Supplementary Information for

ORIGINAL ARTICLE

A facile and universal method to achieve liposomal remote loading of non-ionizable drugs with outstanding safety profiles and therapeutic effect

Shuang Zhou[a], Jinbo Li[a], Jiang Yu[a], Liyuan Yang[a], Xiao Kuang[a], Zhenjie Wang[a], Yingli Wang[a], Hongzhuo Liu[a], Guimei Lin[c], Zhonggui He[a], Dan Liu[b,*], Yongjun Wang[a,*]

[a]*Wuya College of Innovation, Shenyang Pharmaceutical University, Shenyang 110016, China*

[b]*Key Laboratory of Structure-Based Drugs Design & Discovery of Ministry of Education, Shenyang Pharmaceutical University, Shenyang 110016, China*

[c]*School of Pharmaceutical Science, Shandong University, Jinan 250012, China*

Received 25 June 2020; received in revised form 3 August 2020; accepted 5 August 2020

*Corresponding authors.

E-mail addresses: sammyld@163.com (Dan Liu); wangyongjun@syphu.edu.cn (Yongjun Wang).

Running title: Remote loading non-ionizable drugs into liposomes by weak acid derivatization

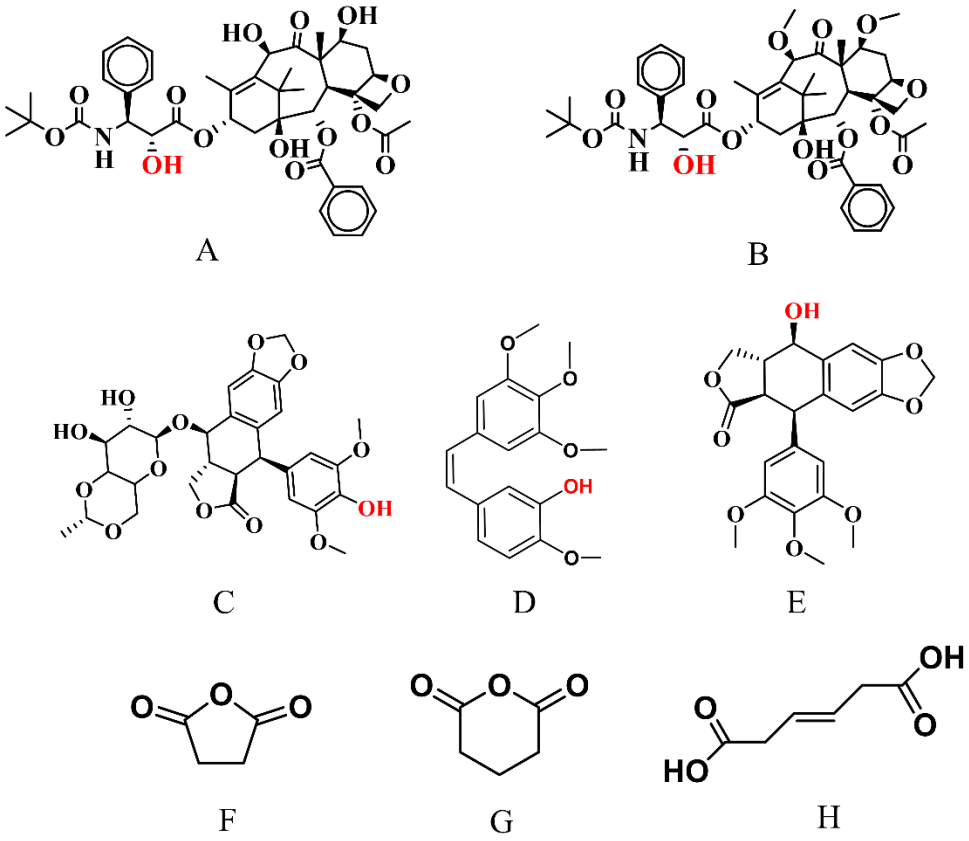


Figure S1 Structures of (A) docetaxel (DTX), (B) cabazitaxel (CTX), (C) etoposide (ETO), (D) combretastatin A4 (CA4), and (E) podophyllotoxin (PPA). (F) succinic anhydride (SA), (G) glutaric anhydride (GA), (H) *trans*-2-butene-1,4-dicarboxylic acid (DA).

MS/^{1}H NMR

a. SA-CTX

b. GA-CTX

c. SA-CTX

d. GA-CTX

e. DA-CTX

f. SA-PPA

g. DA-CA4

h. SA-ETO

i. GA-ETO

^{1}H NMR

SA-DTX: (400 MHz, $CDCl_3$) δ8.10 (2H, d, Ar–H), 7.62 (1H, m, Ar–H), 7.51 (2H, m, Ar–H), 7.39–7.29 (5H, m, Ar–H), 6.21 (1H, t, H13), 5.66 (1H, d, H2), 5.54 (1H, brs, H3′), 5.40 (1H, brs, H2′), 5.24 (1H, s, H10), 4.96 (1H, d, H5), 4.31(1H, d, H20), 4.25 (1H, d, H3),4.18 (1H, d, H20), 3.90 (1H, m, H7), 2.88 (1H, m, H6), 2.62 (m, 4H, –$OCCH_2CH_2CO$–, 2.42 (3H, s, 4-$OCOCH_3$), 2.21 (2H, m, H14), 1.93 (3H, s, 18-CH3), 1.86 (2H, m, H14, H6), 1.74 (3H, s, 19-CH_3), 1.34 (9H, s, $(OCH_3)_3$), 1.21 (3H, s, 16-CH_3), 1.10 (3H, s, 17-CH_3).

GA-DTX: (400 MHz, $CDCl_3$) δ8.10 (2H, d, Ar–H), 7.62 (1H, m, Ar–H), 7.51 (2H, m, Ar–H), 7.39–7.29 (5H, m, Ar–H), 6.23 (1H, t, H13), 5.67 (1H, d, H2), 5.48 (1H, brs, H3′), 5.37 (1H, brs, H2′), 5.23 (1H, s, H10), 4.96 (1H, d, H5), 4.31(1H, d, H20), 4.26 (1H, d, H3),4.19 (1H, d, H20), 3.92 (1H, m, H7), 2.57 (1H, m, H6), 2.44 (3H, s, 4-$OCOCH_3$), 2.39–2.34 (m, 4H, –$OCCH_2CH_2CH_2CO$–), 2.18 (2H, m, H14), 1.94 (3H, s, 18-CH_3), 1.88 (m, 2H, –$OCCH_2CH_2CH_2CO$–), 1.83 (2H, m, H14, H6), 1.75 (3H, s, 19-CH_3), 1.33 (9H, s, $(OCH_3)_3$), 1.22 (3H, s, 16-CH_3), 1.12 (3H, s, 17-CH_3).

SA-CTX: (400 MHz, $CDCl_3$) δ8.10 (2H, d, Ar–H), 7.61 (1H, m, Ar–H), 7.50 (2H, m, Ar–H), 7.40–7.30 (5H, m, Ar–H), 6.25 (1H, t, H13), 5.64 (1H, d, H2), 5.49 (1H, brs, H3′), 5.37 (1H, brs, H2′), 4.99 (1H, d, H5), 4.82 (1H, s, H10), 4.30(1H, d, H20), 4.16 (1H, d, H20), 3.89 (1H, m, H7), 3.83 (1H, d, H3), 3.44 (3H, s, 10-OCH_3), 3.30 (3H, s, 7-OCH_3), 2.73 (1H, m, H6), 2.71–2.65 (m, 4H, –$OCCH_2CH_2CO$–), 2.44 (3H, s, 4-$OCOCH_3$), 2.31 (2H, m, H14), 1.98 (3H, s, 18-CH_3), 1.78 (2H, m, H14, H6), 1.71 (3H, s, 19-CH_3), 1.36 (9H, s, $(OCH_3)_3$), 1.21 (3H, s, 16-CH_3), 1.19 (3H, s, 17-CH_3).

GA-CTX: (400 MHz, $CDCl_3$) δ8.10 (2H, d, Ar–H), 7.61 (1H, m, Ar–H), 7.50 (2H, m, Ar–H), 7.40–7.30 (5H, m, Ar–H), 6.26 (1H, t, H13), 5.64 (1H, d, H2), 5.49 (1H, brs, H3′), 5.35 (1H, brs, H2′), 4.99 (1H, d, H5), 4.83 (1H, s, H10), 4.30(1H, d, H20), 4.18 (1H, d, H20), 3.90 (1H, m, H7), 3.85 (1H, d, H3), 3.44 (3H, s, 10-OCH_3), 3.30 (3H, s, 7-OCH_3), 2.71 (1H, m, H6), 2.45 (3H, s, 4-$OCOCH_3$), 2.372.32 (m, 4H, –$OCCH_2CH_2CH_2CO$–), 2.21 (2H, m, H14), 2.00 (3H, s, 18-CH_3), 1.92 (m, 2H, –OCCH2CH_2CH_2CO–), 1.78 (2H, m, H14, H6), 1.71 (3H, s, 19-CH_3), 1.35 (9H, s, $(OCH_3)_3$), 1.21 (3H, s, 16-CH_3), 1.20 (3H, s, 17-CH_3).

DA-CTX: (400 MHz, CDCl3) δ8.03 (2H, d, Ar–H), 7.54 (1H, m, Ar–H), 7.43 (2H, m, Ar–H), 7.33–7.22 (5H, m, Ar–H), 6.19 (1H, t, H13), 5.57 (1H, d, H2), 5.57–5.47 (s, 2H, –CH=CH–), 5.40

(1H, brs, H3′), 5.25 (1H, brs, H2′), 4.92 (1H, d, H5), 4.75 (1H, s, H10), 4.23 (1H, d, H20), 4.11 (1H, d, H20), 3.82 (1H, m, H7), 3.77 (1H, d, H3), 3.36 (3H, s, 10-OCH_3), 3.23 (3H, s, 7-OCH_3), 3.11–3.03 (4H, –$OCCH_2CH$=$CHCH_2CO$–), 2.63 (1H, m, H6), 2.37 (3H, s, 4-$OCOCH_3$), 2.23 (2H, m, H14), 1.92 (3H, s, 18-CH_3), 1.72 (2H, m, H14, H6), 1.64 (3H, s, 19-CH_3), 1.28 (9H, s, $(OCH_3)_3$), 1.19 (3H, s, 16-CH_3), 1.13 (3H, s, 17-CH_3).

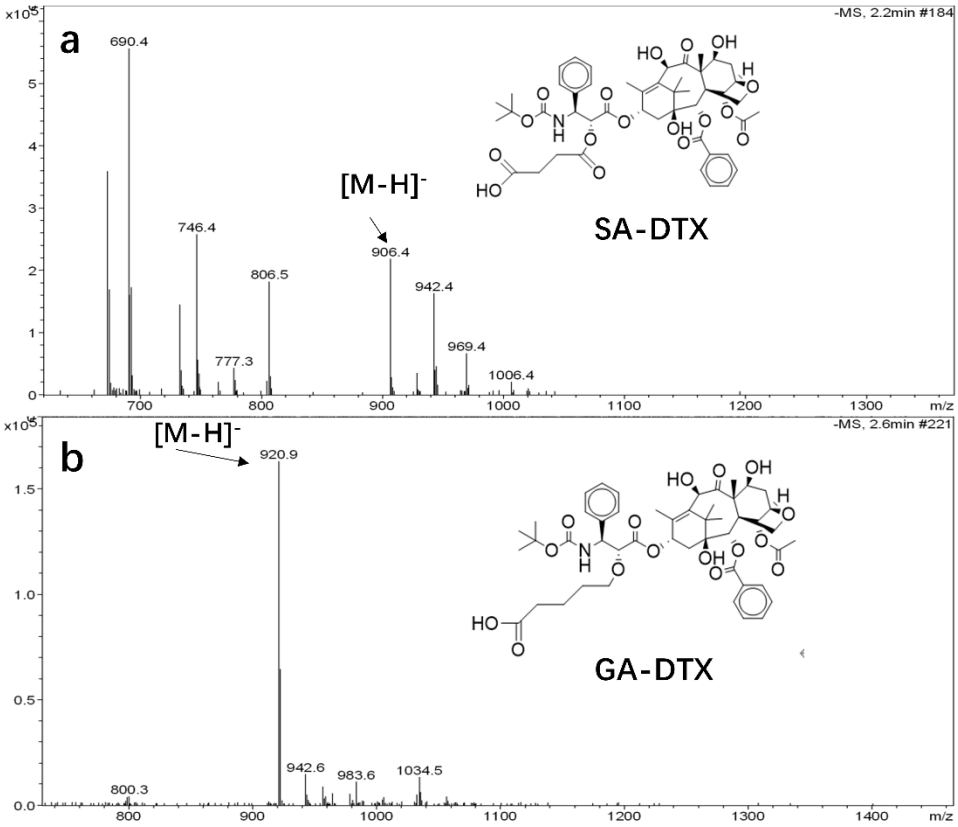
SA-PPA: (400 MHz, DMSO) δ 12.33 (s, 5H), 6.94 (s, 1H), 6.60 (s, 1H), 6.33 (s, 2H), 6.02 (s, 2H), 5.94 (d, J = 9.4 Hz, 1H), 4.56 (d, J = 4.7 Hz, 1H), 4.30 (t, J = 7.8 Hz, 1H), 4.15 (dd, J = 10.4, 8.8 Hz, 1H), 3.65 (s, 6H), 3.62 (s, 3H), 3.40 (d, J = 4.8 Hz, 1H), 2.63 (dd, J = 9.5, 5.8 Hz, 2H), 2.56 (d, J = 5.8 Hz, 2H).

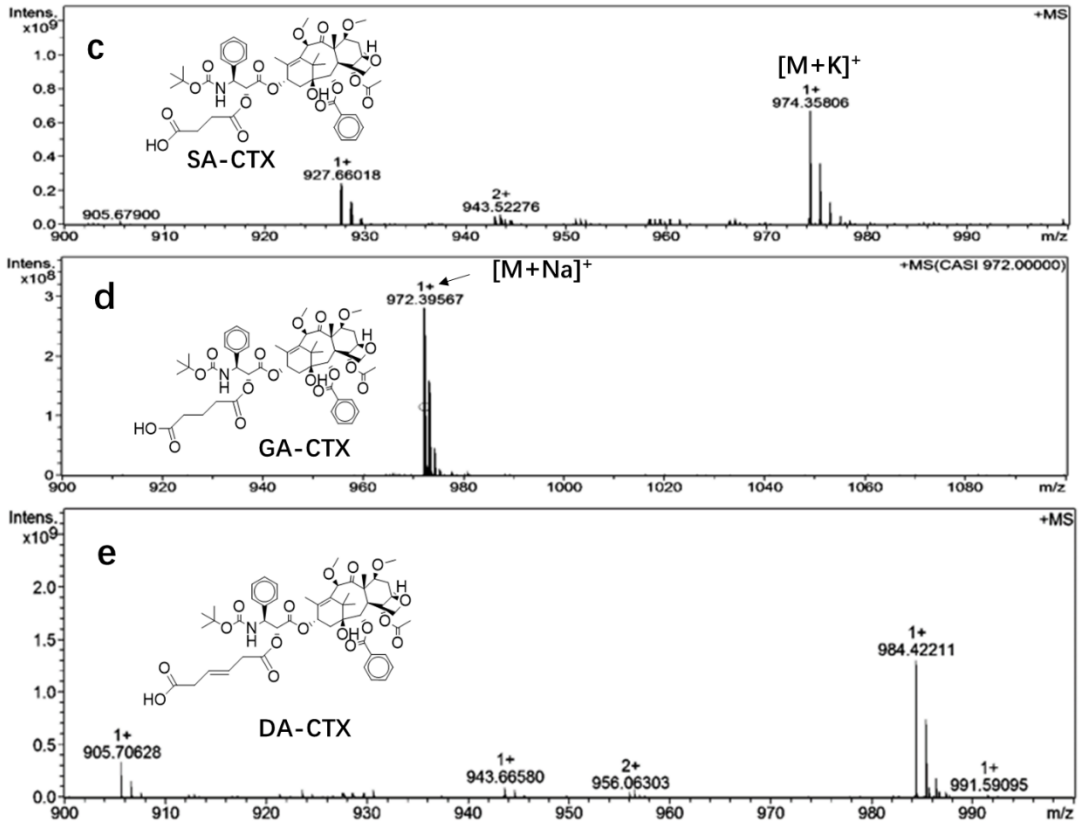
DA-CA4:(400 MHz, DMSO) δ 7.15 (dd, J = 8.5, 1.9 Hz, 1H), 7.07 (d, J = 8.5 Hz, 1H), 6.99 (d, J = 1.8 Hz, 1H), 6.54 (d, J = 2.9 Hz, 2H), 6.50 (d, J = 4.1 Hz, 2H), 5.77–5.57 (m, 2H), 3.74 (s, 3H), 3.64 (s, 3H), 3.60 (s, 6H), 3.33 (s, 2H), 3.04 (d, J = 6.5 Hz, 2H).

SA-ETO: (400 MHz, DMSO) δ 8.25 (s, 1H), 7.05 (s, 1H), 6.52 (s, 1H), 6.18 (s, 2H), 6.02 (d, J = 12.7 Hz, 2H), 5.76 (s, 2H), 4.97 (d, J = 9.0 Hz, 1H), 4.94 (d, J = 3.1 Hz, 1H), 4.77 (d, J = 7.6 Hz, 1H), 4.72 (d, J = 5.0 Hz, 1H), 4.49 (d, J = 5.3 Hz, 1H), 4.26 (d, J = 9.4 Hz, 2H), 4.14–4.08 (m, 1H), 3.59 (d, J = 19.7 Hz, 6H), 3.52 (d, J = 10.4 Hz, 1H), 3.44–3.40 (m, 2H), 3.26 (d, J = 3.3 Hz, 1H), 3.21 (d, J = 5.4 Hz, 1H), 2.94–2.84 (m, 1H), 2.43 (d, J = 6.1 Hz, 2H), 2.22 (d, J = 4.4 Hz, 2H), 1.19 (d, J = 4.9 Hz, 3H).

GA-ETO: (400 MHz, DMSO) δ 8.26 (s, 1H), 7.03 (s, 1H), 6.50 (s, 1H), 6.15 (s, 2H), 6.02 (d, J = 19.3 Hz, 2H), 5.76 (s, 1H), 4.98 (d, J = 8.0 Hz, 1H), 4.85 (d, J = 3.0 Hz, 1H), 4.75 (q, J = 4.9 Hz, 1H), 4.62–4.53 (m, 1H), 4.49 (d, J = 5.0 Hz, 1H), 4.27 (t, J = 7.8 Hz, 1H), 4.22–4.12 (m, 1H), 4.07 (dd, J = 10.0, 4.8 Hz, 1H), 3.60 (s, 6H), 3.55–3.48 (m, 1H), 3.41 (dd, J = 9.6, 4.9 Hz, 2H), 3.28 (s, 2H), 3.01–2.81 (m, 2H), 2.28–1.97 (m, 4H), 1.98–1.78 (m, 2H), 1.25 (d, J = 5.1 Hz, 3H).

MS Figure S2 a–i

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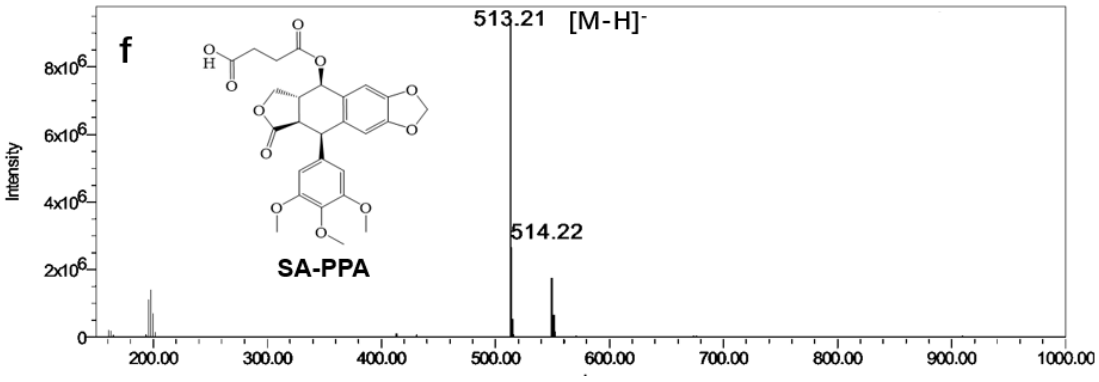
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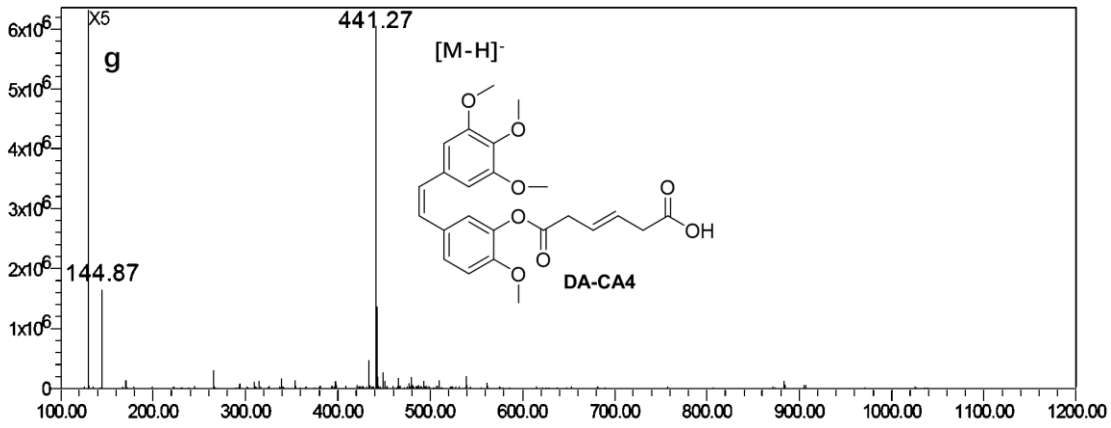
514.22
Intensity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[M-H]-
144.87

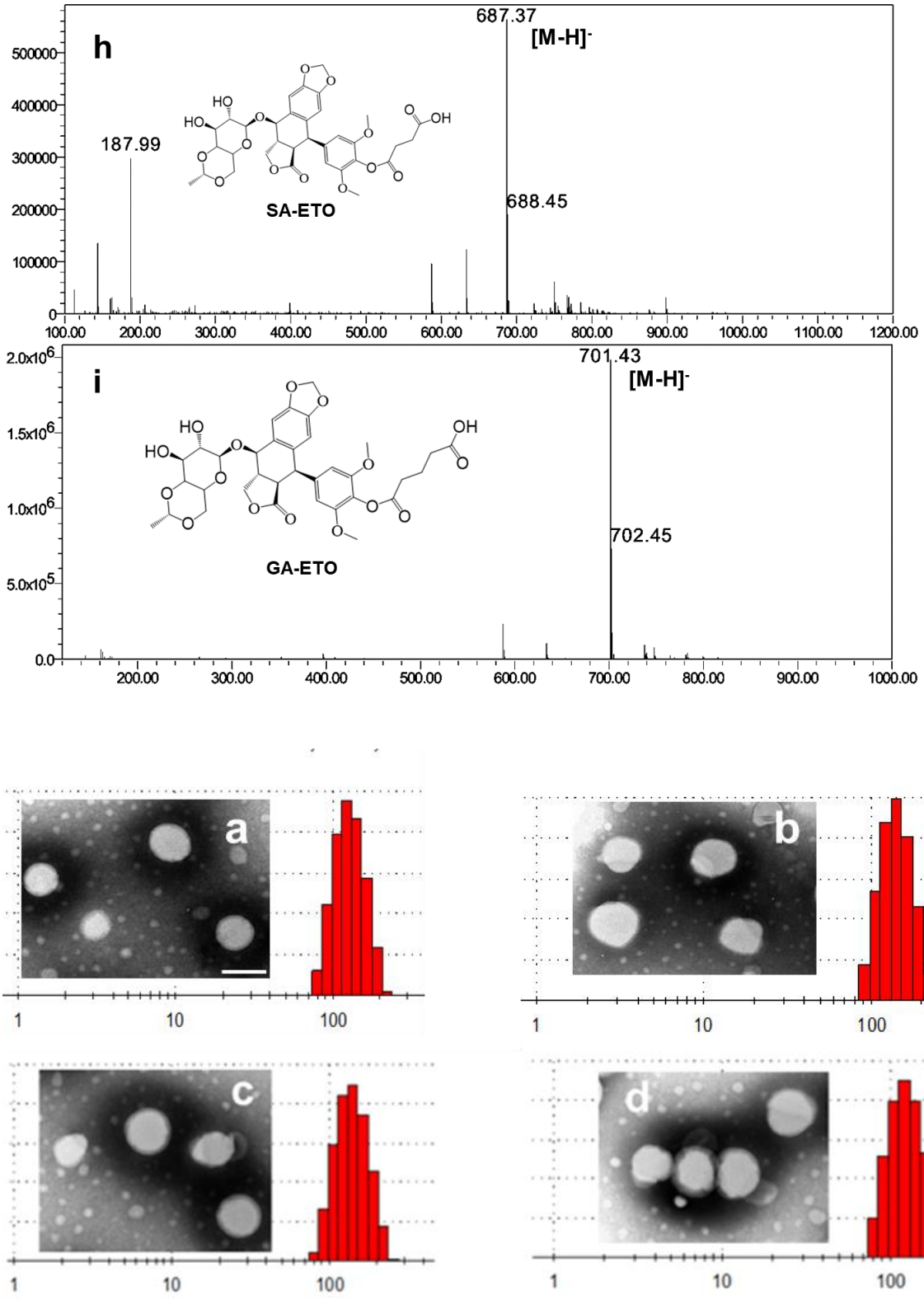

Figure S3 The TEM and size distribution of the (a) blank liposomes, (b) Lipo SA-CTX, (c) Lipo GA-CTX, (d) Lipo DA-CTX. Scar bar=100 nm.

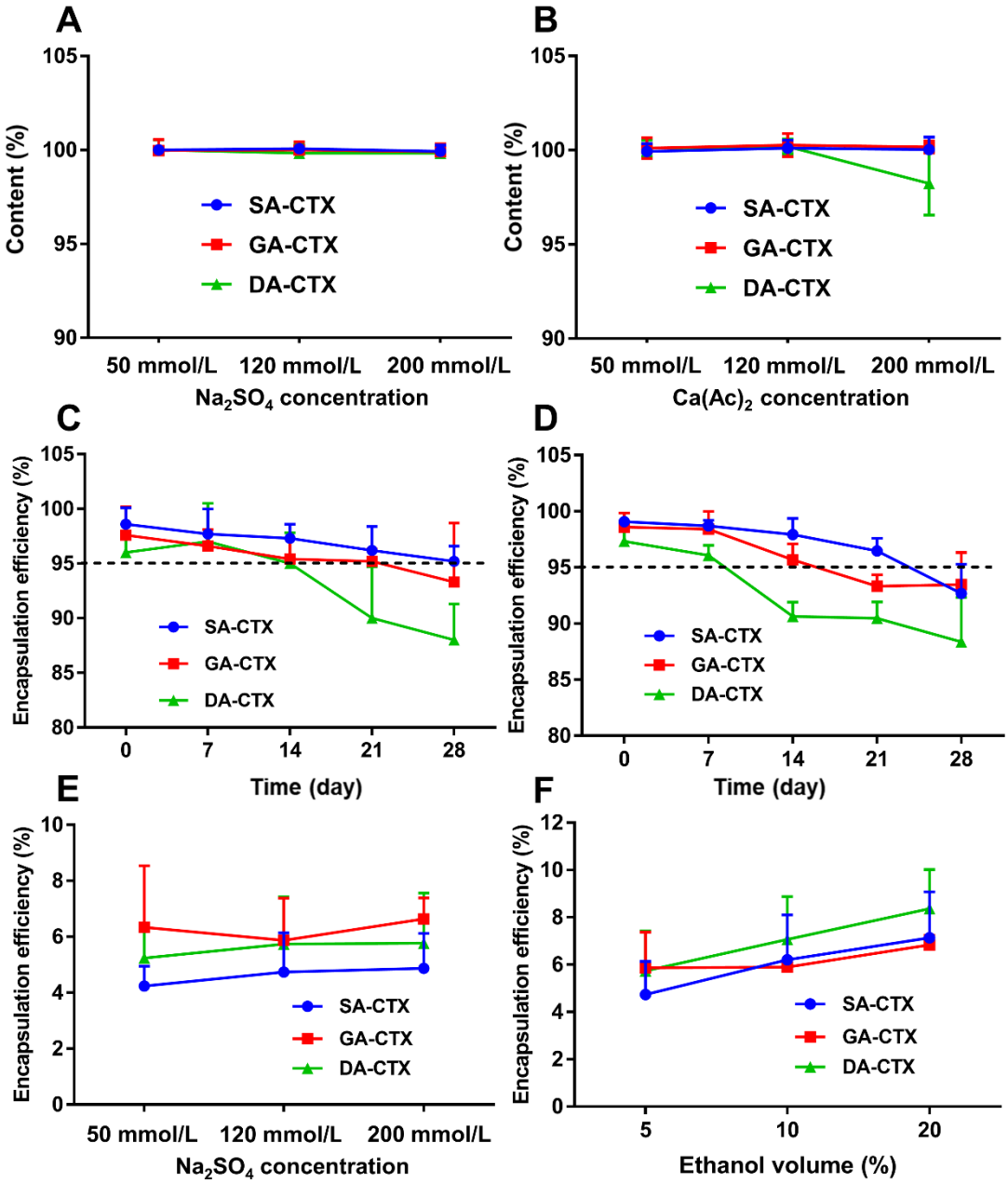

Figure S4 The stability of the three cabazitaxel weak acid derivatives in the outer aqueous phase (A) and inner aqueous phase (B). The storage stability of cabazitaxel (CTX) weak acid derivatives remote loading liposomes at 4 ℃ (C) and 25 ℃ (D) a expressed by the encapsulation efficiency (EE, %). (E) The effect of inner aqueous phase concentration loaded by non-pH gradient liposomes. (F) The effect of ethanol volume on EE loading by non-pH gradient liposomes. Data are shown as the mean±SD, *n*=3.

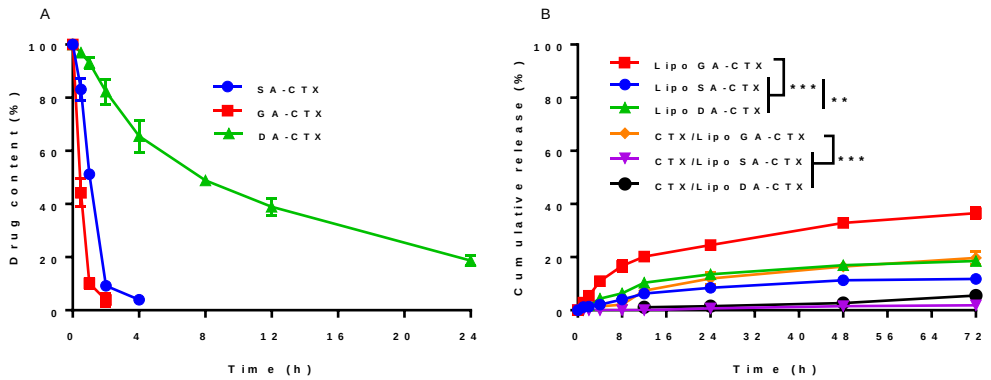

Figure S5 (A)The chemical stability of CTX weak acid derivatives in mouse plasma at 37 ℃ for 24 h. (B) The *in vitro* release profiles of CTX weak acid derivatives loaded liposomes in PBS (pH 7.4) at 37 ℃ for 72 h. Data are shown as the mean±SD, n=3, $^{**}P$<0.01, $^{***}P$<0.001.

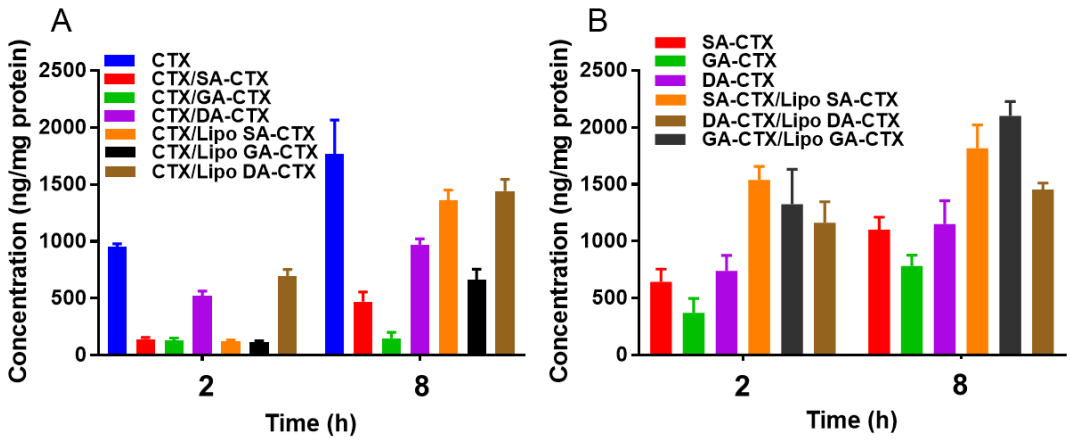

Figure S6 (A) The cellular uptake of CTX released from corresponding CTX derivatives formulations. (B) The cellular uptake of CTX weak acid derivatives and corresponding liposomes. Data are shown as mean±SD, n=3.

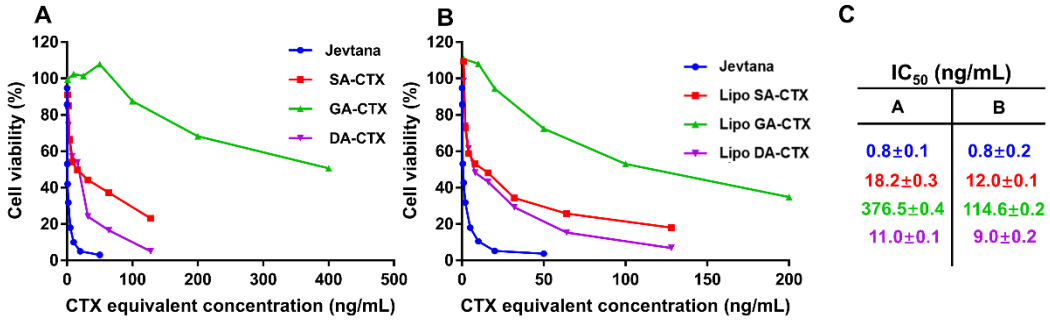

Figure S7 *In vitro* cytotoxicity of cabazitaxel (CTX) weak acid derivatives liposome on 4T1 cells for 48 h (A), 72 h (B), and the IC_{50} (C). Data are shown as the mean ±SD, n=6.

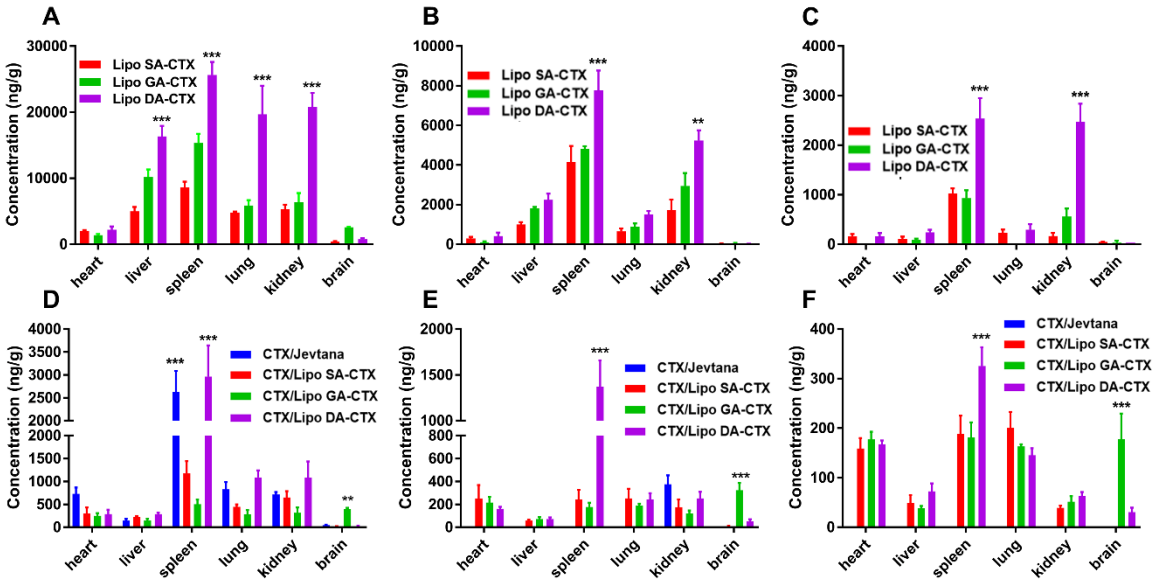

Figure S8 The biodistribution of cabazitaxel (CTX) weak acid derivatives liposomes and CTX released from corresponding formulation on non-targeting tissues (heart, liver, spleen, lung, kidney, and brain) on 6 h (A, D), 24 h (B, E), 48 h (C, F). Data are shown as the mean ±SD, n=3, $^{**}p$<0.01, $^{***}p$<0.001.

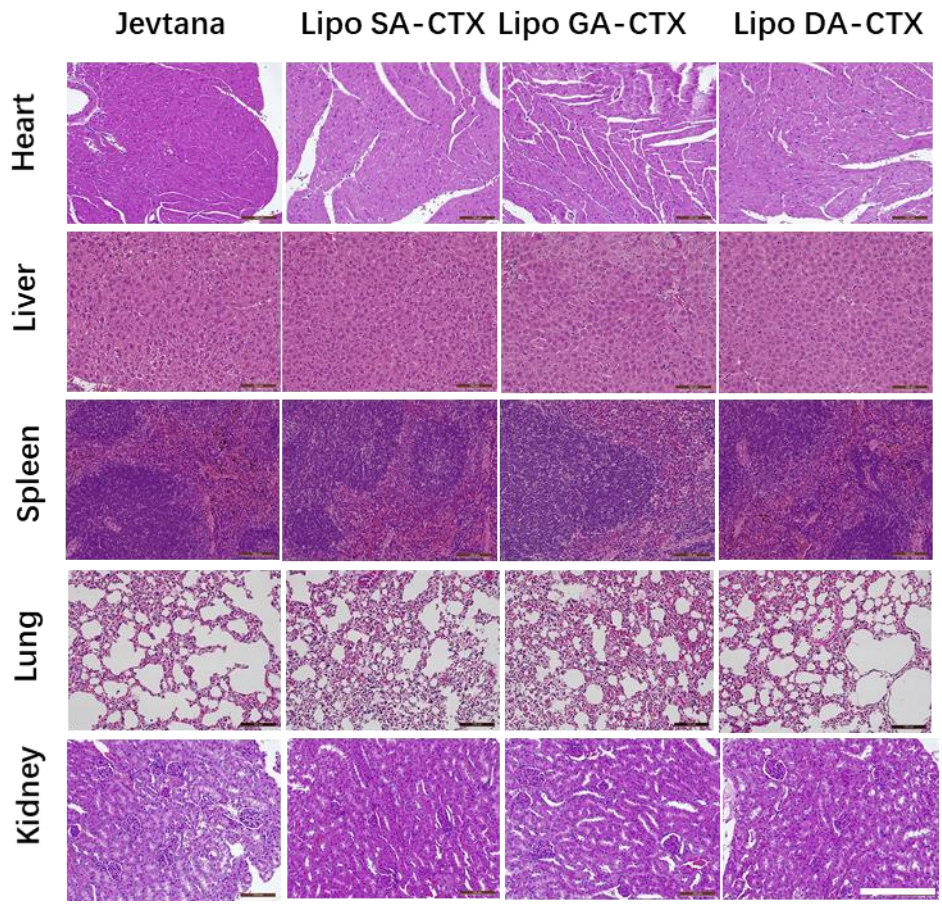

Figure S9 Hematoxylin and eosin (H&E) staining of mice major organs. Scar bar=100μm.

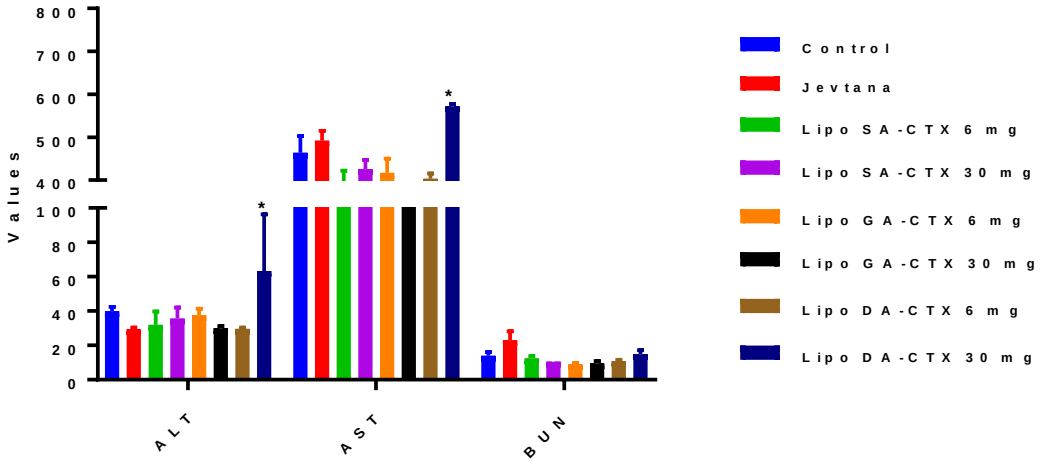

Figure S10 The hematological and blood biochemical parameters of different formulations after treatment (the unit for AST, ALT, and BUN is U/L, U/L, and mmol/L, respectively). Data are shown as the mean ±SD, n=3, * P<0.05

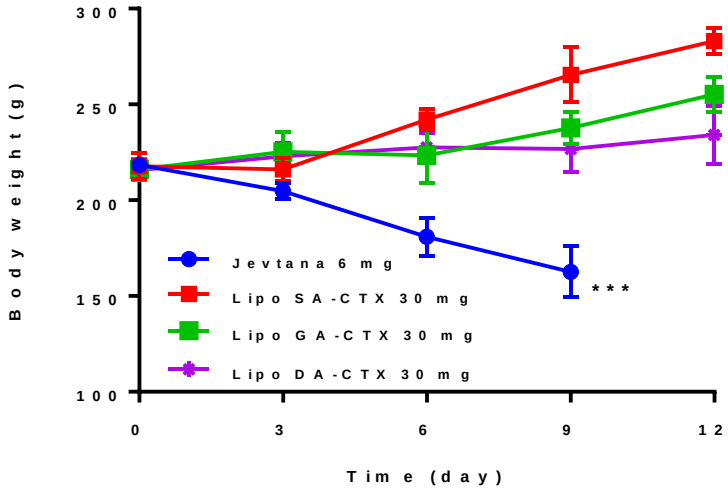

Figure S11 Body weight of Sprague–Dawley (SD) rats which were administered at tolerated doses of mice. Data are shown as the mean ±SD, n=3, $^{***}P$<0.001

Table S1 The characterizations of weak acid drug derivatives remote loading liposomes.

Drug derivate	DSPC:Chol:DSPE-PEG2000 (mol/mol/mol)	size (nm)	PDI	D:L	EE (%)
SA-DTX	68.1:22.2:1.2	120.6	0.068	1:10	90.4
GA-DTX	68.1:22.2:1.2	121.3	0.056	1:10	92.5
SA-PPA	68.1:22.2:1.2	118.7	0.088	1:10	96.0
DA-CA4	68.1:22.2:1.2	120.4	0.089	1:10	82.8
SA-ETO	95:5:0.1	123.4	0.099	1:10	92.3
GA-ETO	95:5:0.1	125.2	0.054	1:10	89.0

EE, encapsulation efficiency.

Table S2 PK parameters of different formulations.

Formulation	Drug	C_{max} (μmol/L)[a]	T_{max} (h)	AUC_{0-t} (μmol/L ħ)[a]	$t_{1/2}$ (h)[a]
Jevtana	CTX	1.5 ±0.3	0.083	1.5 ±0.3	3.4 ±1.6
Lipo	CTX	5.5 ±0.6	2.4 ±1.6	84.3 ±13.2	15.7 ±4.7
SA-CTX	SA-CTX	184.1 ±30.2	0.083	1262.7 ±153.4	9.6 ±2.9
Lipo	CTX	36.4 ±13.0	8.0 ±4.9	542.5 ±174.4	11.4 ±7.6
GA-CTX	GA-CTX	272.5 ±108.0	0.083	1172.6 ±392.5	5.4 ±1.0
Lipo	CTX	38.0 ±8.4	4.8 ±4.3	577.4 ±131.2	16.5 ±6.2
DA-CTX	DA-CTX	281.6 ±63.3	0.083	1464.7 ±333.5	7.4 ±3.4

[a]Data are mean ± SD, n = 5.

Table S3 Hematology measurements for various formulations after administration.

Parameter	Unit	Reference	Jevtana[a]	Lipo SA-CTX[a]	Lipo GA-CTX[a]	Lipo DA-CTX[a]
White blood cell	10^9/L	0.8–6.8	10.4 ±2.6	4.6 ±2.2	5.2 ±1.4	5.7 ±0.6
Lymphocyte	10^9/L	0.7–5.7	6.9 ±1.4	3.5 ±1.8	4.4 ±2.2	4.2 ±1.1
Monocyte	10^9/L	0.0–0.3	0.4 ±0.1	0.1 ±0.1	0.3 ±0.05	0.2 ±0.1
Neutrophil	10^9/L	0.1–1.8	0.08 ±0.04	1 ±0.4	1.5 ±0.2	1.3 ±0.2
Percent lymphocyte	%	55.8–90.6	66.7 ±18.4	75 ±13.6	60.2 ±4.8	74.1 ±13.6
Percent monocyte	%	1.8–6.0	3.8 ±1.1	3.1 ±2.1	4.6 ±2.1	3.7 ±1.7
Percent neutrophil	%	8.6–38.9	29.5 ±4.6	21.9 ±3.8	35.2 ±1.5	22.2 ±0.8
RBC	10^{12}/L	6.36–9.42	10.89 ±3.8	8.16 ±1.1	9.13 ±0.3	8.54 ±1.2
Hemoglobin -HGB	g/L	110–143	159 ±24	124 ±21	130 ±12	140 ±4
Hematocritg-HCT	%	34.6–44.6	51.8 ±10.4	39.4 ±1.8	42.8 ±1.0	36.8 ±4.3
MCV	fl	48.2–58.3	47.6 ±6.8	48.3 ±0.2	47.9 ±3.8	49.3 ±2.6
MCH	pg	15.8–19	14.6 ±3.3	17.9 ±1.2	18.2 ±0.6	16.5 ±2.2
MCHC	g/L	302–353	306 ±20	314 ±10	303 ±10	318 ±10.6
PLT	10^9/L	450–1590	1775 ±204	1046 ±320	894 ±218	1027 ±320

[a]Data are mean ± SD, n = 3.