Supporting Information for

A Dual-targeting Peptide Facilitates Targeting Anti-inflammation to Attenuate Atherosclerosis in ApoE$^{-/-}$ Mice

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Chemical and materials: Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Fmoc-amino acids were obtained from GL Biochem (Shanghai, China). 2-Cl-trityl chloride resin was obtained from Nankai Resin Co. Ltd. (Tianjin). MMP-9 (1 mg/mL) was obtained from Cloud-clone Corp (Wuhan, China). 4-Chloro-7-nitro-1,2,3-benzoxadiazole (NBD-Cl, purity ≥ 99 %) was purchased from Beijing InnoChem Science & Technology Co. Ltd. (Beijing, China). All the other starting materials were obtained from Alfa (Beijing, China).

General methods: HPLC was conducted at the LUMTECH HPLC (Germany) system using a C18 RP column with MeOH (0.05 % of TFA) and water (0.05 % of TFA) as the eluents. TEM was performed on a JEOL JEM-2100F Field Emission Electron Microscope, operating at 200 kV. The confocal images were taken by a confocal laser scanning microscopy (Leica TSC SP8, Germany). CCK-8 assay for cell toxicity test was performed on a Thermo Scientific Varioskan LUX Multimode Microplate Reader.

Cells experiments

Cell culture: HUVECs were cultured in 4.5 g/L Dulbecco's modification of Eagle's 9 medium (DMEM) with 10 % fetal bovine serum (FBS) and 1 % antibiotics in a 5 % CO_2 atmosphere at 37 ℃. For the establishment of in vitro model, HUVECs were pre-treated by 1μg/ml Lipopolysaccharide (LPS, Abcam, USA) for 24 h, then different compounds were added to co-incubate for 2 days.

Cell viability assay: The cell viability was measured by using CCK-8 assay kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, HUVECs were plated in 96-well plates with a density of 3×10^5 cells/well. After treatments with *Comp.TM* alone at various concentrations (0, 1, 5, 10, 25, 50 and 100 μM) for 48 h, medium (90 μl) was incubated with CCK-8 solution (10 μl) for 2 h in darkness at 37°C. Absorbance was measured at 450 nm by a microplate reader (VARIOSKAN LUX, Thermo Scientific, USA).

ELISA: Determination of MMP-9 levels in the cell culture medium by ELISA was performed using a Human matrix metalloproteinase 9 MMP-9 ELISA Kit (mskbio, China). The cell suspension was aspirated into an eppendorf tube, followed by centrifugation at 2000 rpm for 20 mins, and the supernatant was carefully collected after centrifugation. 50 μL of distilled water or standard substance with different concentrations were added into the corresponding wells. 40 μL of diluent and 10 μL of sample were added into sample wells. After that, the plate was sealed with a piece of sealing paper, followed by incubation at 37℃ for 30 mins. The substances in each well were aspirated and washed for 5 times. 50 μL enzyme-linked reagent was added into each well, which was aspirated and washed again after incubation at 37℃ for 30 mins. Then, 50 μL chromogenic agent A and B were added into each well, followed by incubation away from the light at 37℃ for 15 mins. After that, 50 μL stop buffer was added into each well. The optical density at 450 nm was read. The MMP-9 release levels were calculated on the basis of the standard curve.

Immunofluorescence staining: HUVECs were cultured on confocal dishes and harvested at indicated time points. HUVECs were first permeabilized with 0.1 % 10 Triton X-100 after fixed with 4 % paraformaldehyde for 30 mins. Then the cells were washed and blocked with 1 % BSA for 30 mins at room temperature. Next, the cells were incubated overnight at 4 °C with anti-MMP-9 (Affinity, China) and anti-αvβ3 (Bioss, China). Finally, the cells were incubated with Goat Anti-Rabbit IgG (H + L) Dylight 488 (1:200, Bioworld technology, China) and Goat anti-Mouse IgG (H + L)-FITC (1:200, Bioworld technology, China) for 2 h and counterstained with DAPI Staining Solution (Beyotime, Haimen, China) for 30 mins at room temperature. The cells were imaged by a fluorescence microscope.

Quantitative real-time PCR: Total RNA was extracted from HUVECs using TRIzol regents (Invitrogen, USA) and mRNA was reverse-transcribed with the PrimeScript RT reagent kit (TaKaRa, Japan) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed using SYBR Premix (Takara, Japan) in a StepOne Plus real-time PCR system (Applied Biosystems, USA). qPCR primers used in this study were outlined in Table 1. Value was normalized to β-actin and relative gene expression was determined using

comparative $2^{(-\Delta\Delta Ct)}$ method.

Table 1 Primers for qPCR

Primers	Forward	Reverse
TNF-α	CCCGAGTGACAAGAATGTAG	TGAGGTACAGGCCCTCTGAT
IL-1β	GCCCTAAACAGATGAAGTGCTC	GAACCAGCATCTTCCTCAG
IL-8	AACACAGAAATTATTGTAAAG	CACTGATTCTTGGATACC
β-actin	CGTGACATTAAGGAGAAGCTG	CGTGACATTAAGGAGAAGCTG

Western blot analysis: Total protein was extracted from cultured HUVECs using RIPA buffer with protease and phosphatase inhibitors. Protein concentration was determined by BCA™ Protein Assay kit (Thermo Fisher Scientific, USA). Protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinyl difluoride (PVDF) membranes. The membranes were then blocked with 5 % skim milk in Tris-buffered saline at room temperature for 2 h. Afterwards, the membranes were incubated with primary antibodies against VCAM-1 (Abcam, USA), MCP-1 (Boster, China), αvβ3 (Bioss, China), MMP-9 (Bioss, China), NFκB subunit p65 (Cell Signaling Technology, USA), YAP (Boster, china), and β-actin (Bioworld, China) overnight at 4 °C. The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000, Bioworld technology, China) at room temperature for 2 h. Protein signals were detected by the enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, USA). Protein expression levels were semi-quantified by densitometry and normalized to β-actin expression.

THP-1 monocyte adhesion assay: HUVECs were seeded in 48-well plates and were treated with 10 μM of different compounds or 1 μg/ml LPS for 2 days. THP-1 human monocytic leukemia cells (5×10^6 cells/mL) were labeled with 2 μM Calcein-AM for 45 min at 37 °C

and seeded onto confluent HUVECs. One hour after addition of THP-1 cells, unbound THP-1 cells were thoroughly washed with PBS. The number of attached monocytes were counted in three randomly chosen microscopic fields per well after photographed under the fluorescence microscopy.

Animals' experiments

Animals and ethics statement: ApoE$^{-/-}$ mice were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). Animals were fed ad libitum with standard chow diet until surgery at 8–9 weeks of age. Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health) and were approved by the Institutional Animal Care and Use Committee at Southern Medical University.

Ex vivo tissue culture: Mice were anesthetized with pentobarbitone sodium and pressure-perfused with heparinized normal saline, and the aortas were harvested and opened longitudinally under sterile conditions. Aortas were incubated with/without 1 μg/ml LPS in DMEM supplemented with 10 % FBS and 1 % antibiotics for 2 days, then 25 μM ***Comp.TM*** was added to co-incubate for 24 h.

Mouse partial carotid ligation surgery: Mouse partial carotid ligation procedures were performed as previously described. Briefly, mice were anesthetized with 1 % pentobarbital sodium (50 μl/10g). The left carotid artery (LCA) bifurcation was exposed by blunt dissection and three of four caudal LCA branches (left external carotid, internal carotid, and occipital arteries) were ligated with 6-0 silk sutures, leaving the superior thyroid artery intact. The contralateral right carotid artery (RCA) was left intact as an internal control. Following surgery, the surgical incision was then closed with 6-0 monofilament sutures. Mice were randomly divided into six groups (n = 5): control, Pue, ***Comp.TM***, ***Comp.T***, ***Comp.M***, and ***Comp.S***. The control group received an injection of phosphate-buffered saline (PBS). In the other groups, different compounds (20 mg/kg/d) were injected intraperitoneally each day. All mice were fed a high-fat diet for 2 weeks.

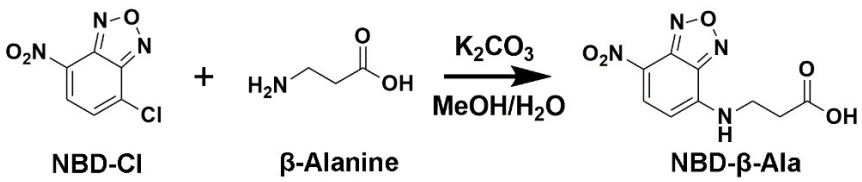
The biological activity and toxicity of *Comp.TM* in vivo: After ligation surgery, ApoE$^{-/-}$ mice were treated with different concentrations of ***Comp.TM*** (5, 20 and 50 mg/kg, daily, intraperitoneally) and fed a high-fat diet for 2 weeks. Mice were sacrificed and Hematoxylin and Eosin (HE) staining on organisms was performed for histological examination.

Plaque lesion analysis: At the end of experiments, mice were sacrificed and aorta and carotid arteries en bloc were isolated. Aortic tree, including the carotid arteries, and cross sections derived from the lesion area of LCA, were stained with oil-red-O. The plaque size was quantified using ImageJ software.

Statistical analysis: Data were analyzed by SPSS 21.0 software and expressed as mean ± SEM. Normality and equal variance were confirmed by Shapiro-Wilk test and then multiple comparisons of means were performed using one-way analysis of variance followed by Tukey's post hoc multiple comparison test. If the sample did not pass normality, statistical analyses were performed by unpaired Mann-Whitney *U* test and non-parametric Kruskal-Wallis test with Dunn's post hoc test. Differences with $P < 0.05$ were considered significant statistically.

Synthesis of peptide *Comp. TM*.

Synthesis of NBD-β-Ala (Scheme S1)**:** 540 mg β-Alanine and 1.38 g K_2CO_3 were dissolved in the mixture of 50 mL MeOH and 10 mL H_2O. 1 g NBD-Cl was dissolved in 20 mL MeOH and was then added to the above solution and reacted overnight at room temperature. Then MeOH was evaporated, and the pH of the residual solution was adjusted to 1-2 using 2 M HCl. The resulting suspension was filtrated and the solid was collected for following synthesis. (87 % yield).



Scheme S1. Synthetic route of NBD-β-Ala.

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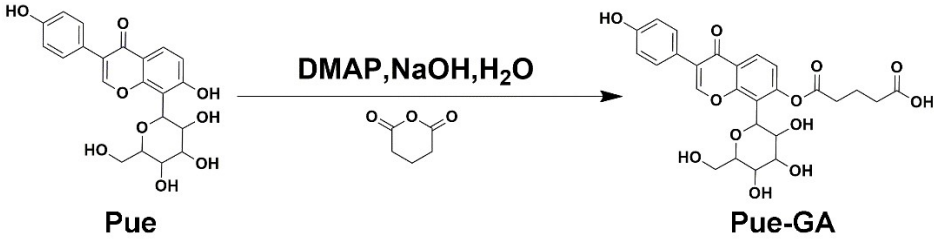
Synthesis of Pue-GA (Scheme S2)**:** 1.0 g Pue, 1.1 g glutaric anhydride (GA), and 29.3 mg 4-Dimethylaminopyridine (DMAP) were dissolved in the mixture of 10 mL H_2O. Then the pH of the solution was adjusted to 8-9 using 2 M NaOH and reacted overnight at room temperature. The resulting solution was purified by 200 mesh silica gel column using H_2O as the eluents. (21 % yield). MS：calc. M^+=530.14, obsvd. $(M+Na)^+$ =553.10. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.74 (s, 1H), 10.32 (s, 0H), 8.50 (s, 1H), 7.96 (d, J = 9.1 Hz, 1H), 7.63 (d, J = 8.2 Hz, 2H), 7.40 (d, J = 8.1 Hz, 0H), 7.19 (d, J = 8.2 Hz, 2H), 7.11 (s, 0H), 7.05 – 6.96 (m, 1H), 6.81 (d, J = 8.1 Hz, 0H), 5.52 (s, 0H), 4.83 (d, J = 9.9 Hz, 1H), 4.03 (t, J = 9.2 Hz, 1H), 3.72 (d, J = 11.8 Hz, 1H), 3.45 (s, 0H), 3.24 (dt, J = 18.2, 8.8 Hz, 3H), 2.88 (s, 0H), 2.73 (s, 0H), 2.65 (t, J = 7.5 Hz, 2H), 2.36 (t, J = 7.4 Hz, 2H), 2.24 (t, J = 7.6 Hz, 1H), 1.87 (p, J = 7.6 Hz, 2H), 1.70 (p, J = 7.4 Hz, 0H), 1.39 (s, 0H).



Scheme S2. Synthetic route of Pue-GA.

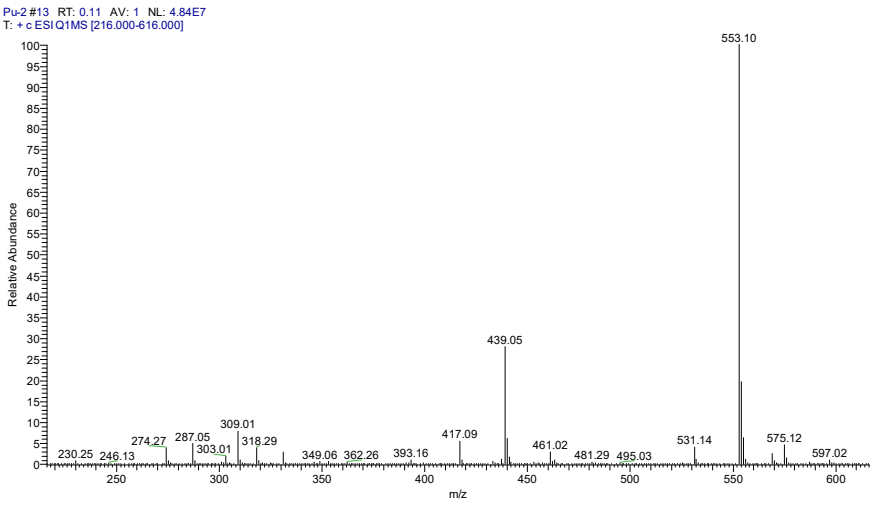


Figure S1. Mass spectrum of Pue-GA.

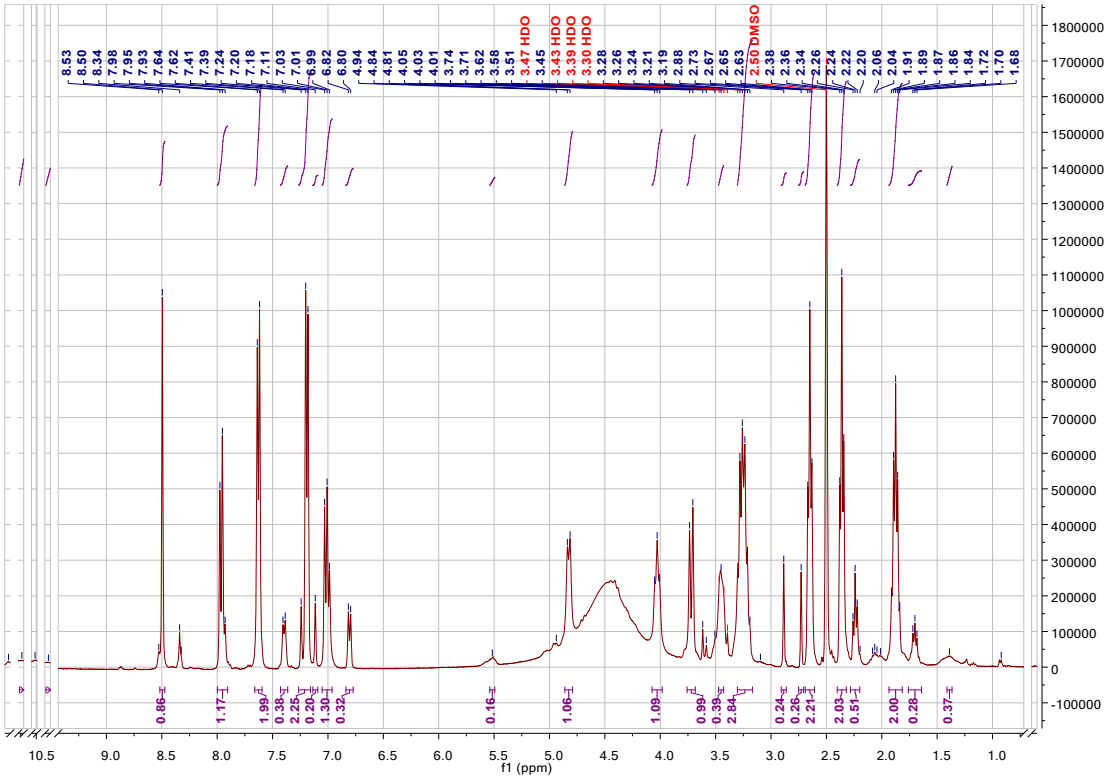
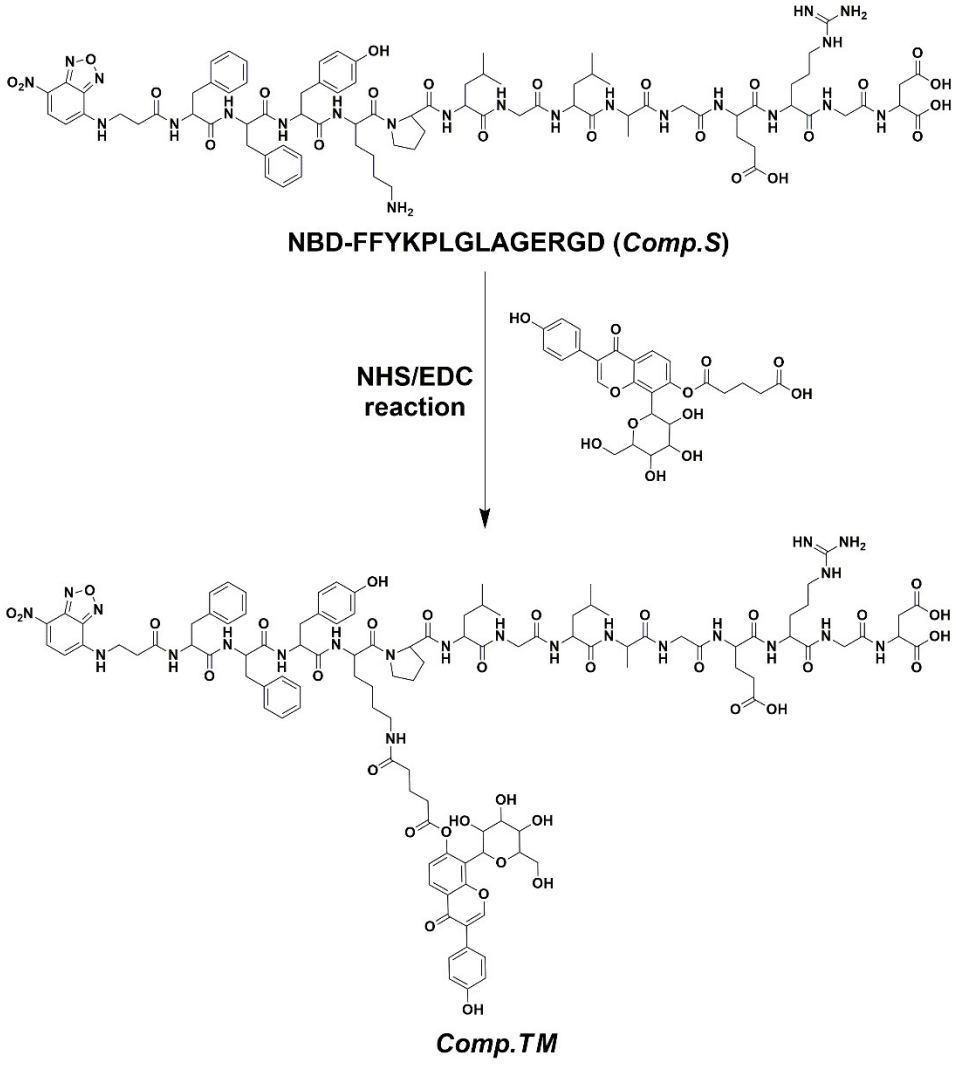


Figure S2. ^{1}H NMR spectrum of Pue-GA in DMSO-d_6.

Synthesis of peptide *Comp. TM* (Scheme S3)**:** Peptide of ***NBD-FFYKPLGLAGERGD (Comp. S)*** was prepared by solid phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin and the corresponding N-Fmoc protected amino acids with side chains properly protected. N,N-Diisopropylethylamine (DIPEA) was used to adjust the pH to alkaline. 20 % piperidine in anhydrous N, N'-dimethylformamide (DMF) was used during the deprotection of the Fmoc group. Then the next Fmoc-protected amino acid was coupled to the free amino group using O-(Benzotriazol-1-yl)-N, N, N', N'-tetramethyluroniumhexafluorophosphate (HBTU) as the coupling reagent. The growth of the peptide chain was according to the established Fmoc SPPS protocol. After the last coupling step, excessive reagents were removed by DMF wash for 5 times (10 mL per time), followed by five steps of washing using DCM (10 mL per time). The peptide derivative was cleaved using 95 % of trifluoroacetic acid (TFA) with 2.5 % of trimethylsilane (TMS) and 2.5 % of H_2O for 30 minutes. The cleavage reagent was then added into 40 mL ice-cold diethylether. The resulting precipitate was centrifuged for 5 min at 4 ^{0}C at 2,000 rpm. Afterward, the supernatant was decanted, and the resulting solid was dried by a vacuum pump. Pue-GA (0.15 mmol), N-hydroxy succinimide

(NHS, 0.18 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 0.18 mmol) were dissolved in 3 mL DMF for stirring 2 h to obtain Pue-GA NHS active ester, then 0.15 mmol peptide was added and the reaction pH was adjusted to basic by diisopropylethylamine (DIPEA). The resulting reaction mixture was stirred at room temperature overnight for HPLC separation using MeOH and H_2O containing 0.1 % of TFA as eluents. (54 % yield for ***Comp. S***, and 22 % yield for ***Comp. TM***). ***Comp. S***, MS：calc. M^+=1802.84, obsvd. 1/2$(M+H)^+$ =902.66. ***Comp. TM***, MS：calc. M^+=2314.97, obsvd. 1/2$(M+H)^+$ =1158.84.



Scheme S3. Synthetic route of ***Comp. TM***.

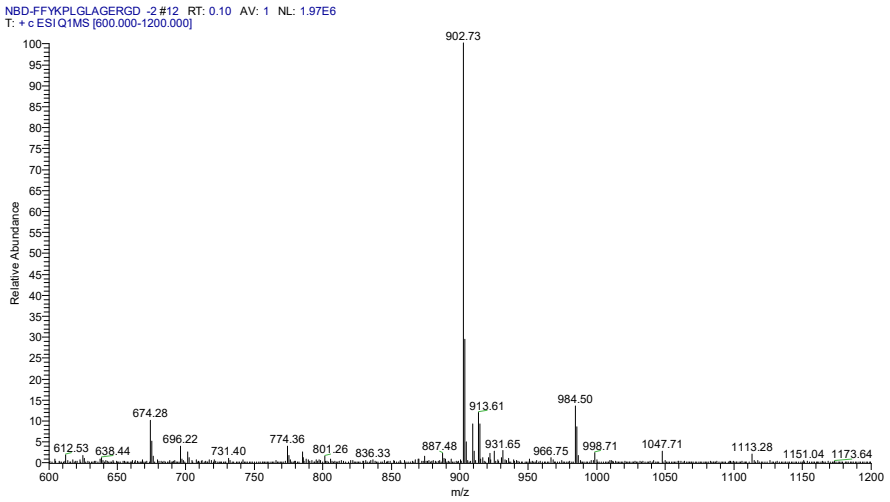


Figure S3. Mass spectrum of ***Comp. S***.

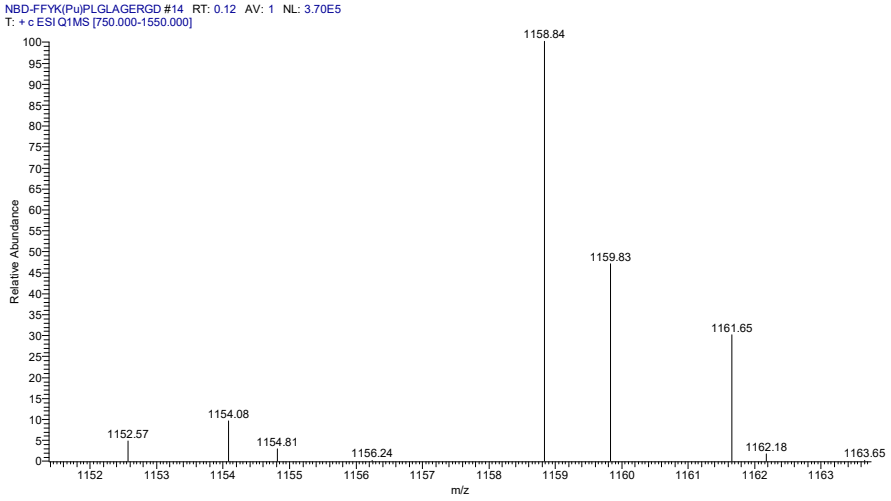


Figure S4. Mass spectrum of ***Comp. TM***.

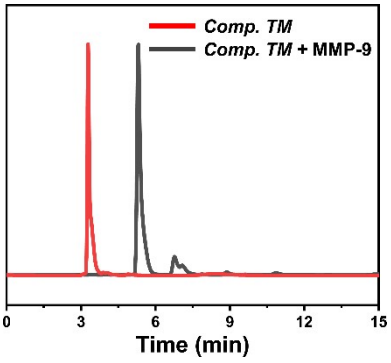


Figure S5. HPLC traces of aliquots before and after incubation with MMP-9.

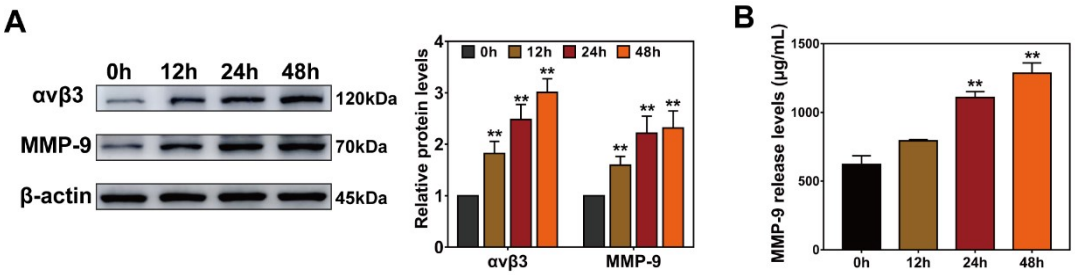


Figure S6. ECs were treated with 1 μg/mL LPS for different times (12, 24 and 48 h). (A) The expression of αvβ3 and MMP-9 detected by Western blot. (B) MMP-9 levels in the cell culture medium detected by ELISA. N = 3. ∗∗ $p < 0.01$ versus the 0 h group.

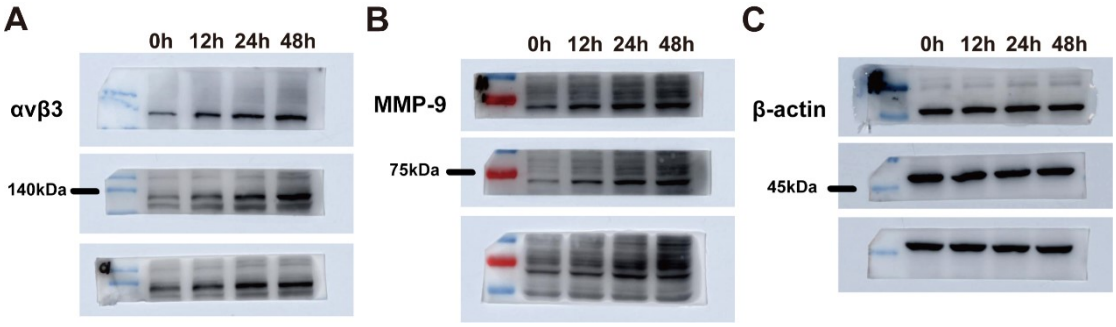


Figure S7. The raw images of Western blot of αvβ3, MMP-9 and β-actin, respectively.

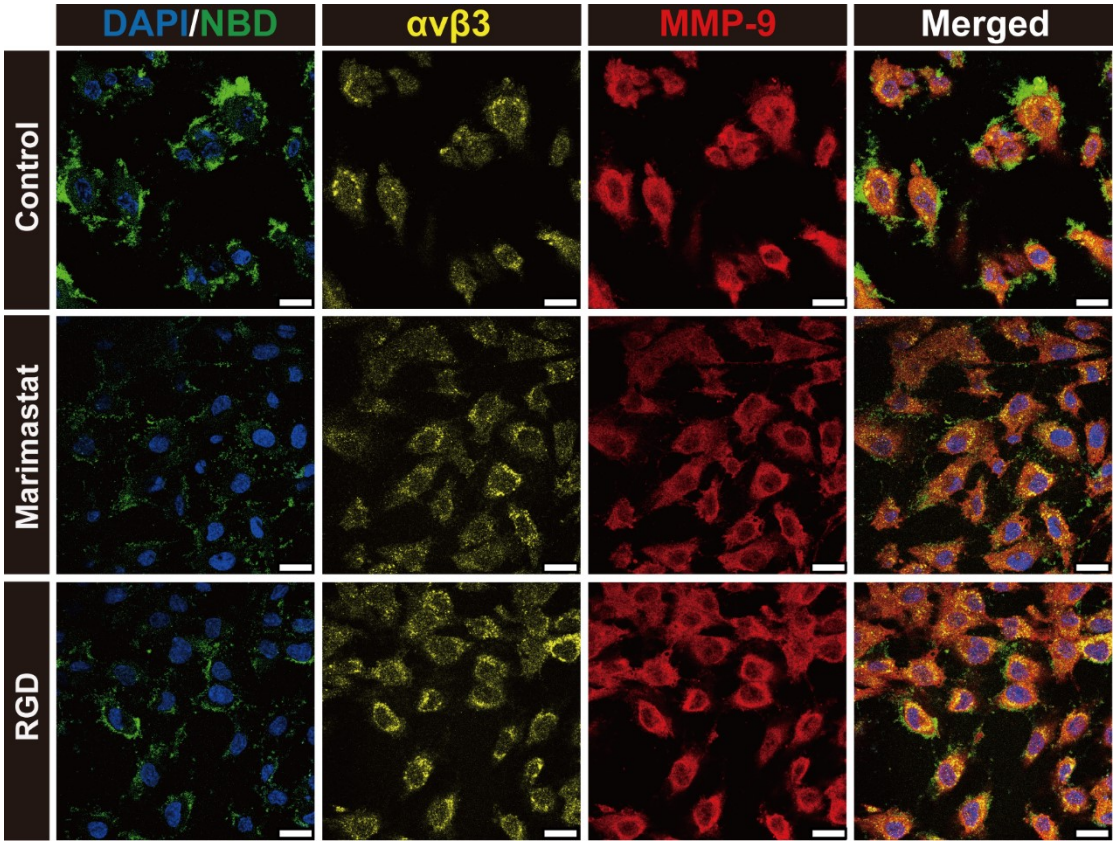


Figure S8. CLSM images of inflamed ECs treated with 25 μM ***Comp.TM*** for 12 h in the absence (Control) or presence of the MMP inhibitor Marimastat (2 μM) and integrin-inhibiting tripeptide RGD (1 mM). Scale bar is 25 μm. Blue fluorescence from DAPI represents the nucleus, yellow fluorescence from Cy3 represents αvβ3, red fluorescence from Alexa Fluor 647 represents MMP-9, and green fluorescence from NBD represents ***Comp.TM***.

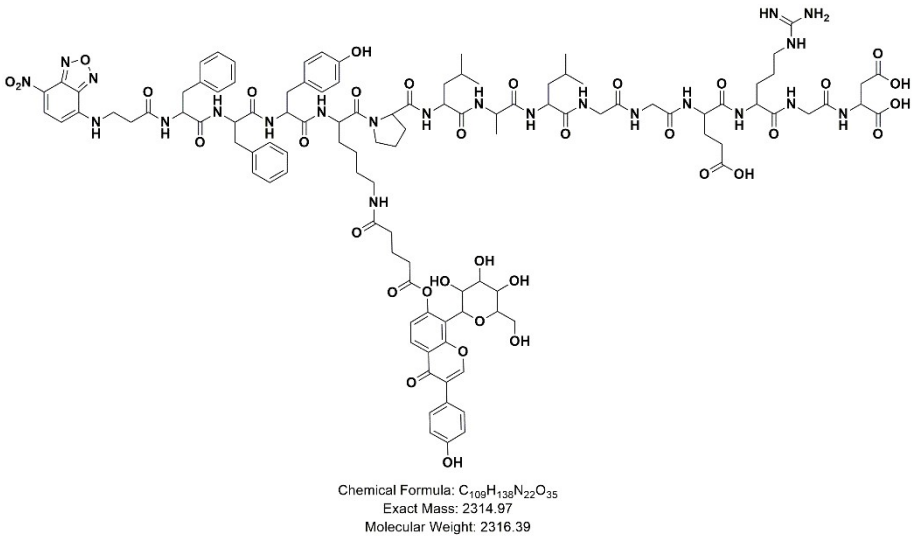


Figure S9. Chemical structure of ***Comp.T***. (23 % yield for ***Comp. T***).

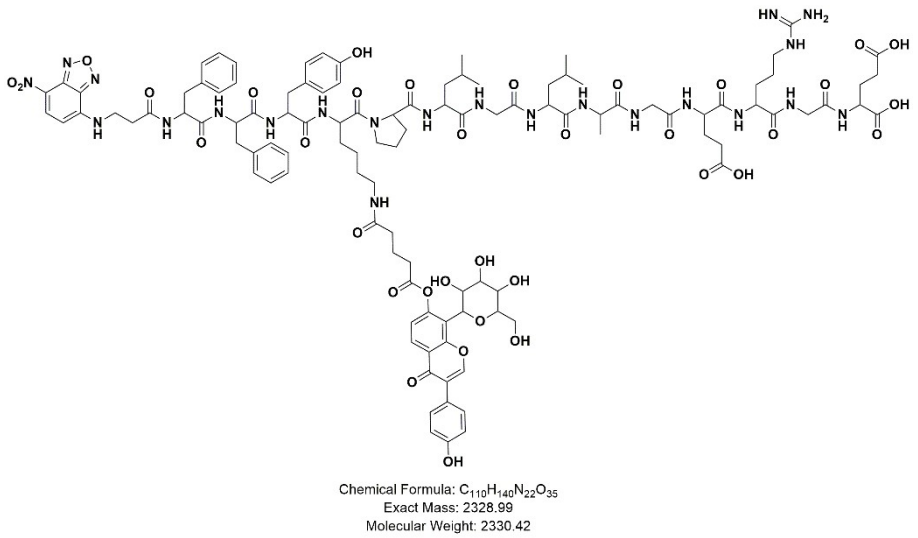


Figure S10. Chemical structure of ***Comp.M***. (16 % yield for ***Comp. M***).

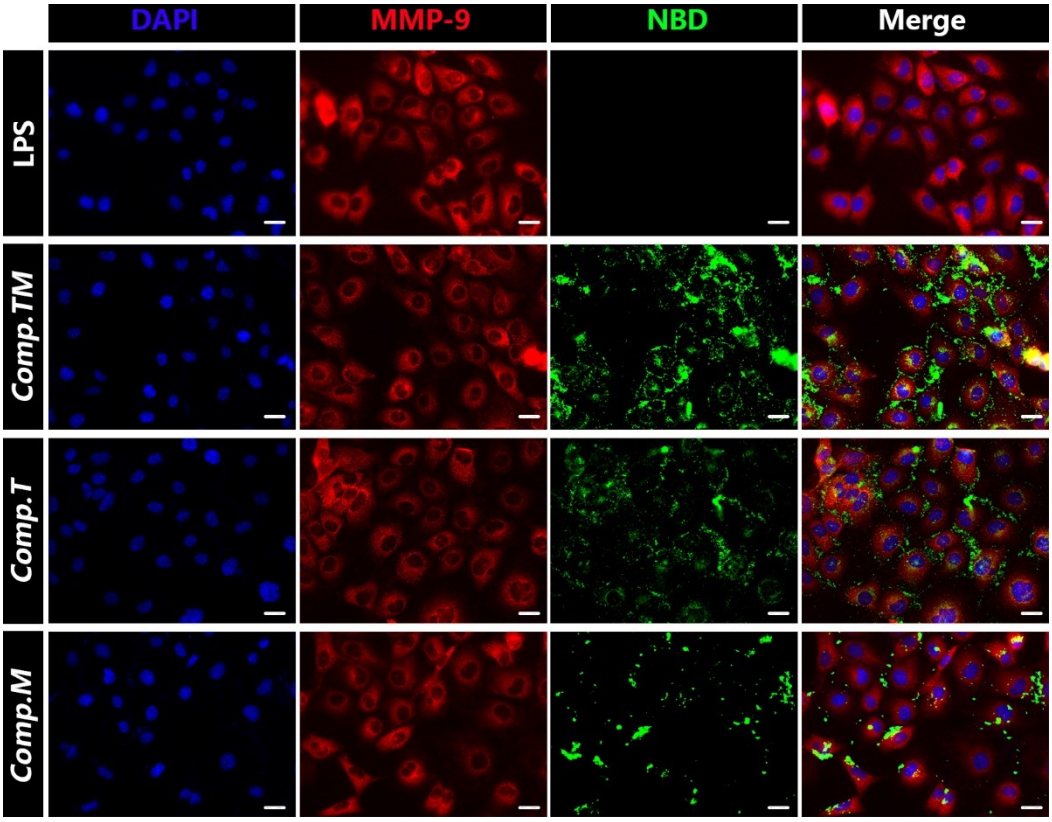


Figure S11. CLSM images of inflamed ECs incubated with 25 μM of ***Comp.TM***, ***Comp.T***, and ***Comp.M*** for 12 h, respectively. Scale bar is 25 μm. Blue fluorescence from DAPI represents the nucleus, red fluorescence from Cy3 represents MMP-9, and green fluorescence from NBD represents NBD peptides.

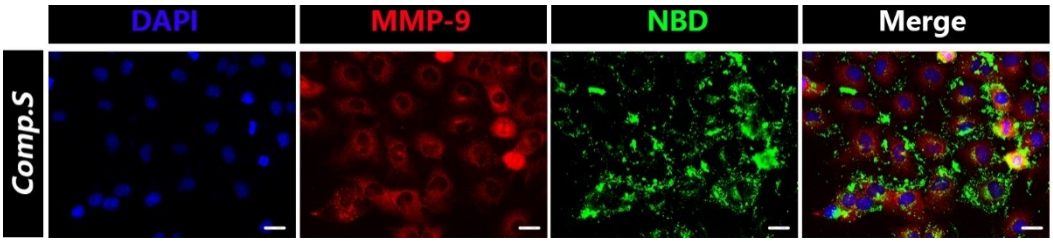


Figure S12. CLSM images of inflamed ECs incubated with 25 μM of ***Comp.S*** for 12 h. Scale bar is 25 μm. Blue fluorescence from DAPI represents the nucleus, red fluorescence from Cy3 represents MMP-9, and green fluorescence from NBD represents NBD peptides.

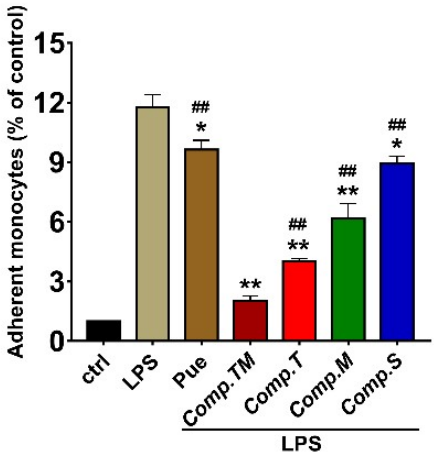


Figure S13. Adherent THP-1 cells were counted by fluorescence microscopy. N = 3. ∗ p < 0.05, ∗∗ p < 0.01 versus the positive control group; # p < 0.05, ## p < 0.01 versus the ***Comp.TM*** group.

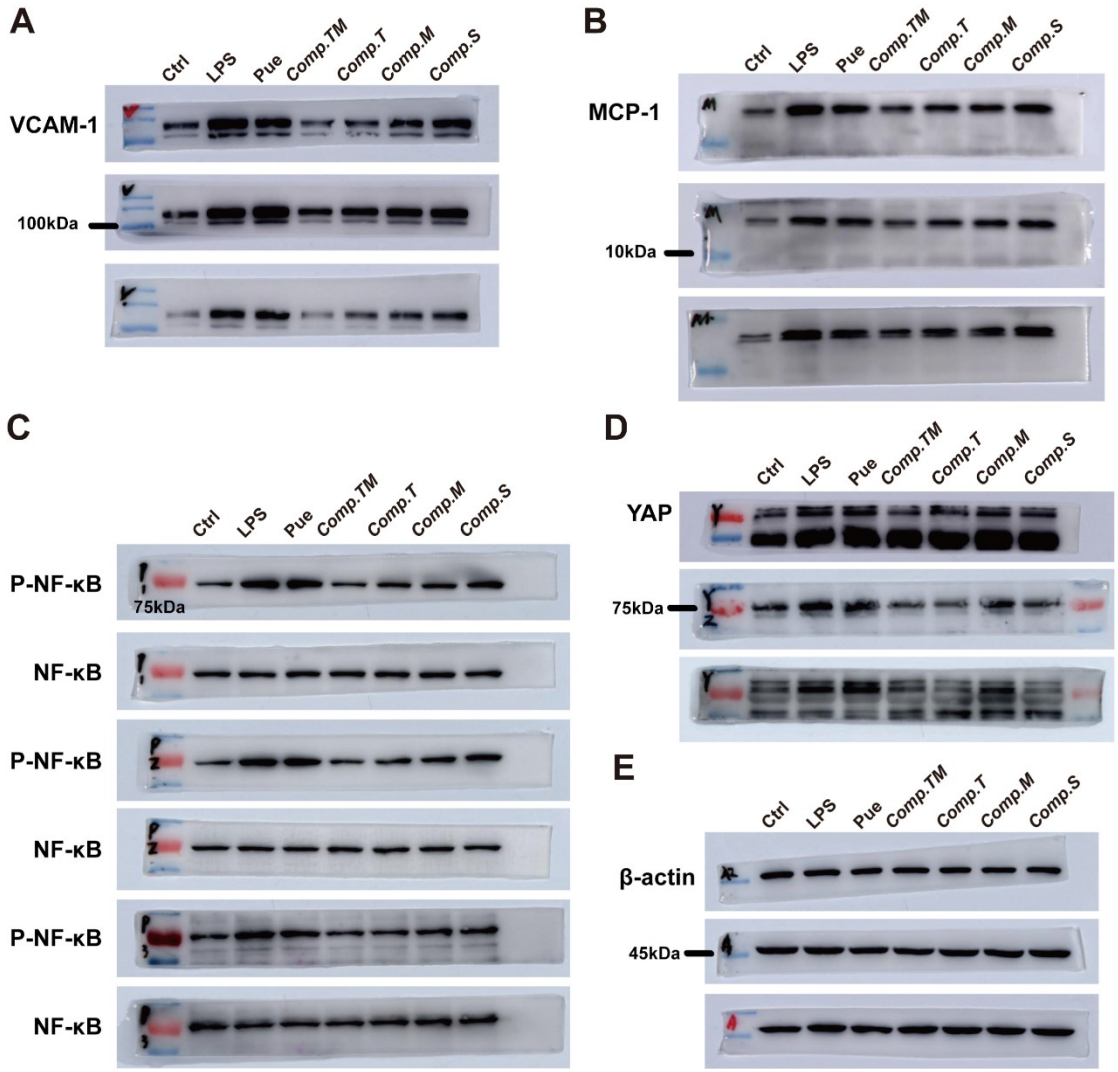


Figure S14. The raw images of Western blot of VCAM-1, MCP-1, P-NF-κB / NF-κB, YAP and β-actin, respectively.

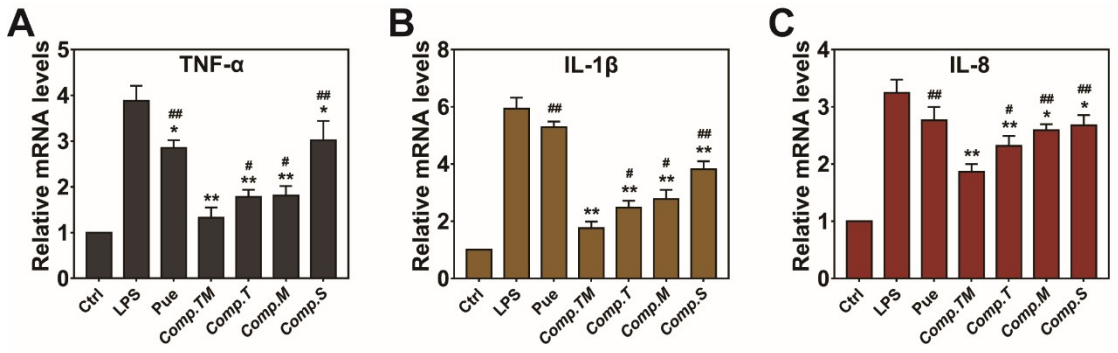


Figure S15. The mRNA levels of inflammatory factors (i.e., TNFα, IL-1β, and IL-8) in ECs were determined by qPCR. N = 3. ∗ $p < 0.05$, ∗∗ $p < 0.01$ versus the positive control group; #

$p < 0.05$, ## $p < 0.01$ versus the ***Comp.TM*** group.

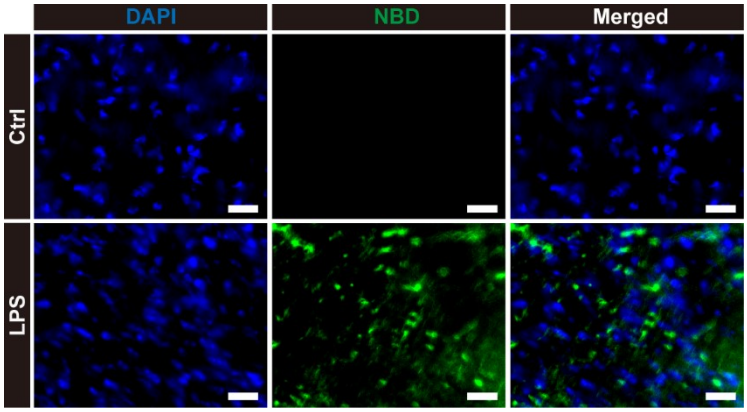


Figure S16. The aortas were incubated ex vivo with 25 μM ***Comp.TM*** for 24 h and en face confocal imaged. Scale bar is 25 μm. Blue fluorescence from DAPI represents the nucleus, and green fluorescence from NBD represents ***Comp.TM***.

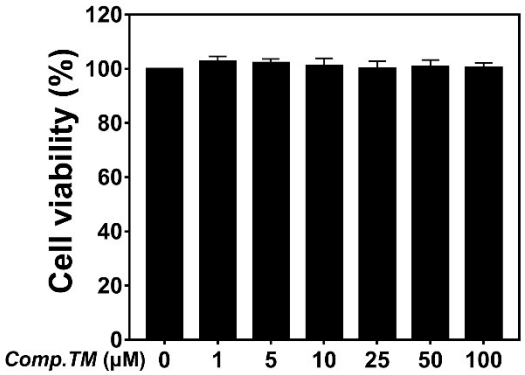


Figure S17. The toxicity of ***Comp.TM***.in vitro. ECs were incubated with ***Comp.TM*** alone at various concentrations (0, 1, 5, 10, 25, 50 and 100 μM) for 48 h. The effect of ***Comp.TM*** on cells viability was detected by CCK-8 assay.

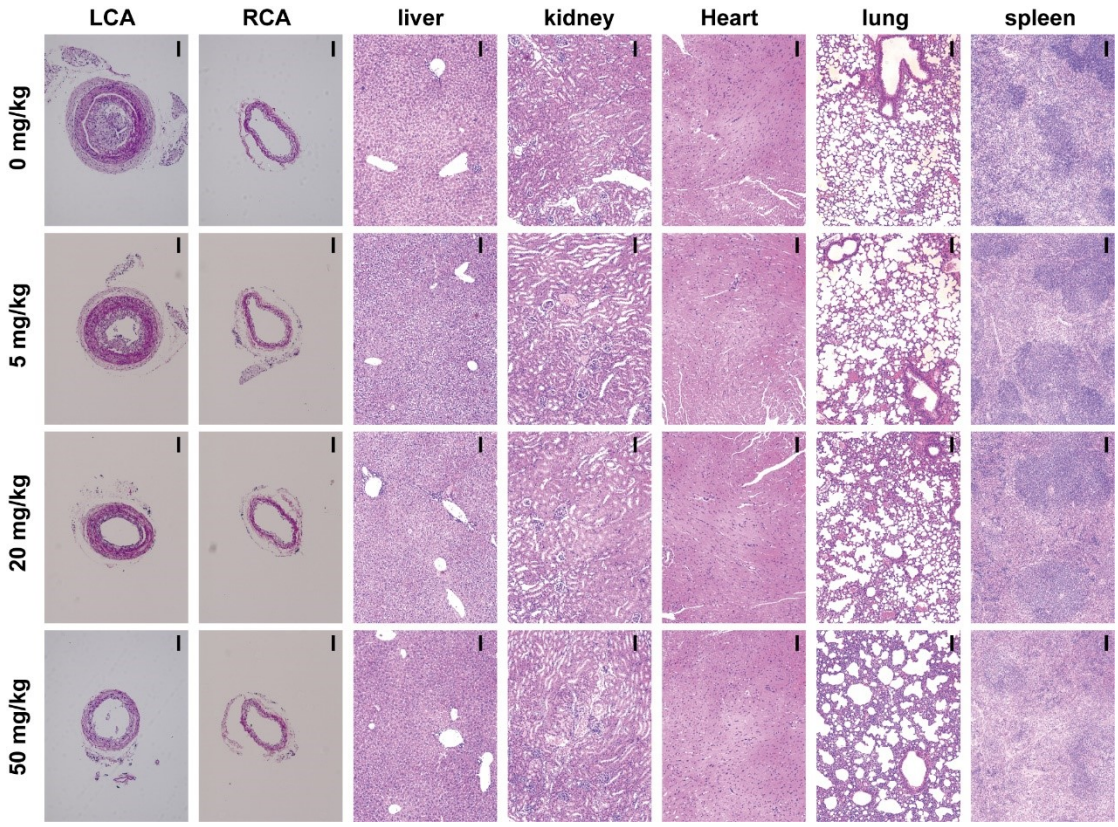


Figure S18. The biological activity and toxicity of ***Comp.TM***.in vivo. Mice were treated with different concentrations of ***Comp.TM*** (5, 20, and 50 mg/kg, daily, intraperitoneally) and partially ligated and fed a high-fat diet for 2 weeks. Hematoxylin and Eosin (HE) staining on organisms was performed for histological examination (scale bar = 100 μm).