Supporting Information

Water soluble multiarm-polyethylene glycol-betulinic acid prodrugs: design, synthesis, and *in vivo* effectiveness

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Synthesis of 4arm-PEG_{40K}-BA and 8arm-PEG_{20K}-BA conjugates

4arm-PEG_{40K}-BA: 4arm-PEG_{40K}-COOH (10.0 g, 0.25 mmol) and BA (0.91 g, 2.0 mmol) were dissolved with 250 mL of tetrahydrofuran (THF). The solution was cooled to 0 °C and added EDC (0.23 g, 1.2 mmol) and DMAP (0.24 g, 2.0 mmol). The mixture was stirred at 0 °C for 1 h and at room temperature overnight. The solvent was evaporated under vacuum. The residue was dissolved in 100 mL of THF, and the crude product was precipitated with ethyl ether (500 mL). After filtration, the resulting solids were recrystallized with a mixture of DMF/IPA (120 mL/480 mL). Then, the solids were filtered, washed with ethyl ether (2 × 500 mL), and dried under vacuum at 40 °C to give 4arm-PEG_{40K}-BA.

8arm-PEG_{20K}-BA: 8arm-PEG_{20K}-COOH (5.0 g, 0.25 mmol) and BA (4.56 g, 10.0 mmol) were dissolved with 250 mL of THF. The solution was cooled to 0 °C and added EDC (1.15 g, 6.0 mmol) and DMAP (1.22 g, 10.0 mmol). The mixture was stirred at 0 °C for 1 h and at room temperature overnight. The solvent was evaporated under vacuum. The residue was dissolved in 100 mL of THF, and the crude product was precipitated with ethyl ether (500 mL). After filtration, the resulting solids were recrystallized with a mixture of DMF/IPA (120 mL/480 mL). Then, the solids were filtered, washed with ethyl ether (2 × 500 mL), and dried under vacuum at 40 °C to give 8arm-PEG_{20K}-BA.



Fig. S1. (A) ¹H-NMR spectra of BA and 4arm-PEG_{40K}-BA, 8arm-PEG_{40K}-BA and 8arm-PEG_{20K}-BA conjugates ($8.00 \sim -1.00$ ppm). They were solubilized in CDCl₃ for ¹H-NMR analysis (600 MHz). (B) ¹H-NMR spectra of BA ($3.40 \sim 2.60$ ppm). (C) ¹H-NMR spectra of PEG-BA conjugates ($4.30 \sim 3.77$ ppm).

¹H-NMR analysis

The chemical construction of BA prodrugs was measured by ¹H-NMR. Samples were dissolved in deuterated chloroform (CDCl₃) for analysis by ¹H-NMR (Bruker DRX-600 Avance III spectrometer).

Fig. S1A shows the ¹H-NMR (CDCl₃) spectra of BA, 4arm-PEG_{40K}-BA, 8arm-PEG_{40K}-BA and 8arm-PEG_{20K}-BA, where the signals at 0.70–2.18 attributed to the most characteristic peaks protons of BA, 3.50-3.85 (4nH, –(CH₂CH₂O)_n–) and 4.20 (2H, –CH₂OC(O)O–) attributed to the methylene protons of PEG.

Due to the formation of ester bond, the 4-methine proton peak δ 3.00 (1H, t) of BA moved to δ 3.84 (1H, t) and the terminal methylene proton peak of PEG δ 4.13 (1H, t) moved to δ 4.17 (1H, t) (Fig. S1B). There is a small terminal methylene proton peak of PEG δ 4.13 (1H, t) which was due to the incompletely reacted functional group of PEG. This result was confirmed by the results of MALDI-TOF and absorbance properties.

MALDI-TOF analysis

Each sample was confirmed to be modified as determined by MALDI-TOF mass spectrometry (Fig. S2). MALDI-TOF mass spectrometry was acquired using an Applied Biosystems Voyager System DE Pro MALDI-TOF mass spectrometer using a nitrogen laser. The matrix was a saturated solution of α -cyano-4-hydroxycinnamic acid in a 50:50 mixture of acetonitrile and water containing 0.1% trifluoroacetic acid (TFA). All solutions were prepared in 50/50 acetonitrile/0.1% TFA.

Samples were prepared at a concentration of 10 mg/mL. Sodium trifluoroacetate (NaTFA) was prepared at 10 mg/mL. 20 μ L of sample solution and 20 μ L of NaTFA solution were mixed together with 160 μ L of 50/50 acetonitrile/0.1% TFA. Equal volumes of polymer sample and matrix solution (α -cyano 4-hydroxycinnamic acid; 5 mg/mL in 50/50 acetonitrile/0.1% TFA) were mixed and 1 μ L was deposited onto a stainless steel MALDI target and allowed to air-dry.

4arm-PEG_{40K}, 8arm-PEG_{40K}, 8arm-PEG_{20K}, and BA have a molecular weight of 40KDa, 40KDa, 20KDa, and 456.71Da, respectively. And, the molar ration of 4arm-PEG_{40K}-BA, 8arm-PEG_{40K}-BA and 8arm-PEG_{20K}-BA conjugates was calculated to be 2.38, 6.21, and 5.78, respectively.



Fig. S2. MALDI-TOF mass spectrum of PEG and BA prodrugs.

HPLC analysis

The final products were monitored by an Agilent 1200 (Agilent, USA) HPLC instrument. It employs a VYDAC 214TP54 (C18, 300A, 5 μ m, 4.6 × 250 mm) with a UV detector, using a gradient of 15–100% of acetonitrile in 0.05% TFA at a flow rate of 1 mL/min.

Sample: 4arm-PEG_{40K}-BA



Fig. S3. HPLC of 4arm-PEG_{40K}-BA

Sample: 8arm-PEG_{40K}-BA



UV 210 nm

Fig. S4. HPLC of 8arm-PEG_{40K}-BA

Sample: 8arm-PEG_{20K}-BA



Fig. S5. HPLC of 8arm-PEG_{20K}-BA

4arm-PEG _{40K} -BA			8arm-PEG _{40K} -BA			8arm-PEG _{20K} -BA		
Time	peak	retention	Time	peak	retention	Time	peak	retention
(h)	area	time	(h)	area	time	(h)	area	time
0	2399854	9.36	0	5072106	11.51	0	8690219	12.34
12	1559876	9.24	12	3702556	11.43	12	6517157	12.26
24	1055236	9.27	24	2688012	11.39	24	5040058	12.21
36	767885	9.23	36	1927190	11.35	36	3649115	12.16
48	503897	9.09	48	1369259	11.32	48	2693724	12.13
60	335909	9.07	60	963662	11.29	60	1911668	12.10
72	215876	9.05	72	710186	11.29	72	1476895	12.08
84	167911	9.03	84	557952	11.27	84	1215761	12.07
96	119992	9.03	96	456367	11.27	96	869022	12.05
108	119796	9.02	108	405252	11.25	108	695005	12.03

100 μ l of BA prodrugs 10 mg/mL in PBS pH 8.1 for incubation at 37 °C, and 100 ul of DMSO at each time point and inject 20 μ L onto HPLC.



Fig. S6. pH stability of BA prodrugs in PBS at pH 8.1

4arm-PEG _{40K} -BA			8arm-PEG _{40K} -BA			8arm-PEG _{20K} -BA		
Time	peak	retention	Time	peak	retention	Time	peak	retention
(h)	area	time	(h)	area	time	(h)	area	time
0	2172085	9.52	0	4589276	11.68	0	7868069	12.45
12	1781852	9.46	12	3900976	11.63	12	6923886	12.42
24	1412261	9.41	24	3212507	11.59	24	5822357	12.40
36	1128064	9.37	36	2615936	11.55	36	4642289	12.33
48	868728	9.33	48	2065189	11.50	48	3776752	12.30
60	695059	9.30	60	1606272	11.48	60	3068602	12.27
72	543106	9.28	72	1376807	11.46	72	2439159	12.25
84	477857	9.26	84	1193256	11.44	84	2203031	12.24
96	434629	9.24	96	1055579	11.42	96	1730864	12.21
108	390960	9.21	108	917885	11.37	108	1573785	12.16

100 μ l of BA prodrugs 10 mg/mL in PBS pH 7.4 for incubation at 37 °C, and 100 ul of DMSO at each time point and inject 20 μ L onto HPLC.



Fig. S7. pH stability of BA prodrugs in PBS at pH 7.4

4arm-PEG _{40K} -BA			8arm-PEG _{40K} -BA			8arm-PEG _{20K} -BA		
Time	peak	retention	Time	peak	retention	Time	peak	retention
(h)	area	time	(h)	area	time	(h)	area	time
0	1956876	9.59	0	4236058	11.72	0	7109316	12.51
12	1818817	9.57	12	4024980	11.70	12	6824795	12.50
24	1741982	9.56	24	3897765	11.70	24	6611269	12.49
36	1662967	9.54	36	3686489	11.69	36	6325087	12.47
48	1584583	9.54	48	3515675	11.68	48	5971259	12.38
60	1507159	9.53	60	3389066	11.67	60	5686423	12.46
72	1388710	9.51	72	3177215	11.65	72	5541882	12.45
84	1329685	9.51	84	3007940	11.65	84	5215027	12.45
96	1213006	9.49	96	2923201	11.64	96	5044525	12.44
108	1173158	9.49	108	2754532	11.62	108	4832983	12.43
132	1056851	9.45	132	2545574	11.61	132	4475935	12.43
156	919290	9.45	156	2244987	11.61	156	4152896	12.41
180	782730	9.42	180	1992670	11.60	180	3764383	12.39
204	684201	9.42	204	1737352	11.58	204	3266657	12.36
228	547341	9.40	228	1524004	11.55	228	2981856	12.34
252	489064	9.35	252	1313156	11.50	252	2700516	12.31

100 μ l of BA prodrugs 10 mg/mL in PBS pH 6.1 for incubation at 37 °C, and 100 ul of DMSO at each time point and inject 20 μ L onto HPLC.



Fig. S8. pH stability of BA prodrugs in PBS at pH 6.1