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Carrier-free metal-organic hybrid nanoassembly with combination anti-viral and hepato-protective activity for hepatitis B treatment

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Supporting materials and methods

Materials

18β-Glycyrrhetinic acid (GA) and zirconium chloride oxide octahydrate ($ZrOCl_2 \cdot 8H_2O$) were purchased from Aladdin Bio-Chem Technology Co., Ltd. Phosphorus oxychloride and TFV was provided by Energy Chemical. Tenofovir diphosphate (TFV-DP) was purchased from Mason Chem, Inc (California, USA). Pluronic F-68 and phosphatase were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. Mouse tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 ELISA kits were purchased from ExCell Bio-Technology Co., Ltd. Hepatitis B surface antigen (HBsAg) and envelop antigen (HBeAg) ELISA kits were purchased from Elabscience Biotechnology Co., Ltd. and Mlbio Co., Ltd., respectively.

Synthesis of glycyrrhetinic acid phosphate (GAP)

GAP was synthesized as shown in Figure S1. In brief, the solution of phosphorus oxychloride (2 mL, 14.6 mmol) in anhydrous tetrahydrofuran (THF) (60 mL) was added to a flame dried flask. Subsequently, the solution of GA (3 g, 6.45 mmol) in anhydrous pyridine (5.1 mL) and anhydrous THF (30 mL) was added dropwise into the above solution in ice bath and kept at 0 °C for 30 min. The reaction mixture was moved to room temperature environment and stirred for additional 3 h, and was concentrated under reduced pressure. The residual oil was dissolved in dichloromethane (DCM) (100 mL), and was then washed with 1 M HCl (3 × 100 mL), water (3 × 100 mL), brine (100 mL) and died over anhydrous sodium sulfate. The crude product, obtained by evaporating to remove the solvent, was purified by column chromatography using the DCM/methanol mixture (10:1, v:v) with 1% acetic acid as eluent. The resulting product, GAP was white solid (2.05 g) with the yield of 57.7%, and was characterized by nuclear magnetic resonance spectroscopy (NMR) and mass spectrum (MS).

^{1}H NMR (300 MHz, DMSO-d_6) δ 5.40 (d, J = 2.9 Hz, 1H), 4.29 (d, J = 5.1 Hz, 1H), 3.31 (s, 3H), 3.02 (dt, J = 9.9, 4.5 Hz, 1H), 2.64-2.53 (m, 1H), 2.33 (d, J = 3.0 Hz, 1H), 2.07 (dd, J = 12.6, 5.2 Hz, 2H), 1.87-1.57 (m, 5H), 1.56-1.21 (m, 11H), 1.21-0.83 (m, 16H), 0.72 (dd, J = 20.7, 3.0 Hz, 8H); ^{13}C NMR (500 MHz, DMSO-d_6) δ 199.46, 178.09, 170.05, 127.80, 77.08, 61.63, 54.60, 48.53, 45.34, 43.54, 43.39, 41.15, 39.25, 39.03, 37.99, 37.16, 32.62, 32.00, 30.85, 28.85, 28.61, 28.29, 27.44, 26.57, 26.30, 23.49, 18.85, 17.63, 16.64, 16.45. ^{31}P NMR (500 MHz, DMSO-d_6) δ -0.29; MS (ESI-) calculated for $C_{30}H_{46}O_7P$ $[M-H]^-$ 549.306, found 549.300.

Cell culture

HepG2.2.15 cells with stable HBV expression and replication, RAW264.7 cells and L-02 cells were purchased from China Center for Type Culture Collection in Wuhan (Hubei, China). All cells were cultured in the DMEM medium supplemented with 10% FBS and 100 U/mL penicillin and streptomycin (Invitrogen, Carlsbad, CA) at 37 °C with 5% CO_2.

***In vitro* cytotoxicity**

RAW264.7 cells (4×10^4 cell/well) were seeded in the 96-well plates, and were cultured for 24 h. After removal of culture media, the free GA, GAP or TFV/GAP/NA in the fresh culture media was added at different concentrations. After incubation for 24 or 48 h, the cell viability was assayed by cell counting kit (CCK)-8.

L-02 cells (5×10^3 cell/well) were seeded in the 96-well plates, and were cultured for 24 h. After removal of culture media, TFV/GAP/NA in the fresh culture media was added at different concentrations. After incubation for 24 or 48 h, the cell viability was assayed by CCK-8.

In vitro cellular uptake

HepG2.2.15 cells (4×10^5 cell/well) were seeded in the 24-well plates, and were cultured for 24 h. After removal of culture media, the free TFV, the TFV/GAP mixture and TFV/GAP/NA in the fresh culture media (25 μM TFV, 6.5 μM GAP) were added. After incubation for different time, the media were removed. The cells were washed with cold PBS three times, and were then lysed to determine the concentrations of TFV and TFV-DP by LC-MS/MS and detect protein concentration by BCA assay, respectively. The area under time-concentration curve (AUC) was calculated using the Phoenix WinNonlin pharmacokinetic program 8.2 (Pharsight, Mountain View, CA).

In vitro cellular targeting

HepG2.2.15 cells or L-02 cells (4×10^5 cell/well) were seeded in the 24-well plates, and were cultured for 24 h. After removal of culture media, TFV/GAP/NA in the fresh culture media (25 μM TFV, 6.5 μM GAP) were added. After incubation for 24 h, the media were removed. The cells were washed with cold PBS three times, and were than lysed to determine the concentrations of TFV by LC-MS/MS and detect protein concentration by BCA assay, respectively.

To demonstrated the GA receptor-mediated uptake of TFV/GAP/NA by HepG2.2.15 cells, a competitive blocking study was performed. HepG2.2.15 cells (4×10^5 cell/well) were seeded in the 24-well plates, and were cultured for 24 h. After removal of culture media, the free GA in the fresh culture media at the concentration of 52 μM was added, and were incubated for 2 h. Subsequently, TFV/GAP/NA in the fresh culture media (25 μM TFV, 6.5 μM GAP) was added. After incubation for 24 h, the media were removed. The cells were washed with cold PBS three

times, and were than lysed to determine the concentrations of TFV by LC-MS/MS and detect protein concentration by BCA assay, respectively.

In vitro anti-viral activity

HepG2.2.15 cells (4×10^4 cell/well) were seeded in the 96-well plates, and were cultured for 24 h. After removal of culture media, the free TFV, the TFV/GAP mixture and TFV/GAP/NA in the fresh culture media (1 or 10 μM TFV) were added. After incubation for different time, the culture media were collected for the HBV DNA analysis. The HBV DNA level was quantified by the laboratory department of Jiangsu Province Hospital of Chinese Medicine using TaqMan probe-based real-time PCR method with reference standards.

In vitro anti-inflammatory activity

RAW264.7 cells (4×10^4 cell/well) were seeded in the 96-well plates, and were cultured for 24 h. After removal of culture media, the cells were incubated with the free GA, the free GAP, the TFV/GAP mixture or TFV/GAP/NA in the absence or presence of lipopolysaccharide (LPS) (1 μg/mL) for 24 h. The expressions of TNF-α, IL-1β and IL-6 in the cell culture supernatants were determined by the ELISA kits according to the manufacturers' protocols.

Supporting figures

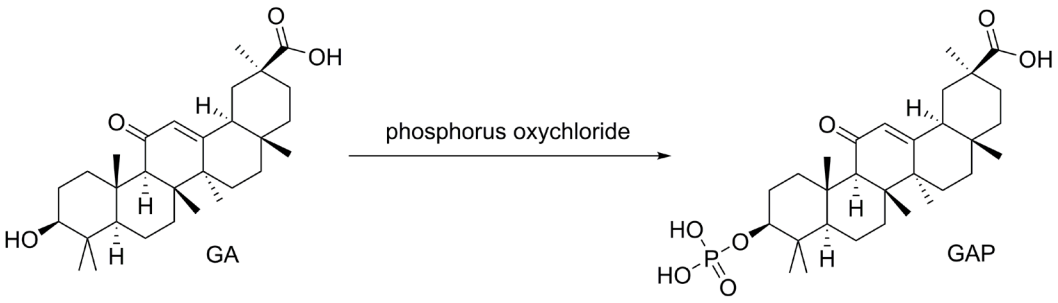

Figure S1 Synthetic route of GAP.

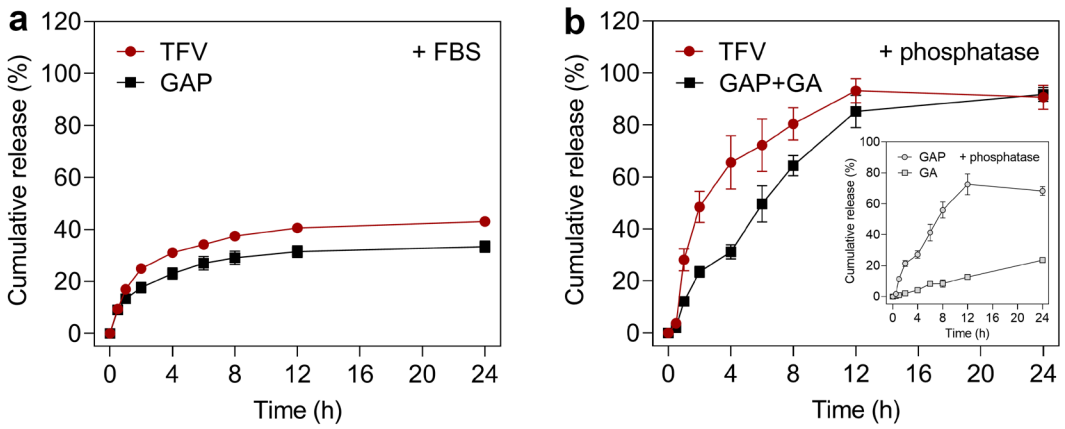

Figure S2 (a) Release profiles of TFV and GAP from TFV/GAP/NA after incubation with FBS (10%) over time (n = 3). (b) Release profiles of TFV, GAP and GA from TFV/GAP/NA after incubation with phosphatase (1 U/mL) over time. Inset: individual release profile of GAP and GA (n = 3).

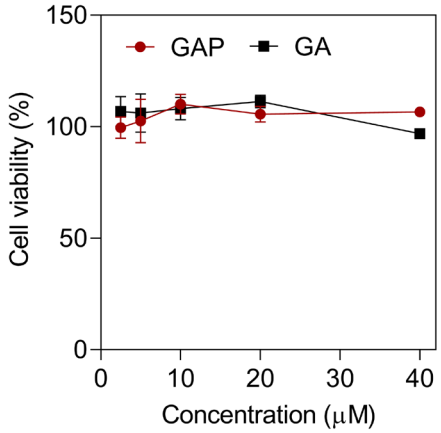

Figure S3 Viabilities of RAW 264.7 cells after treatment with GA or GAP at different concentrations for 24 h (n = 5).

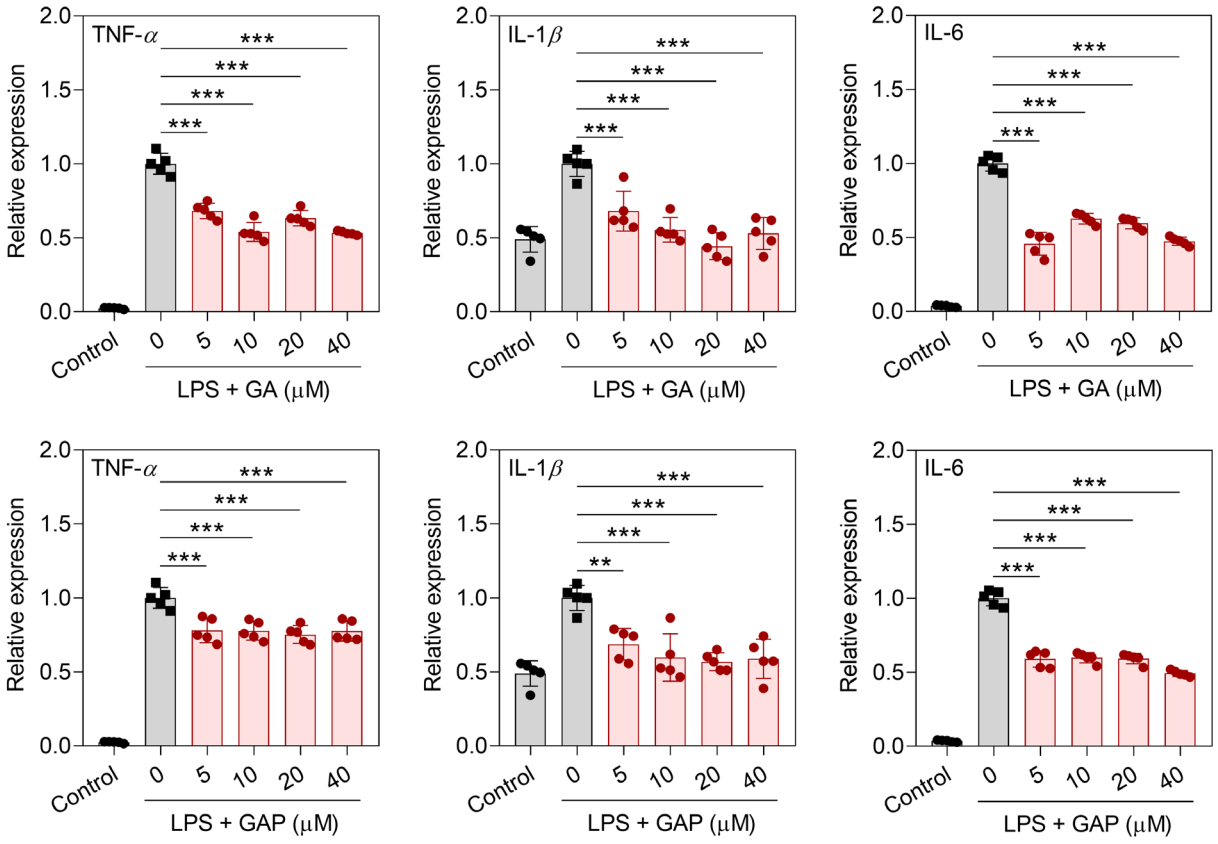

Figure S4 Relative expression of TNF-α, IL-1β and IL-6 from RAW 264.7 cells after incubation with LPS (1 μg/mL) and GA or GAP at different concentrations for 24 h (n = 5). $^{**}P < 0.01$, $^{***}P < 0.001$.

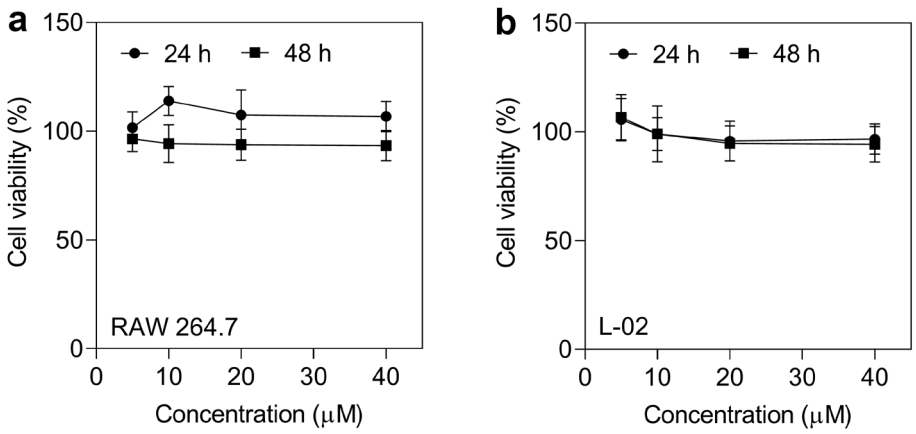

Figure S5 Viabilities of RAW 264.7 (a) and L-02 (b) cells after treatment with TFV/GAP/NA at different concentrations of GAP for 24 or 48 h (n = 8).

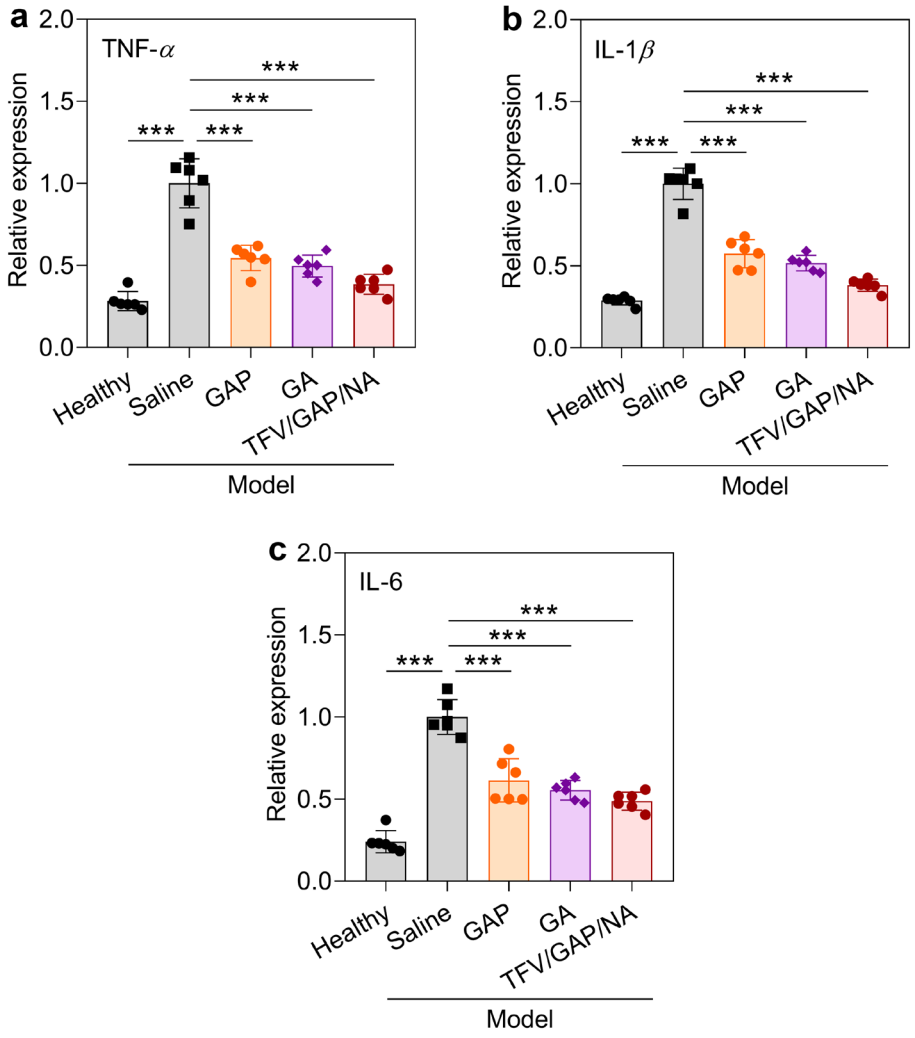

Figure S6 Relative expression of TNF-α (a), IL-1β (b) and IL-6 (c) in the liver of the mouse models of CCl_4-induced liver injury after different treatments ($n = 6$). $^{*}P < 0.05$, $^{***}P < 0.001$.

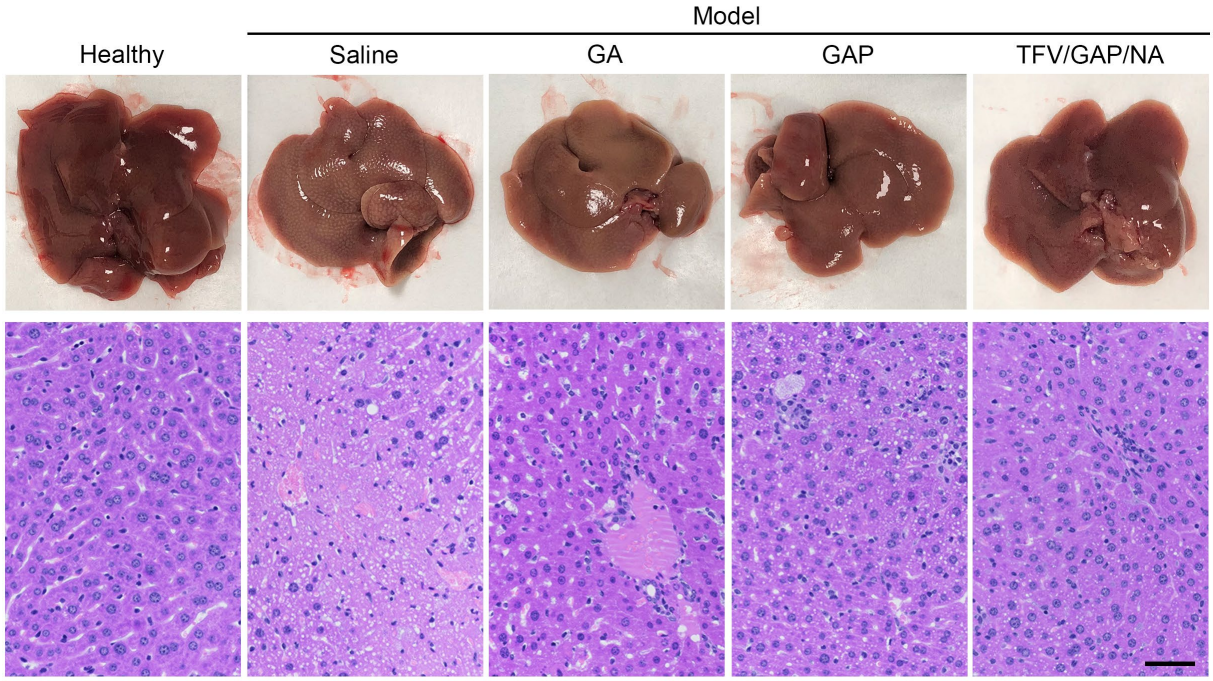

Figure S7 Representative optical and H&E-stained images of the livers collected from the mouse models of CCl_4-induced liver injury after different treatments. Scale bar is 50 μm.

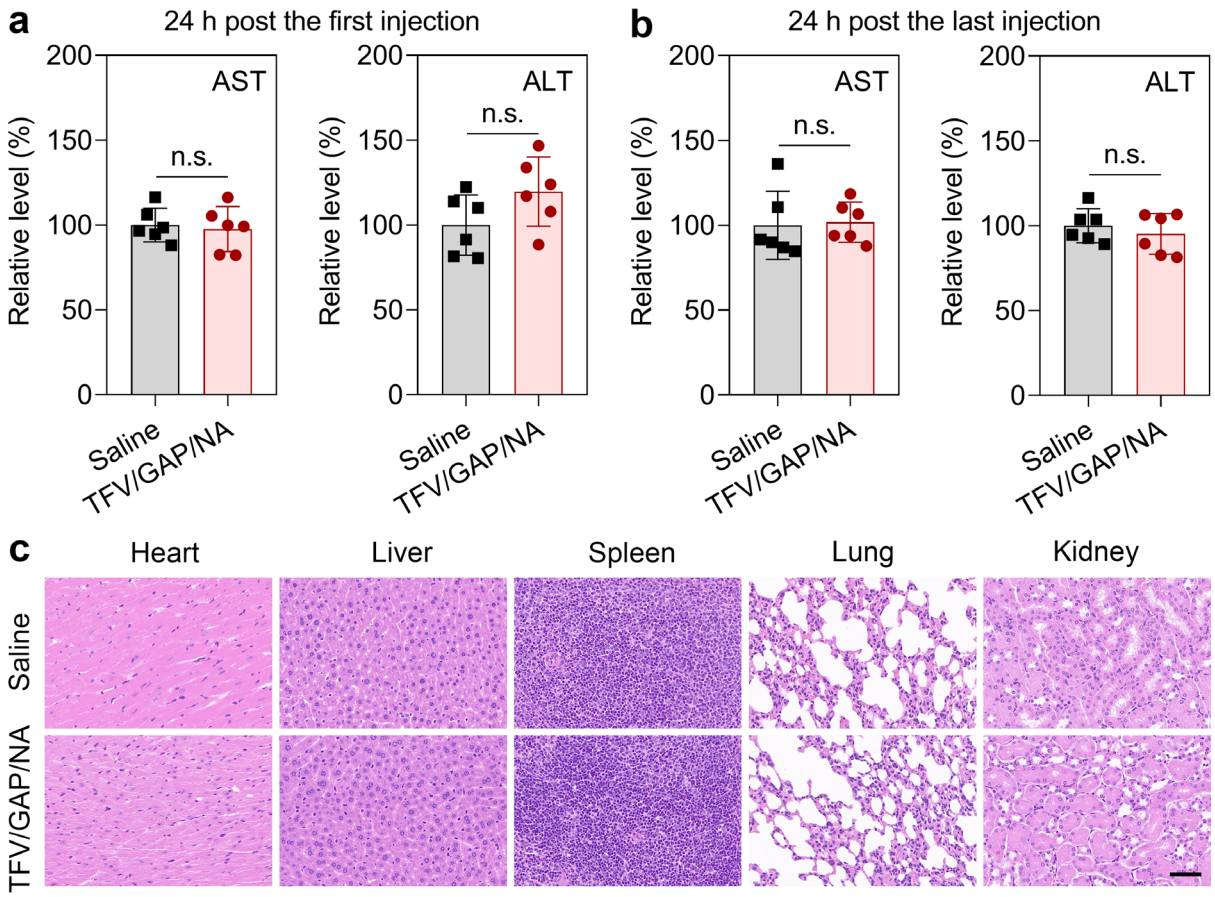

Figure S8 (a, b) Relative level of AST and ALT in the serum of the mice at 24 h post the first (a) and last injection of TFV/GAP/NA (b) (n = 6). P > 0.05 (not significant, n.s.). (c) Representative H&E-stained images of the normal tissue of the mice at 24 h post the last injection of TFV/GAP/NA. Scale bar is 50 μm.

Supporting table

Table S1 Pharmacokinetic parameters of TFV in the mice after intravenous injection of the free TFV, the TFV/GAP mixture and TFV/GAP/NA.

Parameter	TFV	TFV+GAP	TFV/GAP/NA		
	6 mg/kg	6 mg/kg	1 mg/kg	3 mg/kg	6 mg/kg
C_{max} (μg/mL)	42.18	51.04	15.15	27.23	72.15
t_{max} (h)	0.083	0.083	0.083	0.083	0.083
$t_{1/2}$ (h)	2.63	2.55	13.57	11.89	13.81
MRT (h)	0.38	0.33	2.07	1.96	2.98
AUC_{0-t} (h×μg/mL)	14.17	16.99	6.93	12.78	34.97
CL (L/h/kg)	0.42	0.35	0.13	0.22	0.17

C_{max}: maximum concentration; t_{max}: time to C_{max}; $t_{1/2}$: half-life; MRT: mean residence time; AUC: area under the time-concentration curve; CL: clearance