0]	22.7 [8.0, 37.7]	0.406
Δ CRP	1.7 [0.5, 6.1]	-0.4 [-17.3, 13.8]	0.545
MLA (mm²)			
6 weeks	6.3 [5.7, 7.6]	5.5 [4.5, 6.6]	0.364
10 weeks	7.9 [6.5, 8.4]	7.0 [6.0, 7.3]	0.174
Delta	0.7 [0.5, 1.5]	1.1 [-0.6, 2.5]	0.880
PAV (%)			
6 weeks	14.3 [12.6, 17.7]	16.0 [15.3, 19.7]	0.257
10 weeks	21.1 [19.4, 23.1]	14.0 [11.7, 16.0]	***<0.001
Δ PAV	5.7 [4.4, 8.5]	-3.0 [-5.2, -0.6]	**0.003
TAV (mm³)			
6 weeks	145.6 [131.9, 153.6]	165.6 [147.8, 178.8]	0.364
10 weeks	237.5 [221.0, 253.0]	142.1 [120.2, 165.3]	***<0.001
Δ TAV	98.0 [58.8, 123.5]	-19.7 [-43.9, -5.8]	***<0.001
Average PB (%)			
6 weeks	14.2 [12.6, 17.5]	16.2 [14.4, 19.9]	0.326
10 weeks	19.9 [18.6, 22.8]	14.6 [11.7, 17.0]	*0.01
Δ PB	5.8 [4.4, 8.4]	-2.5 [-5.2, 2.4]	*0.013
NIRF (nM)			
6 weeks	19.6 [17.3, 21.2]	17.8 [13.6, 23.8]	0.706
10 weeks	63.4 [55.5, 101.2]	40.2 [33.0, 76.5]	*0.028
Δ NIRF	44.6 [38.9, 74.4]	18.3 [15.1, 58.9]	*0.028

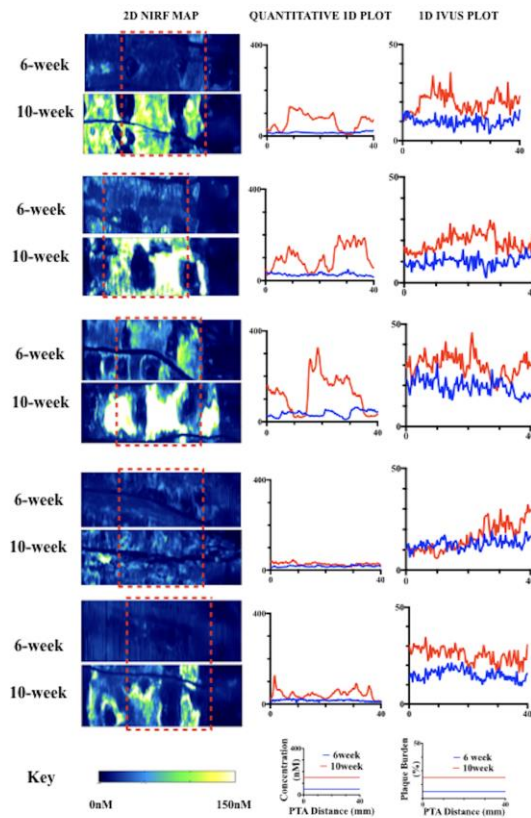
*p<0.05, **p<0.01, ***p<0.001



Supplementary Figure 1. Representative NIRF inflammation and IVUS plaque burden imaging data for all DCB-PTA (N=10) animals. All 2D NIRF maps at 6 week and 10-week timepoints after injection (24 hours prior) with ProSense VM110 for DCB-PTA subjects. The area subject to angioplasty is illustrated by the **red dotted box** (2D NIRF map). The accompanying 1D plots show the NIRF concentration and plaque burden by IVUS at both timepoints. Inset graphs demonstrate 6 week (**blue**) and 10 week (**red**) keys. IVUS image **white scale bar**, 1mm. 2D; 2-dimensional, 1D; 1-dimensional.



Supplementary Figure 2. Representative NIRF inflammation and IVUS plaque burden imaging data for all PTA (N=10) animals. All 2D NIRF maps at 6 week and 10-week timepoints after injection (24 hours prior) with ProSense VM110 for PTA subjects. The area subject to angioplasty is illustrated by the **red dotted box** (2D NIRF map). The accompanying 1D plots show the NIRF concentration and plaque burden by IVUS at both timepoints. Inset graphs demonstrate 6 week (**blue**) and 10 week (**red**) keys. IVUS image **white scale bar**, 1mm. 2D; 2-dimensional, 1D; 1-dimensional.



Supplementary Figure 3. Representative NIRF inflammation and IVUS plaque burden imaging data for all Sham-PTA (N=5) animals. All 2D NIRF maps at 6 week and 10-week timepoints after injection (24 hours prior) with ProSense VM110 for sham-PTA subjects. The area subject to angioplasty is illustrated by the **red dotted box** (2D NIRF map). The accompanying 1D plots show the NIRF concentration and plaque burden by IVUS at both timepoints. Inset graphs demonstrate 6 week (**blue**) and 10 week (**red**) keys. IVUS image **white scale bar**, 1mm. 2D; 2-dimensional, 1D; 1-dimensional.