Supporting Information

Targeted delivery of LXR-agonists to atherosclerotic lesions mediated by polydiacetylene micelles

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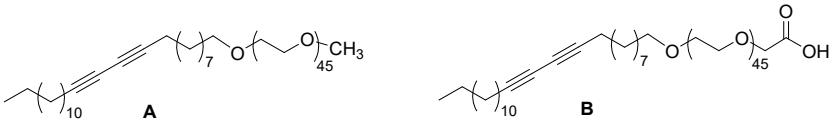
Chemistry

1- General

Unless otherwise specified, chemicals were purchased from Sigma-Aldrich and used without further purification. DiD dye was purchased from PromoKine. CH_2Cl_2 was distilled from calcium hydride before use. ^{1}H-NMR spectra were recorded on a Bruker Avance DPX spectrometer operating at 400 MHz. Chemical shifts (δ) are given in ppm relative to the NMR solvent residual peak and coupling constants (J) in hertz. Mass spectra were recorded using a MarinerTM ESI-TOF spectrometer. Dynamic light scattering (DLS) measurements were carried out using a Vasco Flex instrument by Cordouan Technologies equipped with a laser diode (λ = 450 nm). For ultrasonic mixing, an ultrasonic probe (Branson Sonifier 450, Output 4, Duty cycle 30%) was used. Photo-polymerization experiments were carried out using a low-pressure 40 W mercury UV lamp (Heraeus) at 254 nm.

2- Assembly of pDA-PEG micelles



Polymerizable pegylated amphiphile units **A** and **B** were prepared according to our previously reported procedure (*Nanoscale*, **2020**, *12*, 2452). A mixture of DA-PEG(OMe) **A** (45 mg, 19.5 µmol) and DA-PEG(CO_2H) **B** (5 mg, 2.12 µmol) was suspended in PBS (5 mL). The solution was sonicated using an ultrasonic probe for 10 min (300 ms pulse per second, output 45%) providing a pale-yellow clear colloidal suspension. The colloid was subjected to UV irradiation at 254 nm for 4 h and filtered on 0.2 µm nylon membrane to afford a light-yellow micellar solution.

3- Characterization of pDA-PEG micelles by Dynamic Light Scattering

For the DLS analysis, 10 acquisitions of 60 s were recorded. The hydrodynamic diameter of the polymerized micelles was measured at 12 nm (Figure S1).

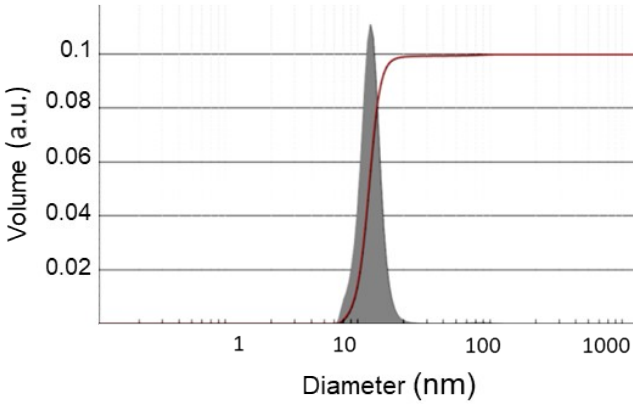
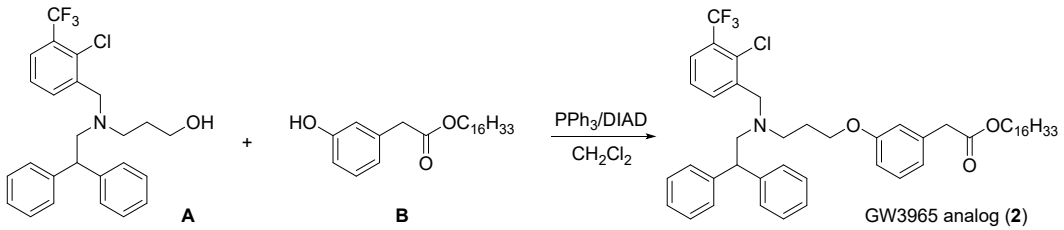


Figure S1. DLS profile of pDA-PEG micelles.

4- DiD loading in pDA-PEG micelles

A stock solution of DiD was prepared in EtOH (10 mg mL^{-1}). 10 µL of this stock solution were added to the suspension of polymerized micelles (1 mL). The mixture was sonicated using an ultrasonic probe for 30 min (300 ms pulse per second, output 45%) and filtered on a 0.2 µm nylon membrane to afford a green colloid suspension. A sample of the obtained fluorescent micelle suspension (10 µL) was diluted in MeOH (1 mL) and quantification of DiD was carried out using UV-vis spectroscopy at 644 nm (molar attenuation coefficient of DiD = 193 000 M^{-1} cm^{-1} in MeOH),

5- Synthesis of the GW3965 analog 2



The synthesis of the GW3965 analog **2** was achieved using, as the key step, a Mitsunobu coupling reaction between amino-alcohol **A** and phenol-ester **B**, according to *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1097.

Under N_2, diisopropyl azodicarboxylate (53 µL, 1.5 equiv.) was added dropwise to a solution of amino-alcohol **A** (80 mg, 0.18 mmol, 1 equiv.) and triphenylphosphine (70 mg, 1.5 equiv.) in anhydrous CH_2Cl_2 (3 mL). The reaction mixture was stirred for 15 min at room temperature before phenol **B** (58 mg, 1 equiv.) was added. The reaction was further stirred for 12 h at room temp. and quenched with 10% aqueous HCl. The aqueous phase was extracted twice with Et_2O, the combined organic layers were washed with saturated K_2CO_3, dried on $MgSO_4$, filtered and concentrated under vacuum. The crude product was purified by column chromatography on silica (cyclohexane/ethyl acetate, 95:5). Compound **2** was obtained as a colorless oil in 43% yield (58 mg).

^{1}H-NMR ($CDCl_3$): δ 7.48 (d, J = 7.6 Hz, 1H), 7.31–7.12 (m, 13H), 6.90–6.80 (m, 2H), 6.72–6.61 (m, 2H), 4.16 (t, J = 7.7 Hz, 1H), 4.11 (t, J = 6.8 Hz, 2H), 3.81 (s, 2H), 3.70 (t, J = 5.9 Hz, 2H), 3.60 (s, 2H), 3.17 (d, J = 7.7 Hz, 2H), 2.73 (t, J = 6.5 Hz, 2H), 1.91–1.81 (m, 2H), 1.68–1.55 (m, 5H), 1.28 (s, 33H), 0.90 (t, J = 6.7 Hz, 3H) ppm.

^{13}C-NMR ($CDCl_3$): δ 171.7, 159.1, 143.6 (2C), 139.8, 135.6, 133.9, 133.8, 129.5, 128.5 (4C), 128.4, 128.4 (4C), 128.3, 126.5 (2C), 126.3, 126.2, 121.6, 115.5, 113.2, 77.4, 65.2, 60.5, 56.2, 50.9, 49.9, 41.6, 32.1, 29.9–29.8 (6C), 29.7, 29.6, 29.5, 29.4, 28.7, 27.0, 26.0, 22.8, 14.3 ppm.

MS (ESI+): *m/z* = 807 $[M+H]^+$.

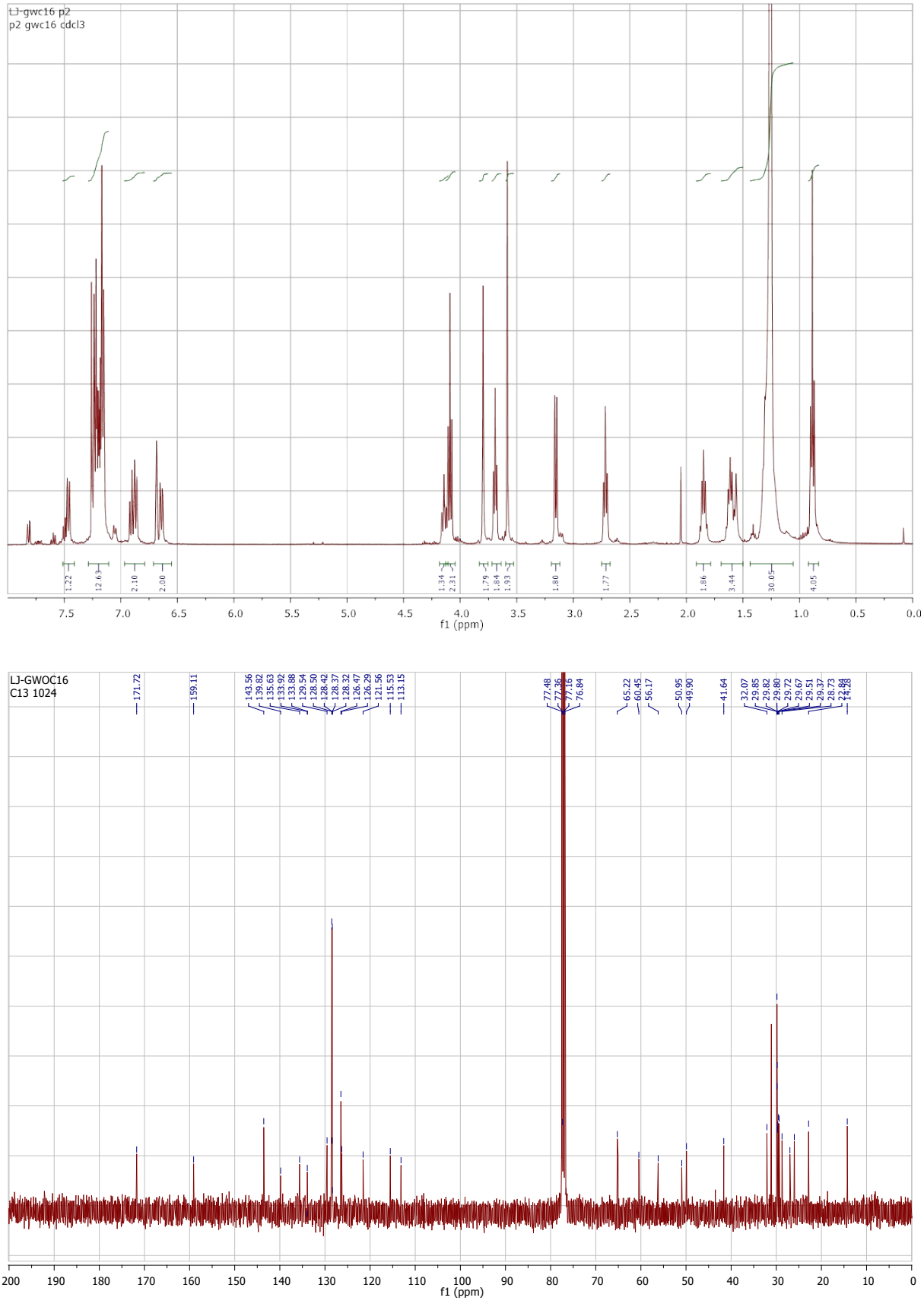


Figure S2. ^{1}H (top) and ^{13}C-NMR (bottom) spectra of GW3965 analog **2**.

6- Drug loading in pDA-PEG micelles

A stock solution of the analog **2** was prepared in $CHCl_3$ (5 mg mL^{-1}). 200 µL of this solution were added to a suspension of polymerized micelles in PBS (1 mL). The biphasic mixture was sonicated using an ultrasonic probe for 30 min (300 ms pulse per second, output 45%) and filtered on a 0.2 µm nylon membrane to afford a yellow clear suspension. Quantification of analog **2** encapsulated in micelles was carried out using UV-visible spectroscopy. The quantification was achieved by diluting samples (10 µL) in EtOH (90 µL) and using the calibration curve plotted using the characteristic absorbance of **2** at 272 nm.

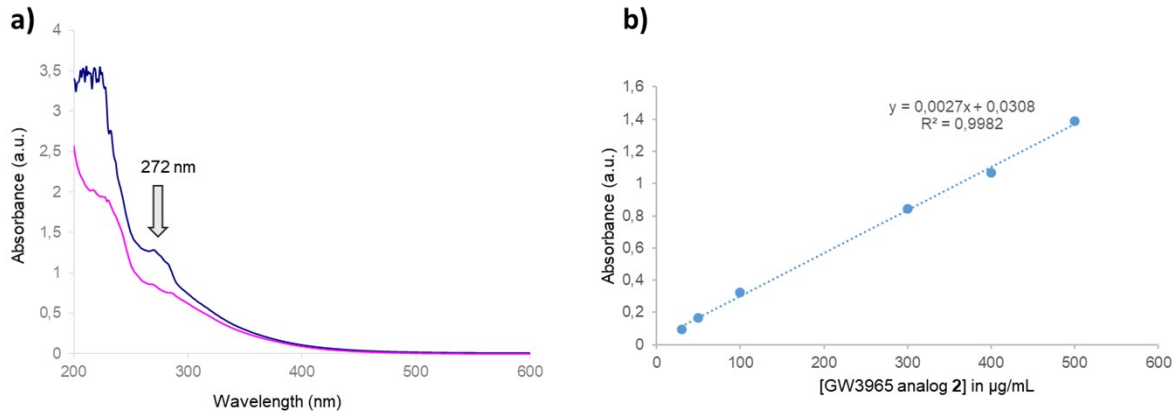


Figure S3. UV-vis spectra in EtOH of: a) pDA-PEG micelles (pink) and GW3265 analog **2** encapsulated in pDA-PEG micelle (blue); b) calibration curve obtained using the characteristic absorbance peak of **2** at 272 nm.

Biology

1- General

In vivo experiments were conducted in compliance with animal welfare regulations. Procedures were carried-out according to the Directive 2010/63/EU of the European Parliament. Animal studies were approved either by the French ethics committee (CEEA, agreement number 12-101) or the regional veterinary services of the "Préfecture de police de Paris" (agreement number 75-751320) / Sorbonne University biology service unit.

8-week old LDLr -/- mice (*Ldlr^{tm1Her}/J* - Jackson Laboratories) were individually housed in a conventional animal facility and had access ad libitum to food and drink. LDLr -/- mice were fed a high-fat rodent diet for 18 weeks to develop atherosclerotic plaques along the aorta and within the brachiocephalic artery.

2- Pharmacokinetics and biodistribution of DiD-loaded micelles

Pharmacokinetics: DiD-loaded micelles (100 µL of a 10 mg/mL micellar solution) were injected intravenously in the tail vein of LDLr -/- mice (*n* = 3) under anesthesia (2% isoflurane). At various time points (1, 5, 15, 30, 60, 120, 240, 360, 1440, 2880 and 4320 min), blood samples (whole blood) were collected and fluorescence measured using 2D fluorescence reflectance with FMT Perkin Elmer 1500's instrument with planar imaging capability. Imaging was conducted with the appropriate excitation (Ex) and emission (Em) filter sets: Ex/Em = 675/720 nm.

Blood fluorescence was converted to % injected dose using a standard curve derived from serial dilutions in mouse blood of DiD-loaded micelle of known concentration.

Biodistribution: Animals were then sacrificed by carbon dioxide asphyxiation after 72 h and whole aorta, liver, spleen, kidneys, lungs, femur bone and brain were harvested post-mortem. Organs and tissues were imaged as above.

Fluorescence was quantified as mean efficiency per pixel and was reported either as % injected dose for aorta (Figure S4a) using the standard curve that was used for mouse blood or as arbitrary unit for other organs (Figure S4b).

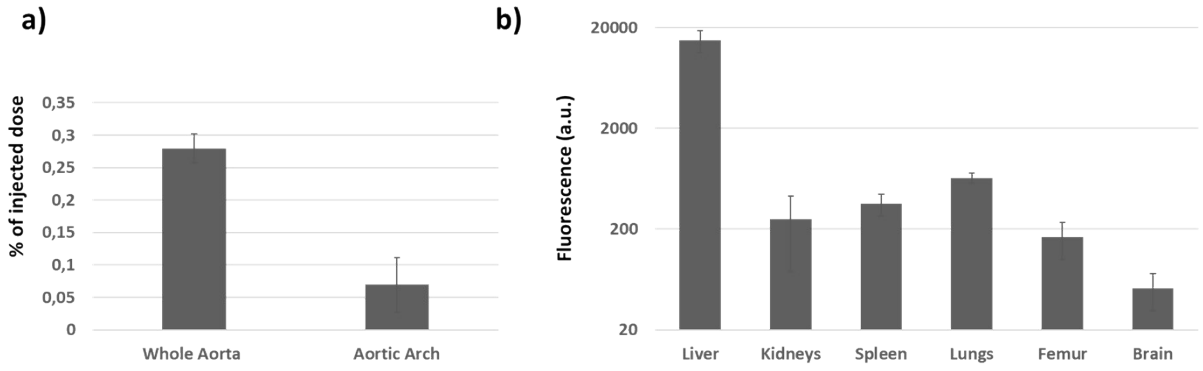


Figure S4: Biodistribution of pDA-micelles in LDLr -/- mice (*n* = 3) 72 h post injection. a) % of injected dose found within the whole aorta and aortic arch. b) Biodistribution and fluorescence intensity patterns observed for pDA-micelles in liver, spleen, kidneys, lungs, femur bone and brain harvested. Fluorescence is expressed as arbitrary units (AU) and reported in logarithm scale.

3- Preparation of cross-section aorta for fluorescence microscopy

Hearts were collected, fixed with 4% PFA followed by overnight incubation in phosphate-buffered 20% sucrose solution at 4 °C and subsequently embedded in Tissue-Tek OCT compound (Sakura Finetek). Aortic root cryosections (10 µm) were fixed in 4% PFA for 5 h. Sections were subsequently counterstained for nuclei with 4',6-diamidino-2-phenylindole (DAPI). Images were captured using a Zeiss AxioImager M2 microscope.

4- Extraction of cells from atherosclerotic plaque and FACS analysis

Tissues were collected from mice perfused with PBS through the heart left ventricle. The aorta was harvested and placed in HBSS (Hank's Balanced Salt Solution, 1 mL) containing an enzyme cocktail (Collagenase II and Elastase for adventitia digestion, Collagenase I and XI, Hyaluronidase type 1-5, DNase I for aorta digestion), minced with scissors and incubated for 20 min at 37 °C with the adventitia digestion enzyme cocktail mix and then 30 min at 37 °C with the aorta mix. Cell suspensions were passed through a 100 µm filter and then stained with appropriate antibodies (CD45, CD11b, F4/80, CD64, CD11c, CD19, CD3) for 30 min on ice. Data were acquired on a BD LSR Fortessa flow cytometer (BD Biosciences) and analysed with FlowJo software (Tree Star). DiD fluorochrome was detected as Cy5.5 with the red laser (640 nm) and a 730/45 nm filter.

5- Pharmacokinetics of GW3965, GW3965 analog 2 and GW3965 analog 2-loaded micelles

GW3965 (100 µg/DMSO), GW3965 analog **2** (100 µg/DMSO), or GW3965 analog **2**-loaded micelles (100 µL of a 10 mg/mL micellar solution containing 9 wt% of **2**) were injected intravenously in the tail vein of LDLr -/- mice (n = 3) under anesthesia (2% isoflurane). Blood samples were collected at different time points and pharmacokinetics of the drugs was assessed according to the protocol below.

Stock solutions for each standard (GW3965, compound **2** or **2**@pDA-micelle) were first prepared at concentrations of 1.7 mM in ethanol and 1.6 mM in water respectively. For establishing calibration curves, known amounts of each standard were spiked into control mouse plasma before performing the extraction according to the following protocol. GW3965, compound **2** or **2**@pDA-micelle were extracted from plasma samples by precipitation and elimination of plasmatic proteins with ice-cold acetone. One volume of plasma sample was thoroughly mixed with four volumes of ice-cold acetone and incubated at −20 °C for 1 h. After centrifugation (15 000 ×g at 4 °C for 10 min), the supernatant was collected. A second extraction was performed using additional cold acetone on the plasma pellet. Both supernatants were gathered and dried at room temperature under hood ventilation. Dried extracts were finally re-suspended in pure ethanol and injected into LC-MS/MS for dosage in MRM scanning mode.

The LC-MS/MS system consisted of an Agilent 1100 HPLC system on-line coupled to an Esquire-HCT ion Trap mass spectrometer (Bruker-Daltonics) equipped with an ESI source for positive ionization mode. LC separation was carried out at 200 µL min^{-1} flow rate on a Thermo Fisher Scientific reverse phase monolithic ProSwift-RP-4H column (1.0 × 50 mm) with a linear gradient 5–100% B over 4 min followed by a 3 min step at 100% B with mobile phase A composed of 0.1% TFA in water and B 0.1% TFA in acetonitrile. ESI conditions were optimized for ionization and detection of GW3965 and compound **2**. Fragmentation energy amplitudes were also optimized for both compounds. The fragmentation transitions followed for dosing of GW3965 and **2** were m/z 582→402 and m/z 806→626, respectively. DataAnalysis software (Bruker Daltonics) was used for qualitative and quantitative processing of raw MS and MS/MS data. The ion chromatograms of MS transitions

were extracted (EIC) and integration of these EICs peaks enabled to plot calibration curves of GW3965 or **2** in plasma and further quantitate their respective plasmatic concentrations in treated mice.

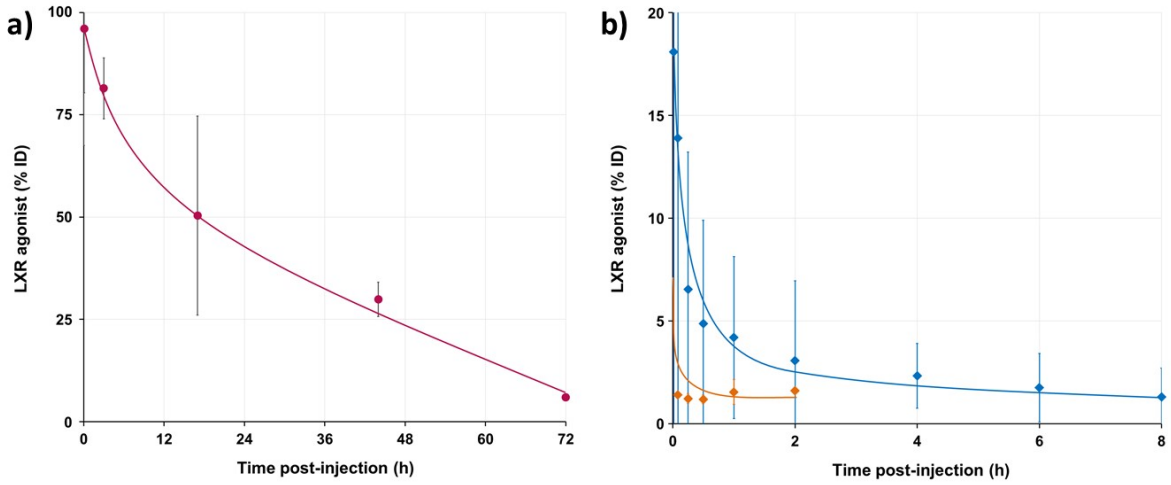


Figure S5: a) Pharmacokinetics of GW3965 analog **2** loaded in micelles (red). b) Pharmacokinetics of GW3965 (blue) and GW3965 analog **2** (orange).

6- Analysis of ABCA1 and ABCG1 expression in primary macrophages

In vitro experiments with macrophages were conducted with primary macrophages isolated from the peritoneal cavity of mice injected (i.p.) with thioglycollate (Sigma-Aldrich, l'Ile d'Abeau, France; 3% wt/vol in PBS). Two days post-injection, mice were euthanised, and ice-cold PBS (3 mL) was injected i.p. to harvest peritoneal cells in tissue culture wells. Adherent monocyte-derived macrophages were then incubated for 4 h at 37 °C in the absence of serum. Then, the cultured macrophages were incubated for 24 h with the appropriate compounds. Cells were washed with PBS, lysed with RA1 lysis buffer (Macherey-Nagel) and frozen at −80 °C. Total RNA was prepared using the NucleoSpin RNAII kit (Macherey-Nagel). RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative PCR analyses were performed using a LightCycler480 real-time PCR system and dedicated software (Roche). Gene expression of ABCA1 and ABCG1 was normalized to the mean expression of Hprt, Rpl13a and Nono housekeeping genes.

7- Triglycerides quantification in liver and gene expression of ABCA1 and ABCG1 in the aorta

Three groups of LDLr -/- mice (*n* = 5) were injected, on a daily basis over 5 days, with either i) 8:2 DMSO/PBS (25 µL), ii) GW3965 (120 µg) in 8:2 DMSO/PBS (25 µL), or iii) **2**@pDA-PEG micelles in PBS (135 µL, corresponding to 120 µg of **2**), respectively.

Mice were anaesthetized with isoflurane (2%) and then euthanized by cervical dislocation. Livers and aorta were collected from mice perfused with PBS. The aorta cells were isolated following the digestion protocol detailed above (section 4). Livers were placed in 3 mL of HBSS containing collagenase D (2.5 mg/mL, Sigma) and DNase (10 U/mL, Sigma), minced with scissors, run on the GentleMACS dissociator and incubated for 30 min at 37 °C under agitation. Samples were then run on the GentleMACS dissociator for a second time. Cell suspensions were passed through a 100 µm filter before staining.

Frozen liver samples (approximatively 50 mg) were homogenized in 3:2 chloroform/methanol mixture (1 mL) using a tissue homogenizer (Precellys, Bertin Technologies) and lipids were extracted overnight. After

centrifugation (20 min × 2200 rpm) at 4 °C, an aliquot of the extract was combined (1:1) with 1% Triton in chloroform. After drying, a commercially available kit (Diasys) was used for colorimetric quantification of triglycerides.

For total aorta RNA isolation, frozen liver samples were homogenized in RA1 lysis buffer (Macherey-Nagel) using a tissue homogenizer (Precellys, Bertin Technologies). Total RNA preparation was performed using the NucleoSpin RNA Plus kit (MACHEREY-NAGEL). RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative PCR analyses were performed using a LightCycler480 real-time PCR system and dedicated software (Roche). Gene expression of ABCA1 and ABCG1 was normalized to 3 housekeeping genes (Hprt, Rpl13a, and Nono).