Supporting Information for

ORIGINAL ARTICLE

Enhanced stability and efficacy of GEM-TOS prodrug by co-assembly with antimetastatic shell LMWH-TOS

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Experimental section

1. Materials

Gemcitabine hydrochloride (＞99%), *N*-hydroxy-succinimide (NHS) (>98%) and 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride (EDCI) (>99%) were obtained from J&K Scientific (Beijing, China). 4-(Dimethylamino) pyridine (DMAP) (>99%), D-*α*-tocopherol polyethylene glycol 1000 succinate (TPGS 1000) and cathepsin B were obtained from Sigma–Aldrich. D-*α*-Tocopherol succinate (TOS) (>95%) and low molecular weight heparin (LMWH, MW 3800–5000) were purchased from Melonepharma (Dalian, China). 4,6-Diamidino-2-phenylindole (DAPI), 3-(4,5-dimethyl-2-tiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and

1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine (DiD) were purchased from Beyotime Biotechnology (Shanghai, China). Plastic cell culture dishes and plates were purchased from Wuxi NEST Biotechnology Co. (Wuxi, China). Annexin V-FITC/PI apoptosis kit was purchased from Jiangsu KeyGEN Biotechnology Co. (Jiangsu, China), Anti-MMP-9 antibody was purchased from R&D systems (Minnesota, USA). Other reagents and chemicals were analytical level.

2. Cell lines and animals.

Mouse breast cancer cells (4T1) were purchased from the Shanghai Institutes for Biological Sciences, CAS (SIBS, Shanghai, China). Cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% FBS (HyClone), 100 μg/mL streptomycin and 100 U/mL penicillin at 37 ℃ in a humidified atmosphere of 5% CO_2 in air. BALB/c female mice (about 5-week-old, 18–22 g, SPF) were purchased from Dashuo Experimental Animal Company (Chengdu, China). All animal experiments were performed according to the rules of Experimental Animals Administrative Committee of Sichuan University.

3. Synthesis and characterization of GEM-TOS and LMWH-TOS

The synthetic route of GEM-TOS (GT) is shown in Fig. S1A. Gemcitabine hydrochloride (GEM·HCl) (36 mg) was dissolved in 3.5 mL DMSO, and triethylamine (TEA) (50 μL) was added into the solution and stirred overnight at 33 ℃ under a nitrogen atmosphere. In addition, TOS (32.1 mg), EDCI (23 mg) and NHS (13.8 mg) were dissolved in 3.5 mL DMSO and stirred at 33 ℃ for 4 h. Subsequently, add the GEM solution dropwise into the mixture mentioned above and stir at 33 ℃ for another 48 h. The obtained solution was directly used to prepare the GLT micellar NPs.

The synthetic route of LMWH-TOS (LT) is shown in Fig. S1B. TOS (67 mg), EDCI (42 mg), NHS (25.3 mg) and DMAP (6.7 mg) were dissolved in 9.5 mL DMF, stirred at 33 ℃ for 3.5 h. LMWH (100 mg) was dissolved in 4 mL formamide and then added dropwise into the mixture mentioned above, stirred at 33 ℃ for another 48

h. After that, the solution was mixed with 27 mL acetone and centrifuged at 1200 g for 4 min to remove the unconnected TOS. The obtained precipitate was dissolved in ultrapure water, dialyzed exhaustively with dialysis membrane (MW 1000) to remove catalysts and freeze-dried.

The structures of GT and LT were determined by ^{1}H NMR and IR.

4. Critical micelle concentration (CMC) of GLT.

The CMC of GLT was investigated by using pyrene as a fluorescent probe. Briefly, aliquots of the pyrene solution (1.5×10^{-5} mol/L, 80 μL) in acetone were added to volumetric flasks and the acetone was removed by vacuum drying. After that, 2 mL GLT solutions with concentrations ranging from 0.0002 to 0.5 mg/mL were added, and then incubated in the dark for 24 h (37 ℃, 75 rpm). Fluorescence of soluble pyrene was measured using a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Waltham, MA, USA) at an excitation wavelength of 336 nm and emission wavelengths of 372 nm (I_1) and 384 nm (I_3). The CMC was determined by plotting intensity ratio of (I_1/I_3) *versus* the logarithm of the GLT concentration.

5. Hemolysis assay

Hemolysis assay was conducted to investigate the interactions of GLT with blood erythrocytes. Briefly, GLT (1 mg/mL) was incubated with 2% red blood cell (RBC) suspension from mouse, RBC treated with PBS and 0.3% Triton was used as negative and positive controls, respectively. At 0, 1, 2 and 4 h, samples were centrifuged (800 g, 5 min). The absorbance of supernatant was evaluated at 540 nm by a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Waltham, MA, USA) and the precipitated erythrocytes were observed under microscope. Hemolysis (%) was calculated according to the following formula. Less than 5% hemolysis was deemed as nontoxic.

$$\text{Hemolysis (\%)} = \frac{A_{\text{sample}}}{(A_{\text{Triton}} - A_{\text{PBS}})} \times 100$$

6. Lysosomal distribution of GLT

The lysosomal distribution of GLT was confirmed by colocalization of lysosome and GLT. In detail, 4T1 cells were seeded into 6-well plates and incubated for 24 h. Then, replace the culture media with 1 mL of fresh medium containing GLT for 2 h, and GLT was labeled with DiD *via* physical entrapment. And 1 μL Lyso-Tracker Red was added to each well 30 min before the end of the incubation. After that, remove the culture medium and fix the cells with 4% paraformaldehyde. Finally, incubate cells with 1 μg/mL DAPI for 5 min, and the cells were observed by laser scanning confocal microscope (FV1000, Olympus, USA) to confirm the distribution of GLT in the lysosome.

7. Cytotoxicity assay

The cytotoxicity of GEM and GLT was determined by MTT assay. 1×10^{4} 4T1 cells were seeded into 96-well plates and incubated for 12 h. The culture media was then replaced by 200 μL of fresh medium containing PBS, GEM, LT or GLT, respectively. Fourty eight hours later, 20 μL MTT solution (5 mg/mL in PBS) was added into each well and incubated for another 4 h. Finally, remove the culture medium and dissolve the residue with 150 μL DMSO. The absorbance of each well was measured using a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Waltham, MA, USA) at 490 nm. Cell viability (%) was calculated according the following formula.

$$\text{Cell viability (\%)} = (A_{\text{test}}/A_{\text{control}}) \times 100$$

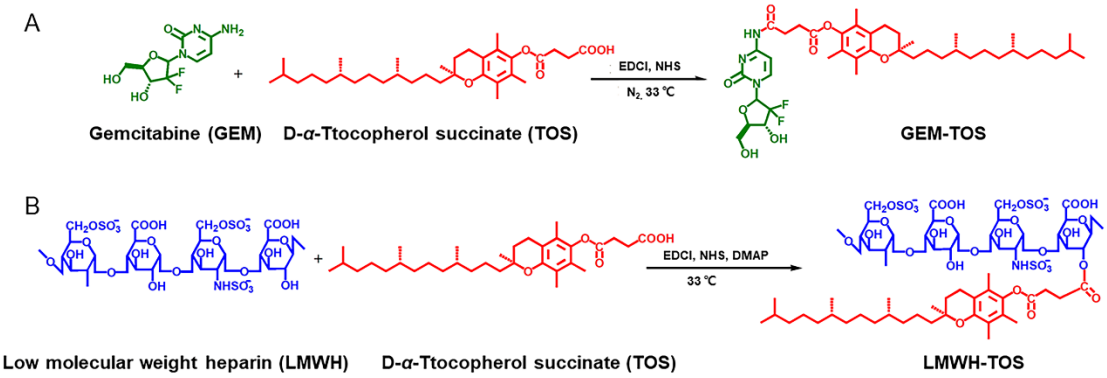
8. Apoptosis assay

The ability of GLT to induce apoptosis was explored by Annexin V-FITC/PI apoptosis detection kit. Briefly, 4T1 cells were seeded into 6-well plates and incubated to near 60% confluency. Then, replace the culture media with 2 mL of fresh medium containing PBS, GEM or GLT (GEM-equivalent 0.012 μg/mL), respectively, for 48 h. After the incubation, cells were harvested and washed with PBS. 500 μL of binding buffer was then added to the cells. Additionally, 5 μL of Annexin-V-FITC and 5 μL of Propidium Iodide were added and incubated at room temperature in the dark for 10 min. The cells were analyzed finally in flow cytometer (Cytomics™ FC 500,

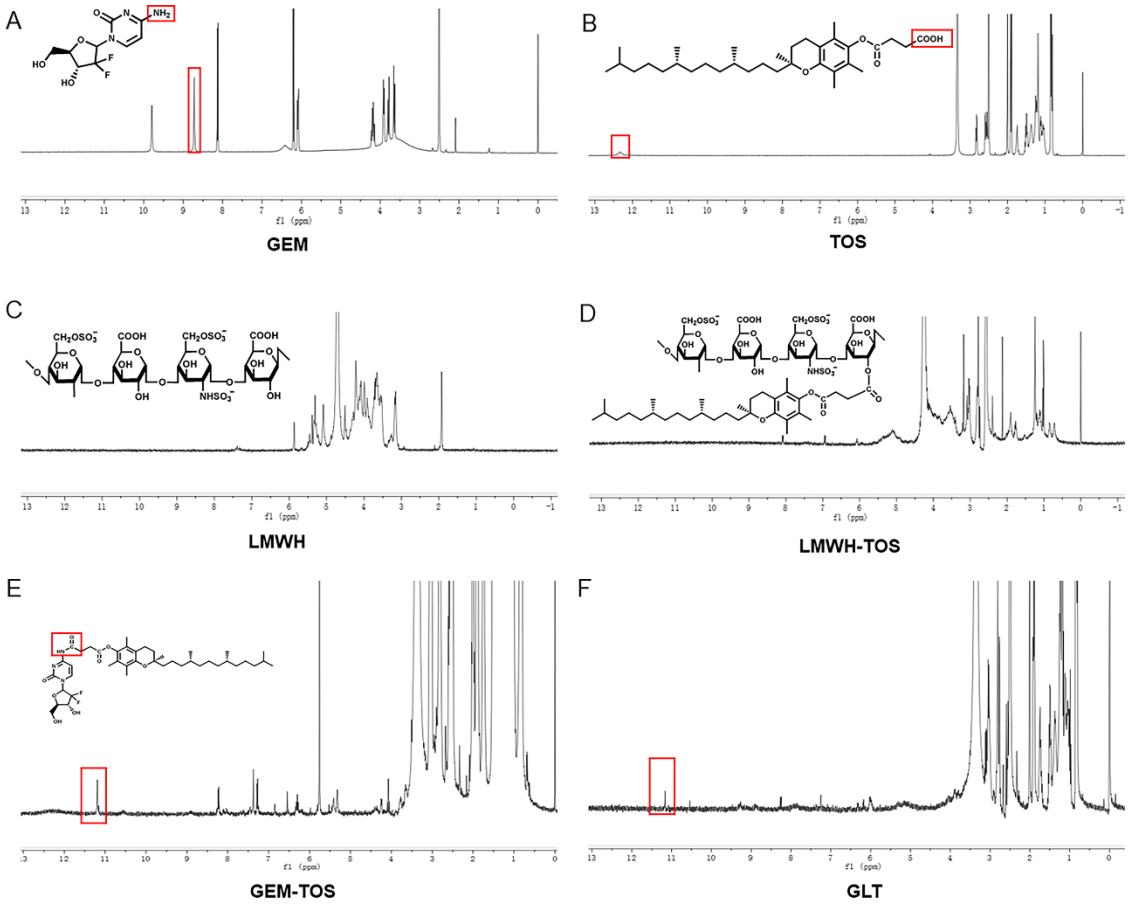
Beckman Coulter, Miami, FL, USA).

9. Statistical analysis

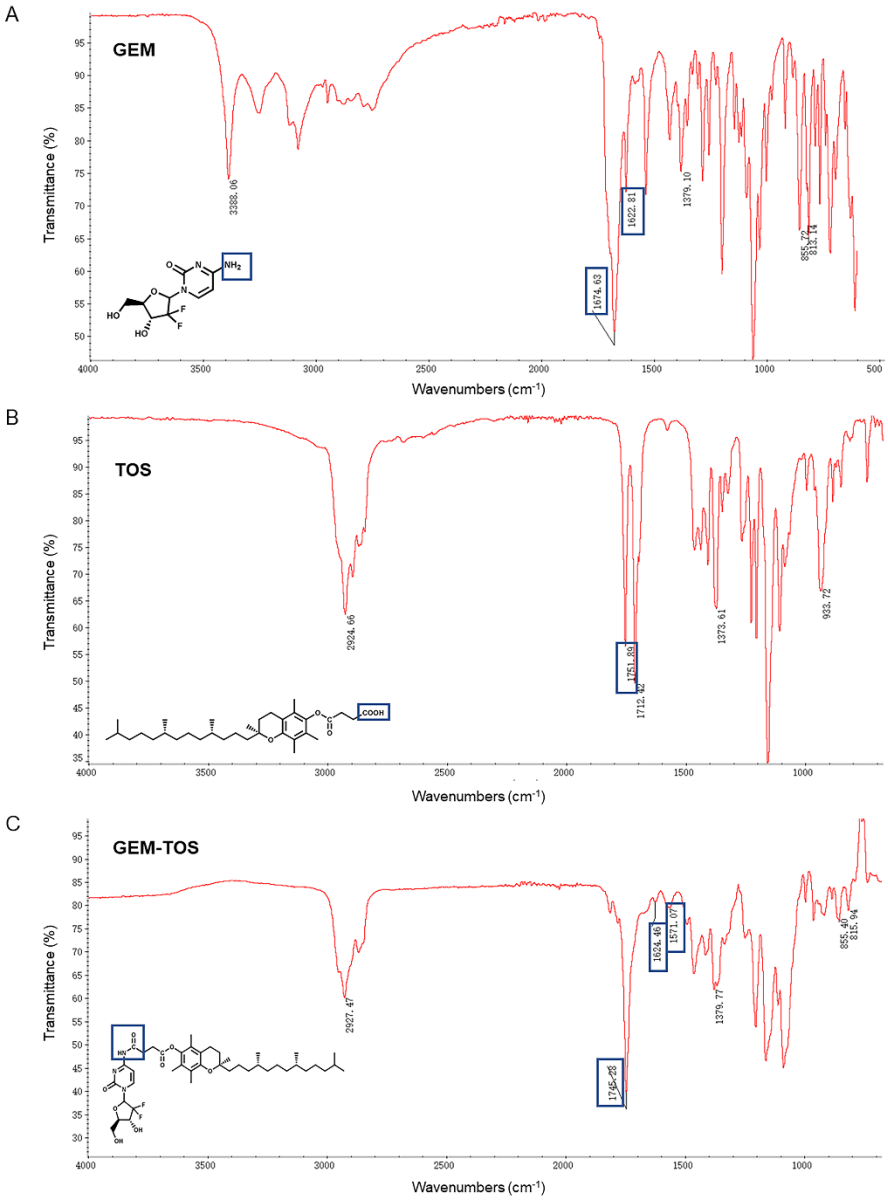
Data were expressed as the means±standard deviations (SD). Statistical analysis was performed by one-way ANOVA or Student's *t*-test. Significant differences between groups are indicated by $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.



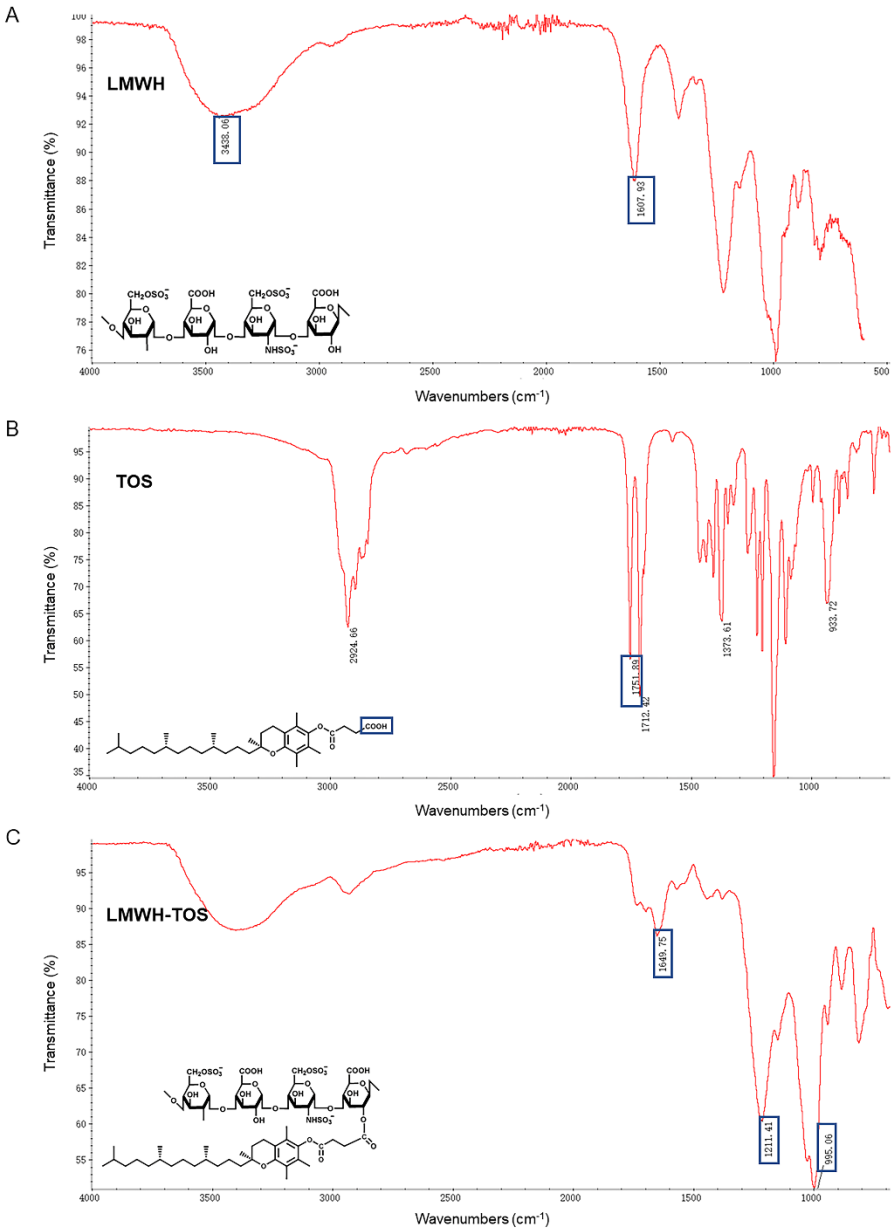
Scheme S1 Synthetic route of GEM-TOS (A) and LMWH-TOS (B).



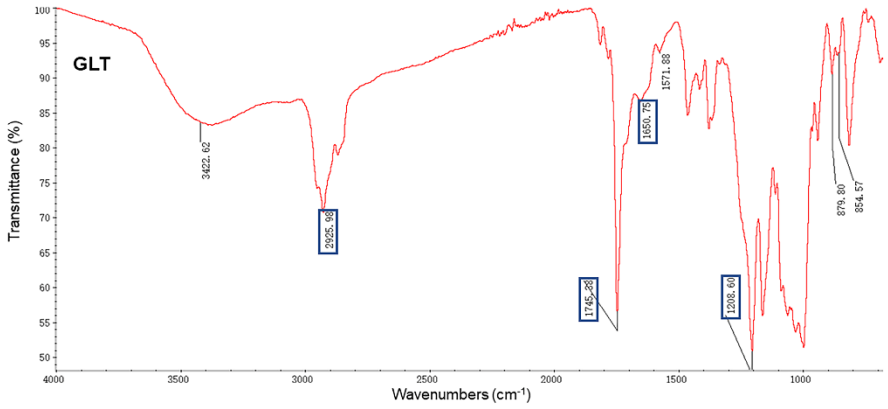
Figures S1 The ^{1}H NMR spectrums of (A) GEM, (B) TOS, (C) LMWH, (D) LMWH-TOS, (E) GEM-TOS and (F) GLT.



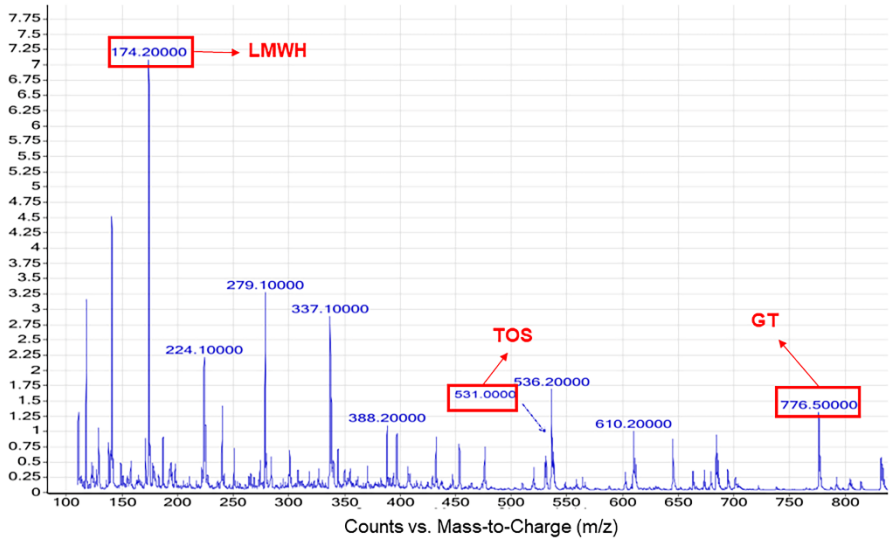
Figures S2 The IR spectrums of (A) GEM, (B) TOS, and (C) GEM-TOS.



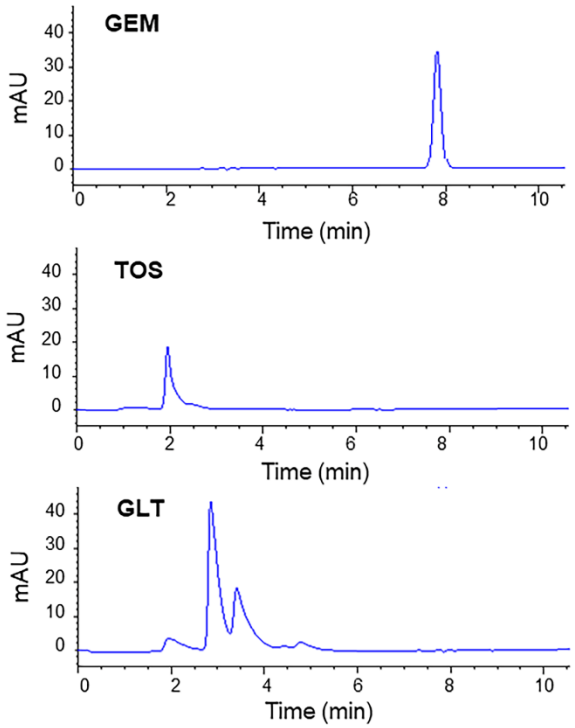
Figures S3 The IR spectrums of (A) LMWH, (B) TOS, and (C) LMWH-TOS.



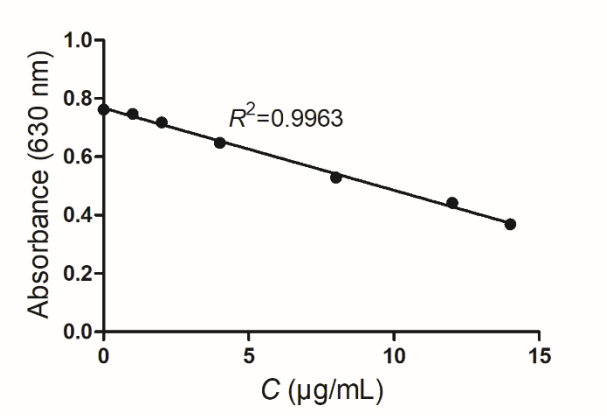
Figures S4 The IR spectrums of GLT.



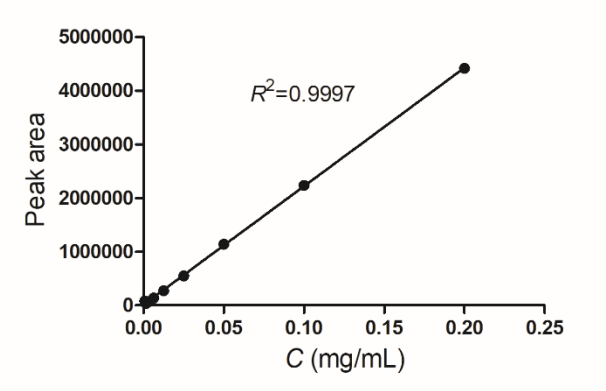
Figures S5 The LC–MS spectrums of GLT.



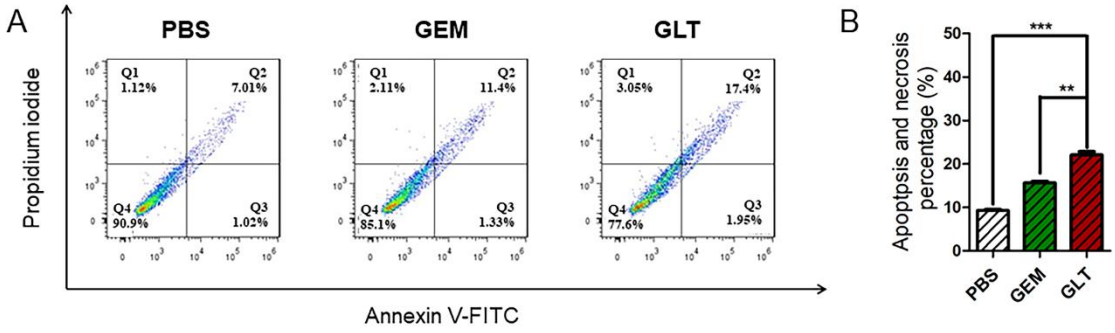
Figures S6 The HPLC spectra of GEM, TOS and GLT



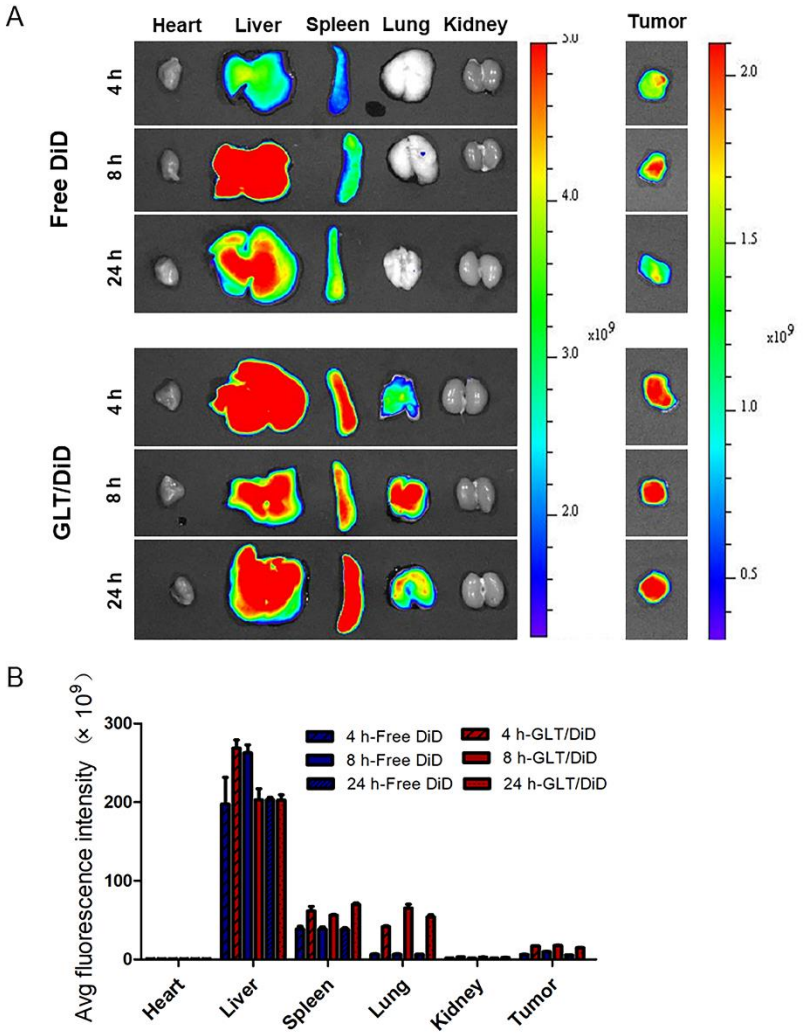
Figures S7 The standard curve of LMWH determined by toluidine blue assay.



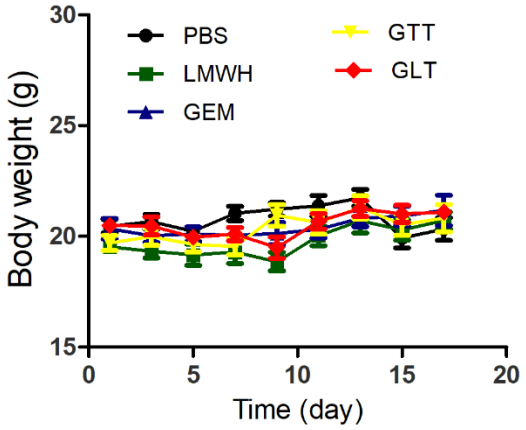
Figures S8 The standard curve of GEM determined by HPLC.



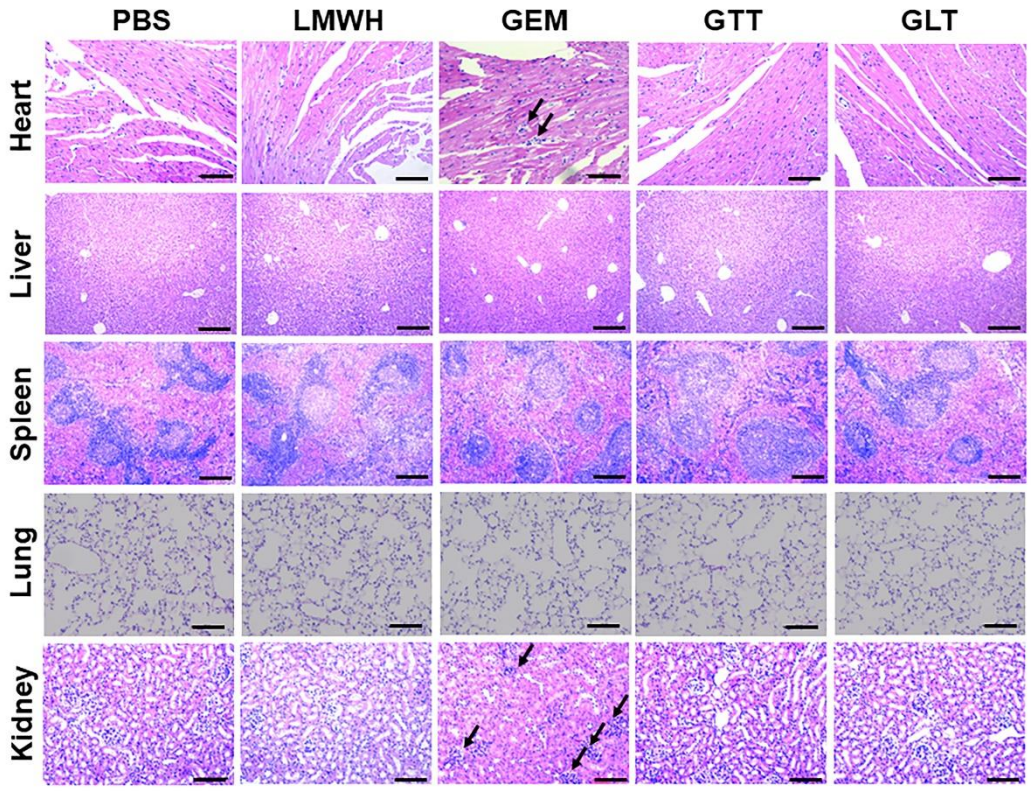
Figures S9 (A) The ability of GEM and GLT to induce apoptosis on 4T1 cells. (B) Apoptosis and necrosis percentage of 4T1 cells treated with GEM and GLT (means±SD, n=3; $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$).



Figures S10 (A) The biodistribution of GLT at 4, 8 and 24 h (mean±SD, *n*=3). (B) Semiquantitative mean fluorescence intensity results showing the organ distribution of DiD in 4T1 tumor-bearing mice at 4, 8 and 24 h (mean±SD, *n*=3).



Figures S11 Body weight of 4T1 orthotopic tumor-bearing mice during treatment (Data are mean ±SD, *n*=6).



Figures S12 HE staining of major organs collected from 4T1 tumor-bearing mice after administration with PBS, free LMWH, free GEM, GTT and GLT, respectively. Scale bars indicated 100 μm.