Supplementary material

Cancer Nanomedicines Stabilized by π-π Stacking between Heterodimeric Prodrugs Enable Exceptionally High Drug Loading Capacity and Safer Delivery of Drug Combinations

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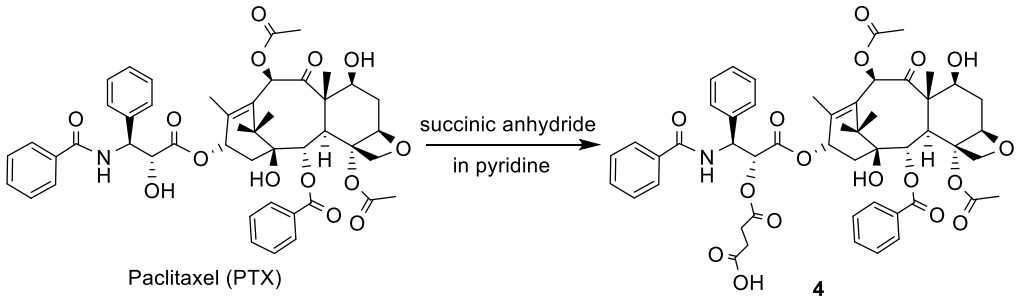
Synthesis of PTX-SN38 conjugate 1



To a solution of paclitaxel (500 mg, 0.59 mmol) and succinic anhydride (176 mg, 1.77 mmol) in 8 mL of anhydrous pyridine were added DMAP (7.2 mg, 0.06 mmol). The reaction mixture was stirred at 25 ºC for 3 hours. After removing the solvent, dichloromethane was added and washed with 0.1 N HCl and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum. The residue was purified by flash column chromatography on silica gel (DCM:MeOH=80:1) to give compound **4** (550mg, 98%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (d, 2H, *J* = 7.3 Hz), 7.76 (d, 2H, *J* = 7.4 Hz), 7.61 (t, 1H, *J* = 7.4 Hz), 7.55–7.46 (m, 3H), 7.44–7.37 (m, 6H), 7.34 (d, 1H, *J* = 6.7 Hz), 7.06–6.97 (m, 1H), 6.29 (s, 1H), 6.22 (t, 1H, *J* = 8.8 Hz), 5.98 (dd, 1H, *J* = 9.1, 3.2 Hz), 5.68 (d, 1H, *J* = 7.1 Hz), 5.54 (d, 1H, *J* = 3.5 Hz), 4.97 (d, 1H, *J* = 8.0 Hz), 4.43 (dd, 1H, *J* = 10.8, 6.7 Hz), 4.31 (d, 1H, *J* = 8.5 Hz), 4.19 (d, 1H, *J* = 8.4 Hz), 3.81 (s, 1H), 3.79 (s, 1H), 2.80–2.65 (m, 2H), 2.65–2.59 (m, 2H), 2.54 (dd, 1H, *J* = 15.0, 9.4 Hz), 2.44 (s, 3H), 2.38 (d, 1H, *J* = 4.3 Hz), 2.36–2.30 (m, 1H), 2.21 (s, 3H), 2.19–2.10 (m, 2H), 1.90 (s, 3H), 1.68 (s, 3H), 1.22 (s, 3H), 1.13 (s, 3H).

HRMS: calcd for $[C_{51}H_{56}NO_{17}]^+$ $[M+H]^+$ = 954.3548; obsd: 954.3564.

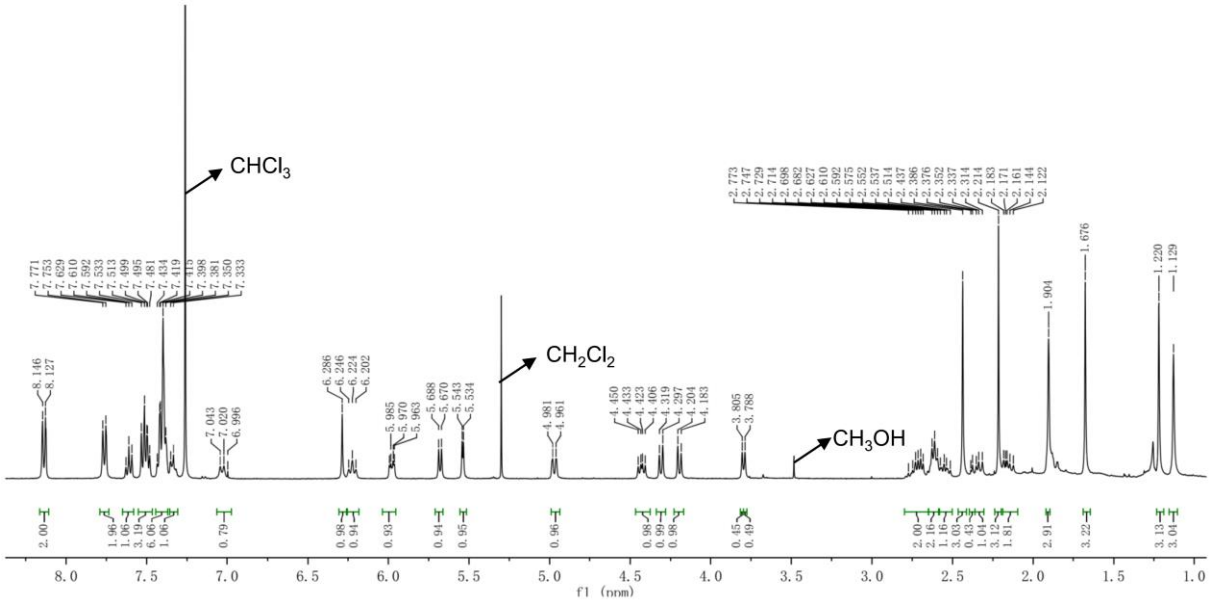


Figure S1. ^{1}H NMR spectrum of PTX derivative **4** in $CDCl_3$.

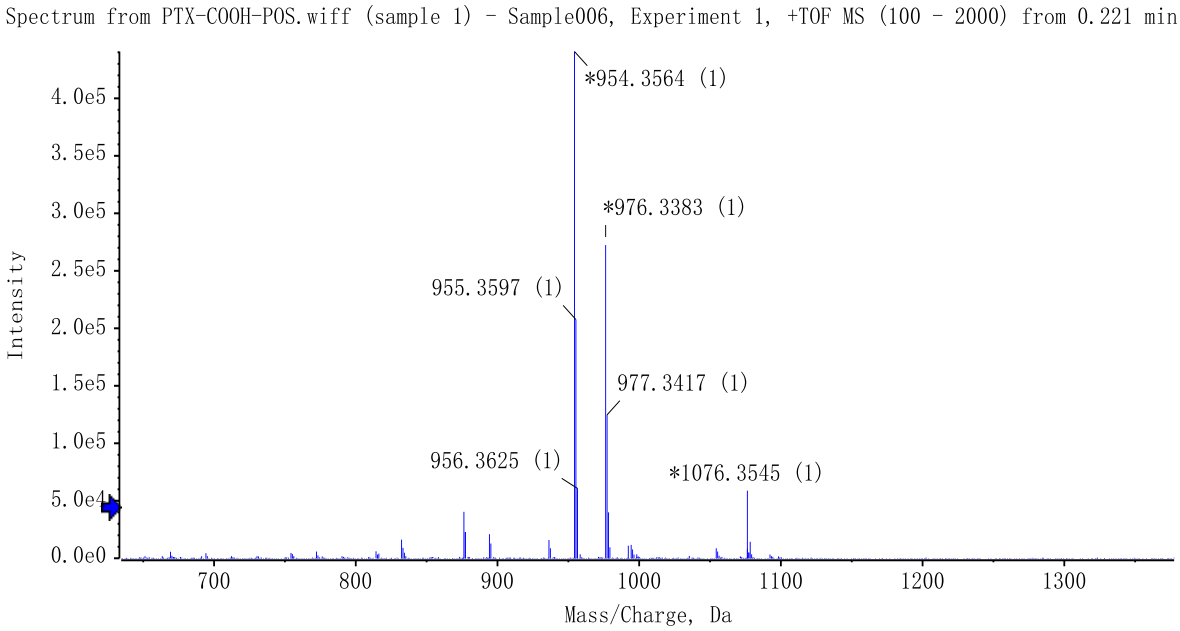
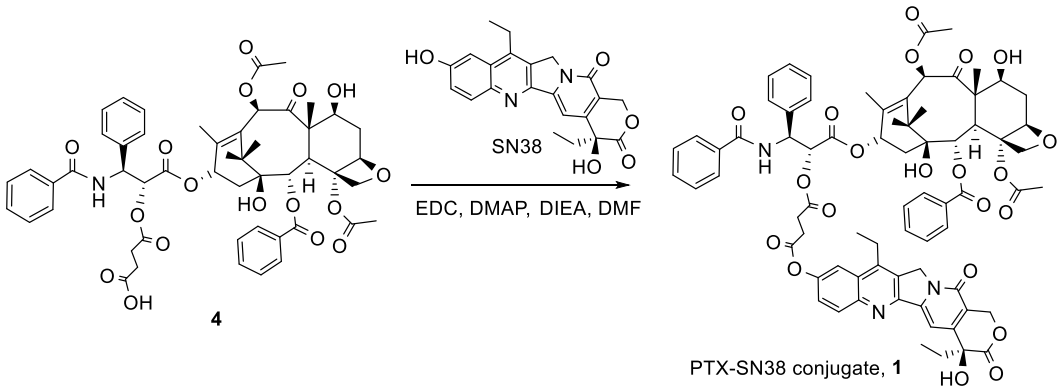


Figure S2. High-resolution mass spectrum of PTX derivative **4**.



To a solution of compound **4** (550 mg, 0.58 mmol) and SN-38 (226 mg, 0.58 mmol) in 18 mL of anhydrous DMF were added EDC·HCl (122 mg, 0.64 mmol) and DMAP (78 mg, 0.64 mmol) and DIEA (144 μL, 0.87 mmol). The reaction mixture was stirred at 25 ºC overnight. After removing the solvent, DCM was added and washed with 5% citric acid, saturated $NaHCO_3$ and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum. The residue was purified by flash column chromatography on silica gel (DCM:MeOH = 100:1) to give PTX-SN38 conjugate, compound **1** (320 mg, 42%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17 (dd, 3H, *J* = 8.2, 4.2 Hz), 7.86 (d, 1H, *J* = 2.3 Hz), 7.64 (s, 1H), 7.60 (d, 3H, *J* = 7.2 Hz), 7.51 (dd, 4H, *J* = 8.2, 4.6 Hz), 7.43 (d, 4H, *J* = 4.2 Hz), 7.13 (t, 1H, *J* = 7.2 Hz), 7.02 (t, 3H, *J* = 7.7 Hz), 6.31 (s, 2H), 6.06 (d, 1H, *J* = 9.3 Hz), 5.76 (d, 1H, *J* = 16.4 Hz), 5.72 (d, 1H, *J* = 7.2 Hz), 5.53 (d, 1H, *J* = 2.6 Hz), 5.34 (s, 1H), 5.30 (s, 1H), 5.19 (t, 2H, *J* = 7.6 Hz), 5.00 (d, 1H, *J* = 9.0 Hz), 4.47 (s, 1H), 4.33 (d, 1H, *J* = 8.5 Hz), 4.22 (d, 1H, *J* = 8.4 Hz), 3.85 (d, 1H, *J* = 6.8 Hz), 3.77 (s, 1H), 3.05–2.90 (m, 6H), 2.62 (s, 6H), 2.50 (s, 3H), 2.24 (s, 3H), 1.94–1.87 (m, 4H), 1.27 (s, 3H), 1.25 (s, 9H), 1.06 (t, 3H, *J* = 7.3 Hz).

^{13}C NMR (100 MHz, $CDCl_3$) δ 203.78, 173.86, 171.23, 171.18, 170.61, 169.91, 168.00, 167.03, 166.83, 157.62, 151.88, 150.22, 149.32, 147.51, 146.76, 145.47, 142.60, 136.89, 133.64, 132.99, 132.89, 132.04, 131.51, 130.25, 130.25, 129.23, 129.13, 129.13, 128.72, 128.72, 128.51, 128.13, 128.13, 127.45, 127.25, 127.00, 127.00, 126.54, 126.54, 125.23, 118.67, 114.91, 98.09, 84.46, 81.13, 79.13, 76.46, 75.60, 75.16, 74.78, 72.79, 72.09, 66.32, 58.52, 53.44, 52.68, 49.35, 45.67, 43.26, 35.60, 35.60, 31.60, 29.78, 26.86, 26.86, 22.94, 22.77, 22.17, 20.84, 14.82, 13.96, 9.65, 7.83.

HRMS: calcd for $[C_{73}H_{74}N_3O_{21}]^+$ $[M+H]^+$ = 1328.4815; obsd: 1328.4798.

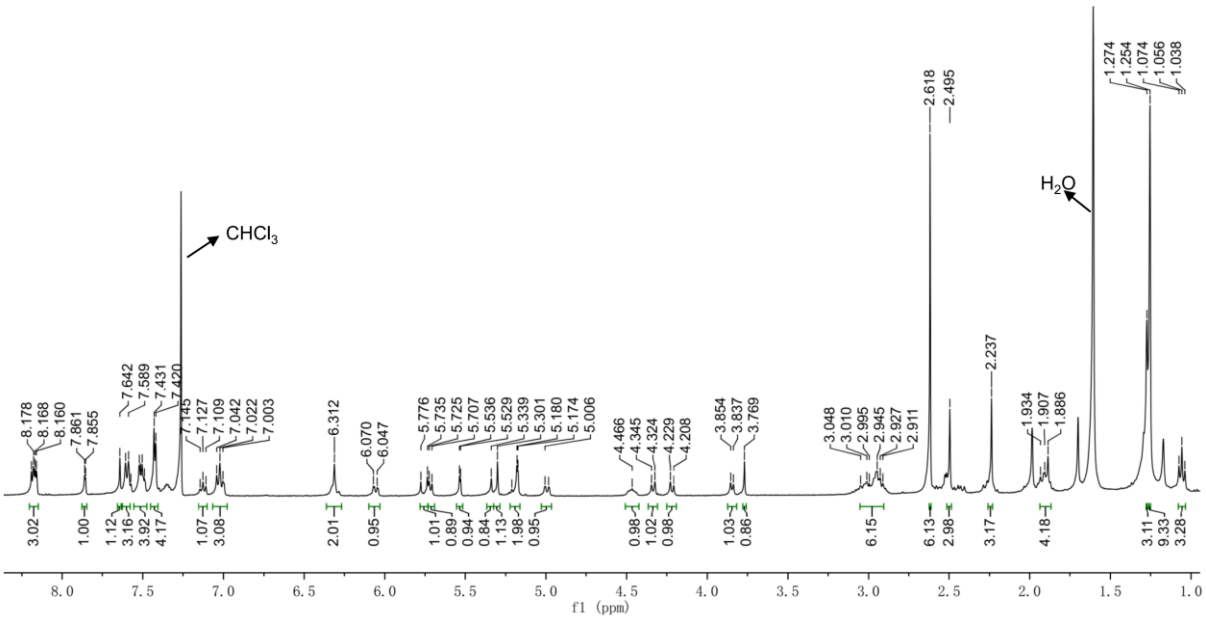


Figure S3. ^{1}H NMR spectrum of PTX-SN38 conjugate **1** in $CDCl_3$.

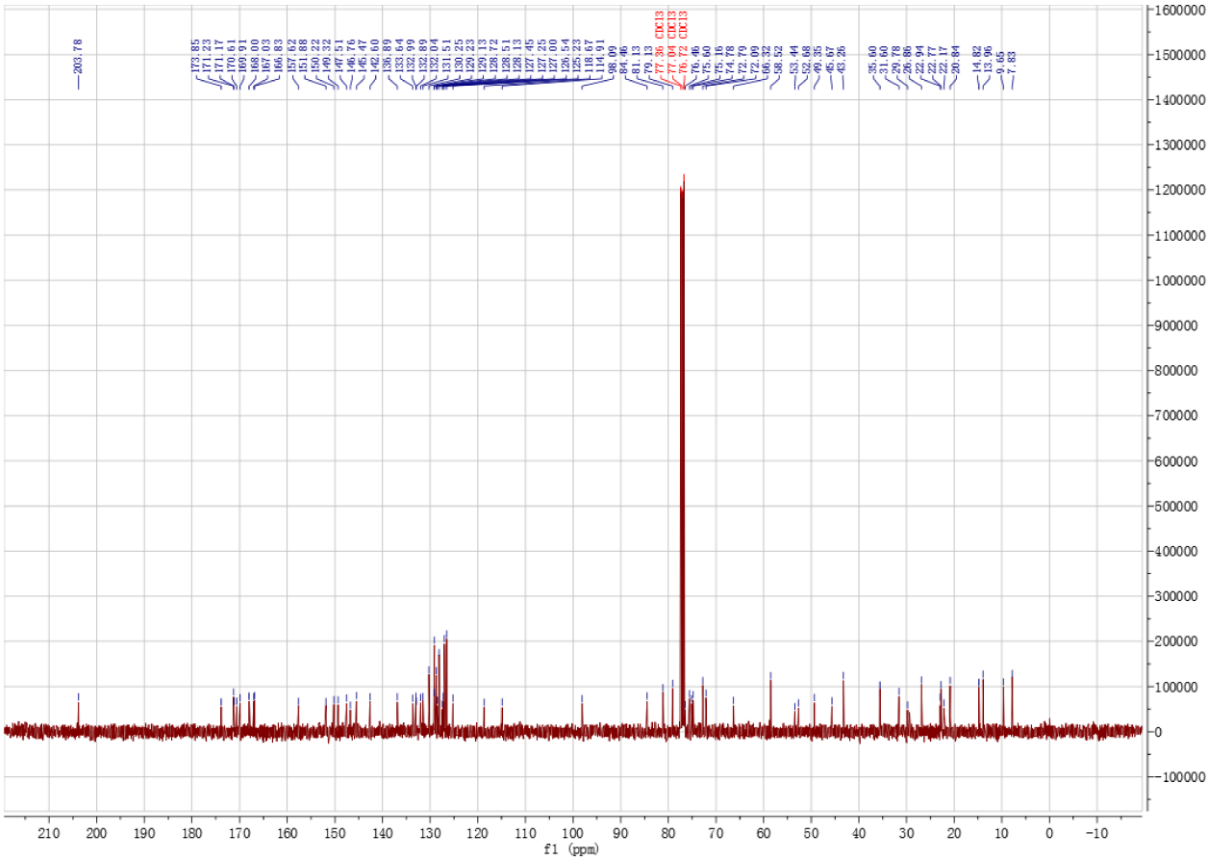


Figure S4. ^{13}C NMR spectrum of PTX-SN38 conjugate **1** in $CDCl_3$.

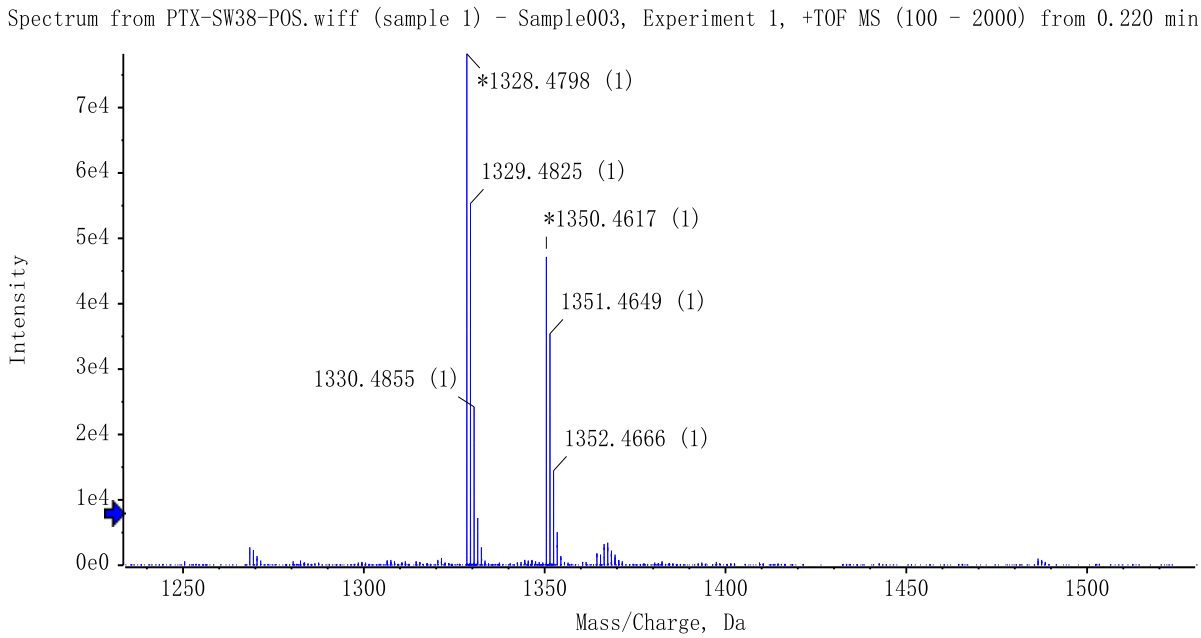


Figure S5. High-resolution mass spectrum of PTX-SN38 conjugate **4**.

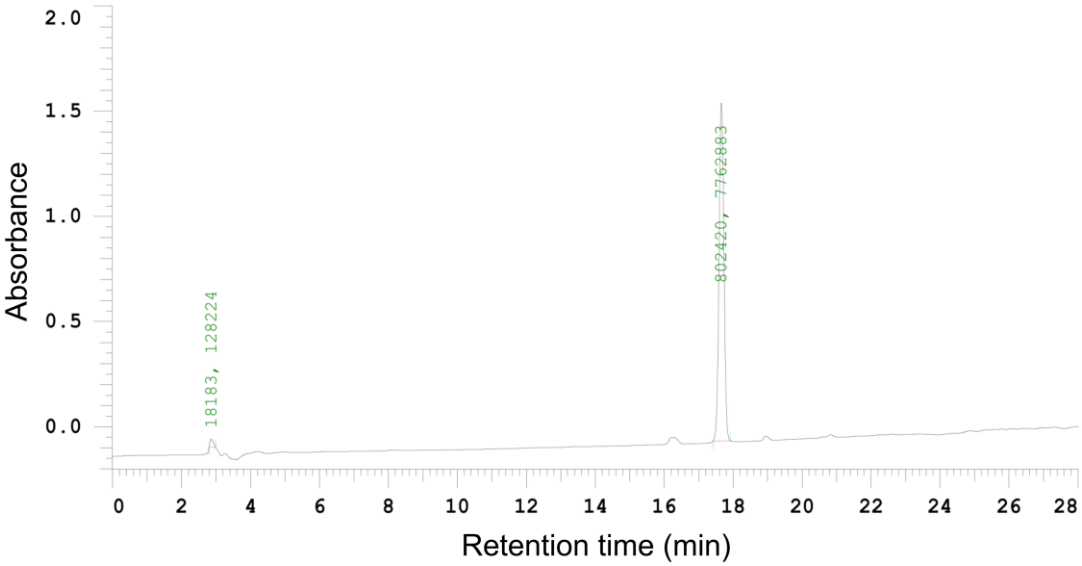
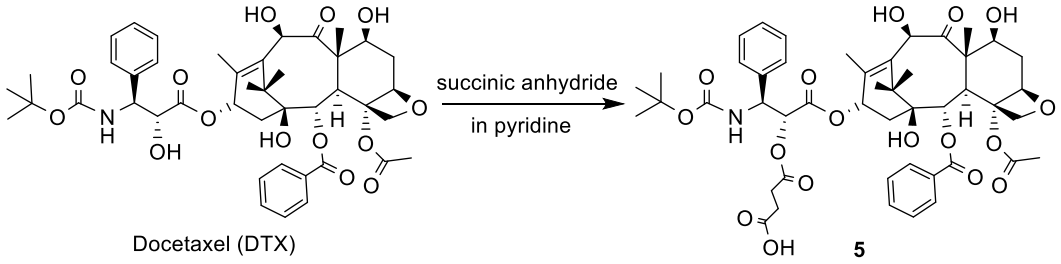


Figure S6. Reverse phase (RP)-HPLC chromatogram of PTX-SN38 conjugate **1**. The purified **1** was subjected to analytical HPLC using a C18 reverse-phase column (5 μm, 250 mm × 4.6 mm). A gradient of 20-100% acetonitrile in water within 20 min was adopted as the mobile phase at a flow rate of 1 mL/min together with UV detection at a wavelength of 220 nm.

Synthesis of DTX-SN38 conjugate 2



To a solution of docetaxel (500 mg, 0.62 mmol) and succinic anhydride (186 mg, 1.86 mmol) in 8 mL of anhydrous pyridine were added DMAP (7.2 mg, 0.06 mmol). The reaction mixture was stirred at 25 ºC for 3 hours. After removing the solvent, dichloromethane was added and washed with 0.1 N HCl and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum. The residue was purified by flash column chromatography on silica gel (DCM:MeOH=80:1) to give compound **5** (500 mg, 91%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (s, 2H), 7.62 (t, 1H, *J* = 7.3 Hz), 7.50 (t, 2H, *J* = 7.6 Hz), 7.42–7.35 (m, 2H), 7.30 (d, 3H, *J* = 7.7 Hz), 6.20 (s, 1H), 5.67 (d, 1H, *J* = 6.9 Hz), 5.48 (d, 1H, *J* = 27.3 Hz), 5.42 (s, 1H), 5.21 (s, 1H), 4.95 (d, 1H, *J* = 9.0 Hz), 4.33–4.16 (m, 3H), 3.90 (s, 1H), 2.81 (s, 1H), 2.69 (d, 3H, *J* = 45.7 Hz), 2.61 (s, 1H), 2.59 (d, 1H, *J* = 3.1 Hz), 2.58 (s, 1H), 2.57–2.53 (m, 1H), 2.42 (s, 2H), 2.17 (dd, 4H, *J* = 44.7, 30.0 Hz), 1.94 (s, 3H), 1.84 (d, 1H, *J* = 13.6 Hz), 1.74 (s, 3H), 1.33 (s, 9H), 1.21 (s, 3H), 1.11 (s, 3H).

HRMS: calcd for $[C_{47}H_{58}NO_{17}]^+$ $[M+H]^+$ = 908.3704; obsd: 908.3713.

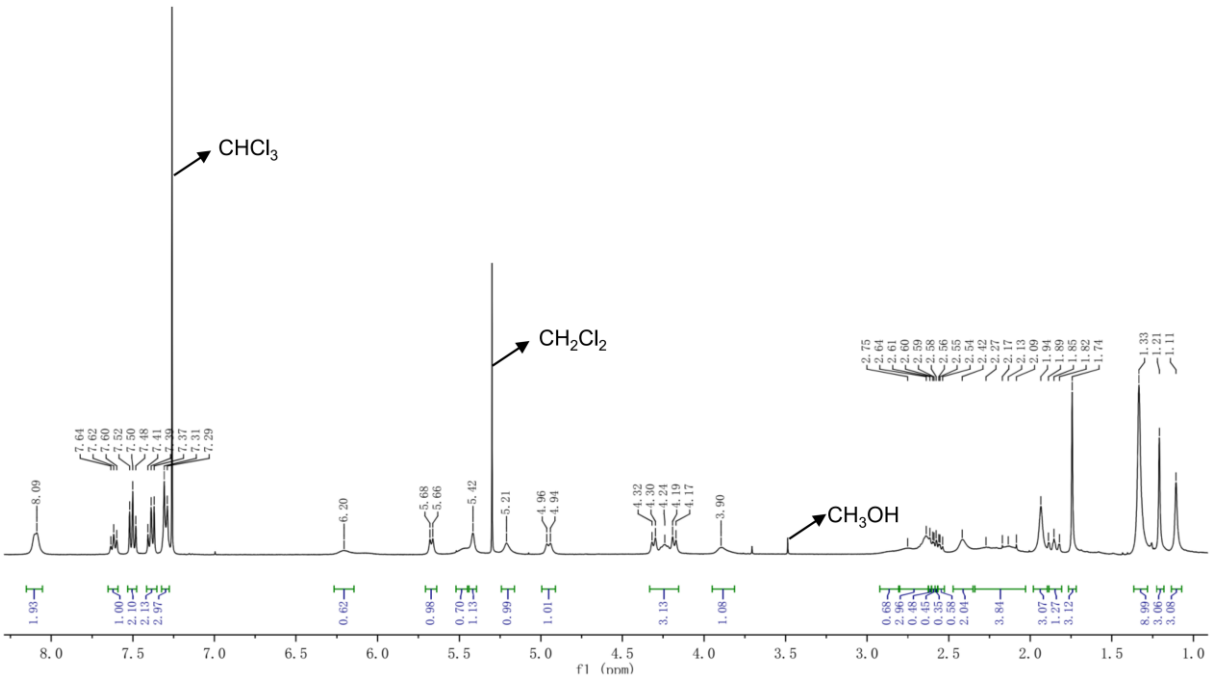


Figure S7. ^{1}H NMR spectrum of DTX derivative **5**.

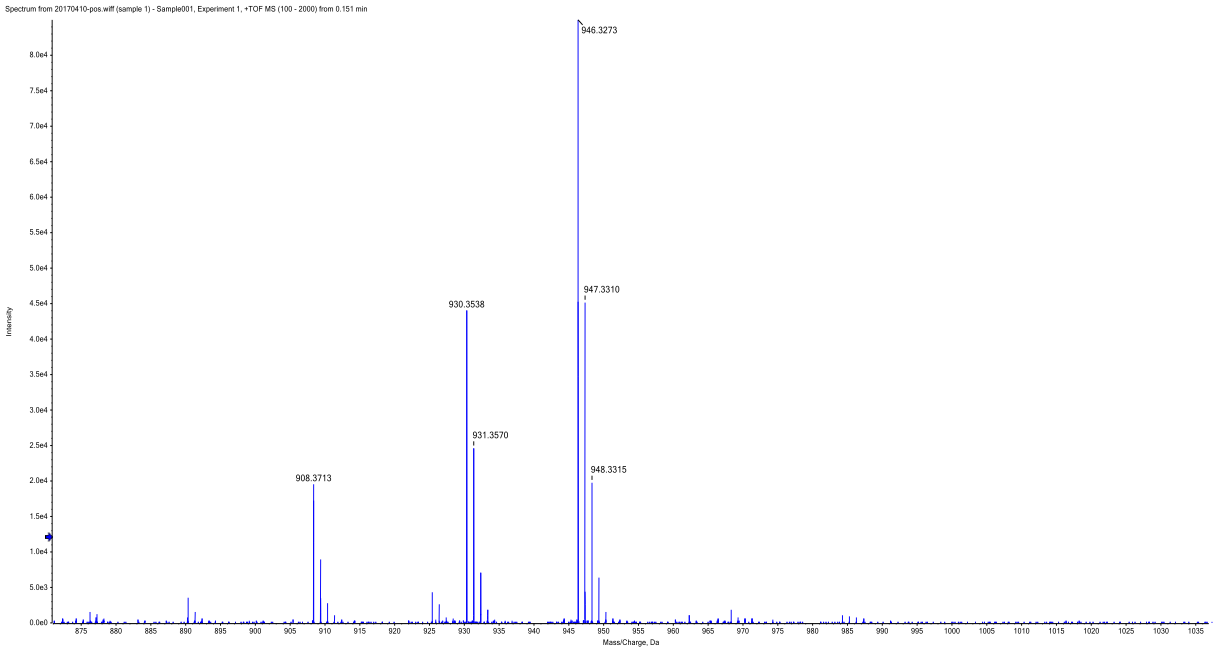
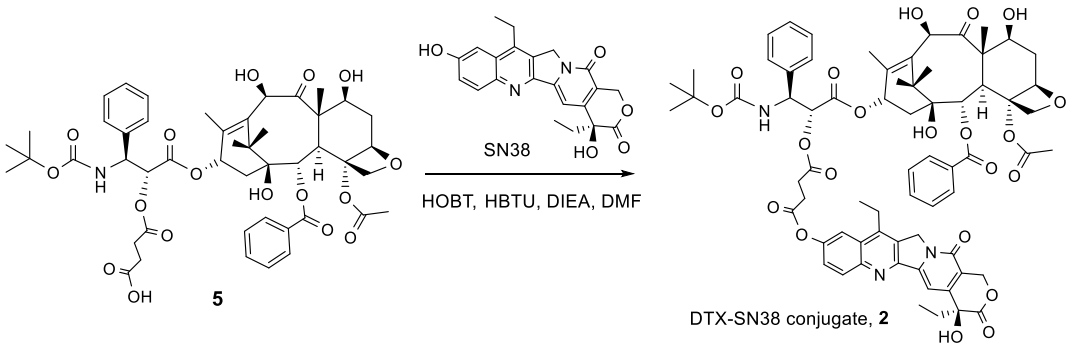


Figure S8. High-resolution mass spectrum of DTX derivative **5**.



To a solution of compound **5** (420 mg, 0.46 mmol) and SN38 (180 mg, 0.46 mmol) in 15 mL of anhydrous DMF were added HOBt (68 mg, 0.51 mmol) and HBTU (193 mg, 0.51 mmol) and DIEA (69 μL, 0.69 mmol). The reaction mixture was stirred at 25 ºC overnight. After removing the solvent, DCM was added and washed with 5% citric acid, saturated $NaHCO_3$ and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum. The residue was purified by flash column chromatography on silica gel (DCM:MeOH = 100:1) to give DTX-SN38 conjugate, compound **2** (220 mg, 37%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18 (d, 1H, *J* = 9.1 Hz), 8.10 (d, 2H, *J* = 7.4 Hz), 7.80 (d, 1H, *J* = 2.3 Hz), 7.61 (d, 2H, *J* = 5.6 Hz), 7.51 (d, 1H, *J* = 3.1 Hz), 7.49 (s, 1H), 7.48 (s, 1H), 7.37 (d, 2H, *J* = 7.4 Hz), 7.32 (s, 2H), 7.30 (s, 1H), 6.22 (s, 1H), 5.71–5.64 (m, 2H), 5.48 (s, 2H), 5.43 (s, 1H), 5.30 (d, 1H, *J* = 4.6 Hz), 5.22 (s, 2H), 5.19 (s, 1H), 4.98 (d, 1H, *J* = 9.6 Hz), 4.30

(dd, 3H, *J* = 15.1, 7.5 Hz), 4.19 (d, 1H, *J* = 8.5 Hz), 3.92 (d, 1H, *J* = 6.7 Hz), 3.12 (dd, 2H, *J* = 15.2, 7.5 Hz), 2.95 (s, 3H), 2.83 (dt, 2H, *J* = 16.6, 10.2 Hz), 2.60 (dd, 2H, *J* = 10.5, 4.3 Hz), 2.44 (s, 3H), 1.90 (dd, 6H, *J* = 13.3, 9.3 Hz), 1.75 (s, 3H), 1.37 (t, 3H, *J* = 7.6 Hz), 1.31 (s, 12H), 1.21 (s, 3H), 1.12 (s, 3H), 1.02 (t, 3H, *J* = 7.4 Hz).

^{13}C NMR (100 MHz, $CDCl_3$) δ 211.46, 173.80, 171.39, 170.54, 169.82, 168.22, 167.02, 157.61, 155.26, 151.68, 150.46, 149.43, 147.15, 146.57, 145.55, 138.81, 137.25, 135.80, 133.65, 131.80, 130.17, 130.17, 129.26, 128.94, 128.94, 128.68, 128.68, 128.28, 127.35, 127.35, 126.44, 126.44, 125.36, 118.66, 114.59, 98.43, 84.37, 81.07, 80.45, 78.80, 77.28, 75.06, 74.91, 74.46, 72.91, 72.14, 71.78, 66.19, 57.69, 53.48, 49.47, 46.50, 43.13, 31.55, 31.55, 29.16, 28.13, 28.13, 28.13, 26.36, 23.11, 22.70, 22.70, 22.70, 20.88, 14.19, 14.00, 9.99, 7.86.

HRMS: calcd for $[C_{69}H_{76}N_3O_{21}]^+$ $[M+H]^+$ = 1282.4971; obsd: 1282.4947.

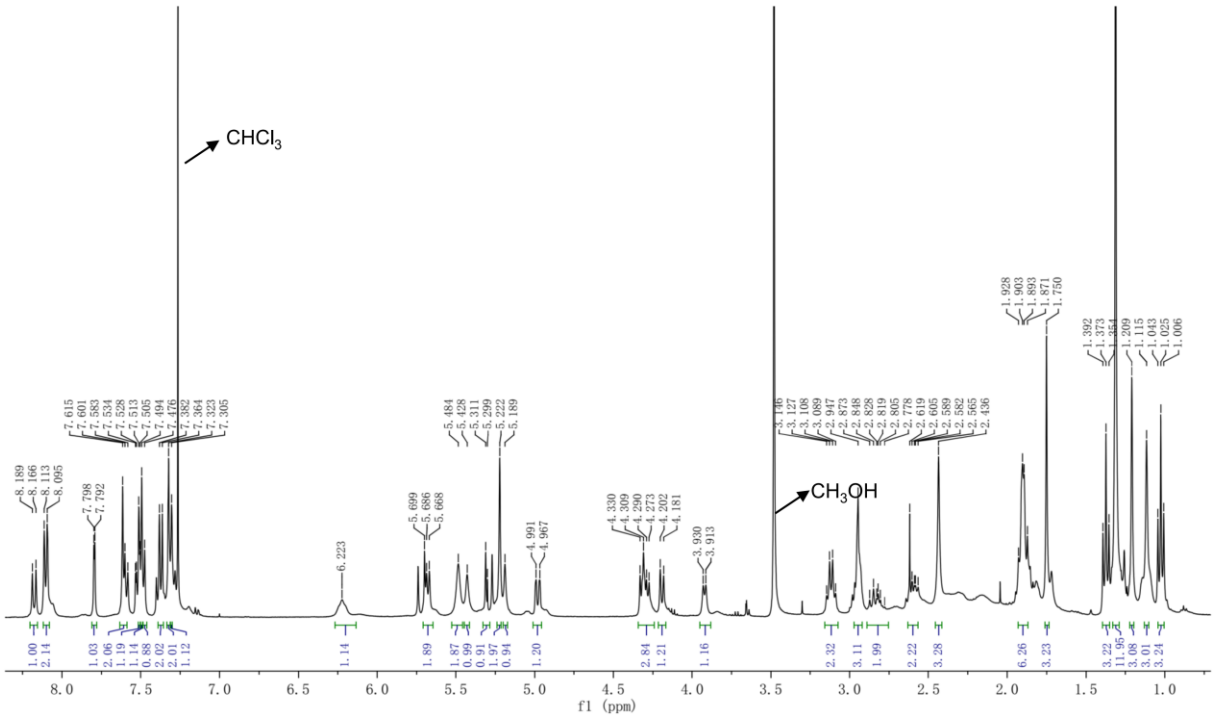


Figure S9. ^{1}H NMR spectrum of DTX-SN38 conjugate **2** in $CDCl_3$.

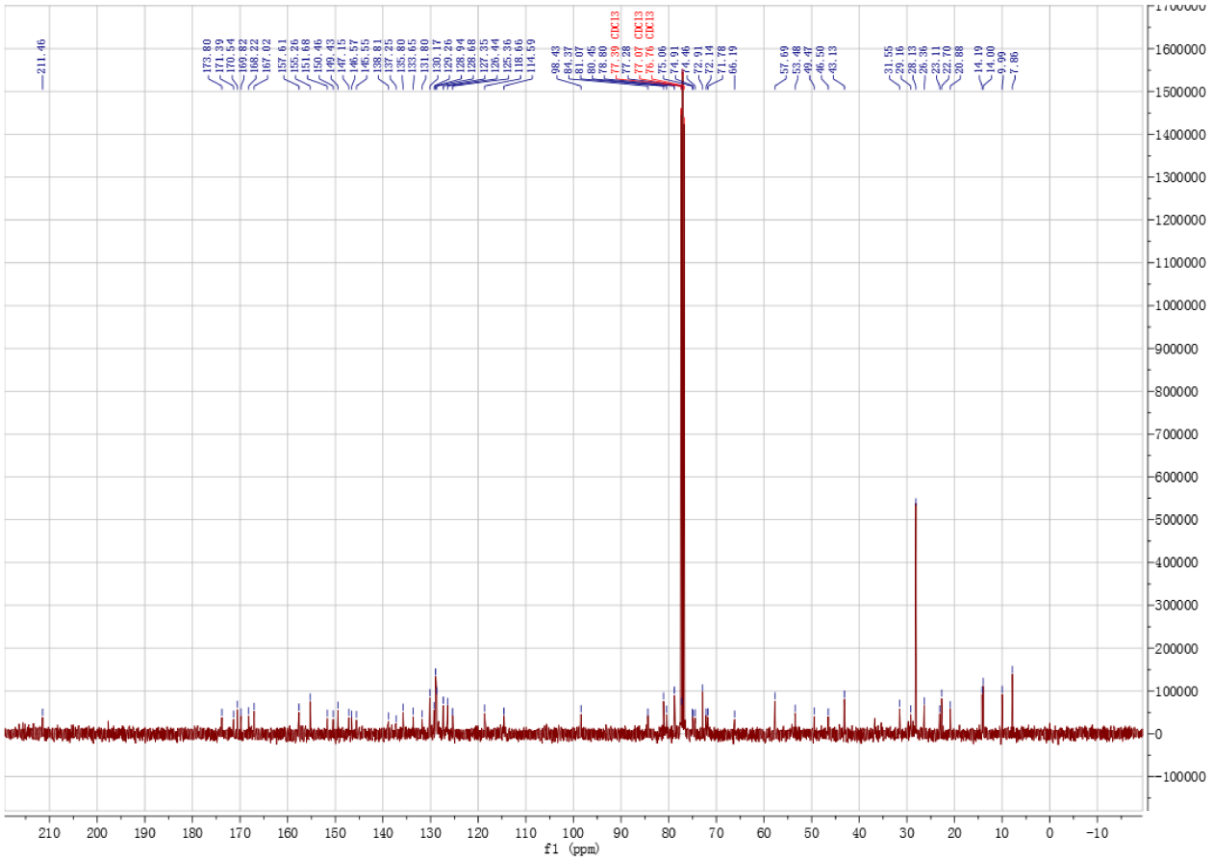


Figure S10. ^{13}C NMR spectrum of DTX-SN38 conjugate **2** in $CDCl_3$.

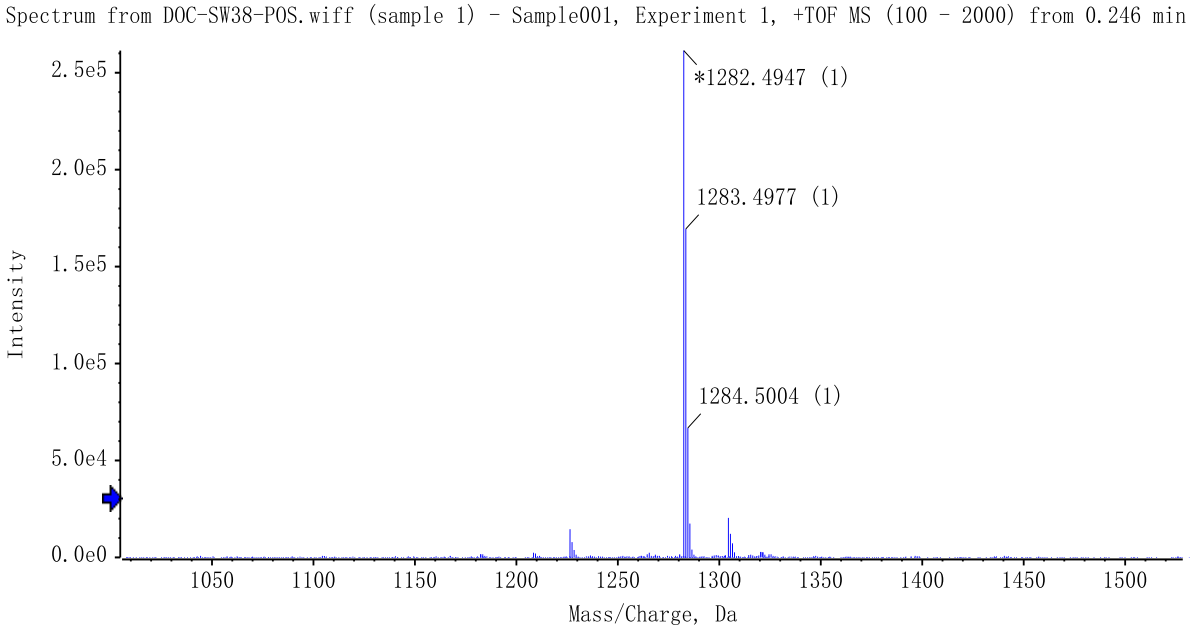


Figure S11. High-resolution mass spectrum of DTX-SN38 conjugate **2**.

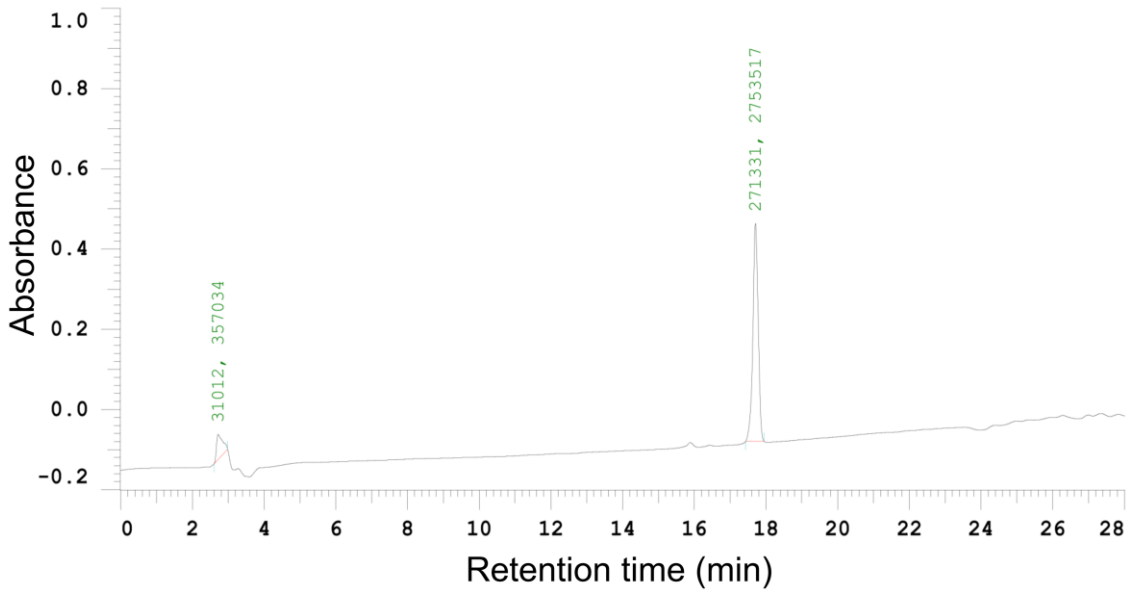
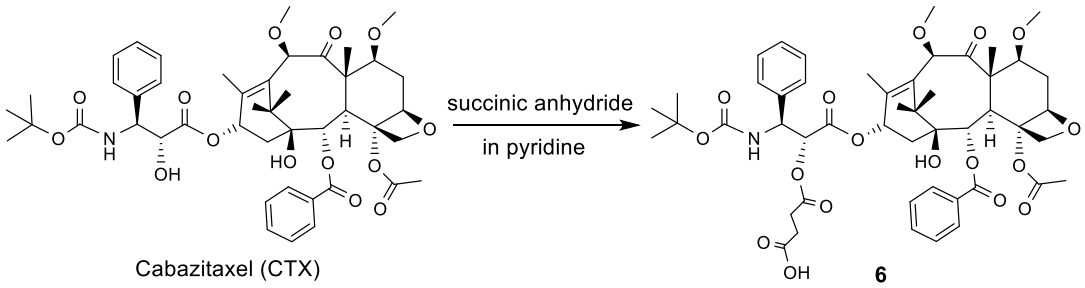


Figure S12. RP-HPLC chromatogram of DTX-SN38 conjugate **2**. The purified **2** was subjected to analytical HPLC. A gradient of 20-100% acetonitrile in water within 20 min was adopted as the mobile phase at a flow rate of 1 mL/min together with UV detection at a wavelength of 220 nm.

Synthesis of CTX-SN38 conjugate 3



To a solution of cabazitaxel (500 mg, 0.60 mmol) and succinic anhydride (180 mg, 1.80 mmol) in 8 mL of anhydrous pyridine were added DMAP (7.2 mg, 0.06 mmol). The reaction mixture was stirred at 25 ºC for 3 hours. After removing the solvent, dichloromethane was added and washed with 0.1 N HCl and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum. The residue was purified by flash column chromatography on silica gel (DCM:MeOH=80:1) to give compound **6** (549mg, 98%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, 2H, *J* = 7.0 Hz), 7.61 (t, 1H, *J* = 7.2 Hz), 7.49 (t, 2H, *J* = 7.7 Hz), 7.44–7.37 (m, 2H), 7.31 (d, 3H, *J* = 7.7 Hz), 6.23 (s, 1H), 5.64 (d, 1H, *J* = 6.8 Hz), 5.46 (s, 1H), 5.36 (s, 1H), 4.99 (d, 1H, *J* = 8.6 Hz), 4.81 (s, 1H), 4.30 (d, 1H, *J* = 8.5 Hz), 4.16

(d, 1H, *J* = 8.7 Hz), 3.89 (dd, 1H, *J* = 10.7, 6.4 Hz), 3.82 (s, 1H), 3.43 (s, 3H), 3.30 (s, 3H), 2.75–2.61 (m, 4H), 2.43 (s, 3H), 2.30 (s, 1H), 2.21 (s, 2H), 2.04 (s, 1H), 1.97 (s, 3H), 1.82–1.75 (m, 1H), 1.71 (s, 3H), 1.35 (s, 9H), 1.26 (s, 1H), 1.20 (s, 6H).

HRMS: calcd for $[C_{49}H_{62}NO_{17}]^+$ $[M+H]^+$ = 936.4017; obsd: 936.4026.

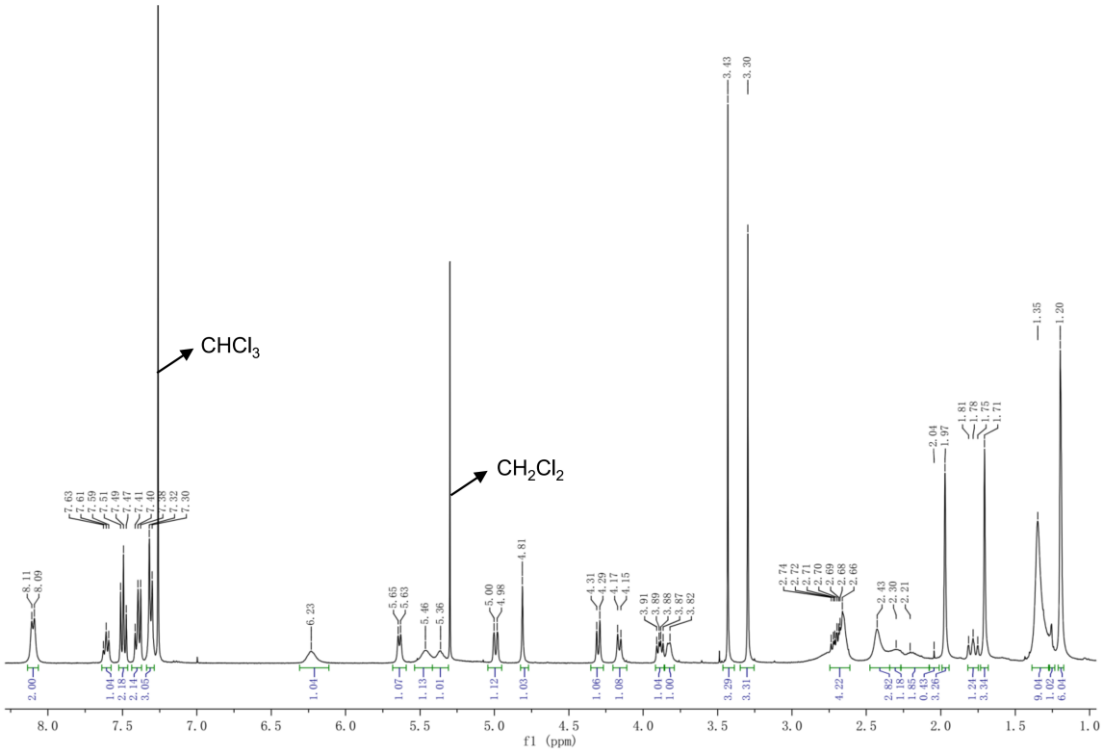


Figure S13. ^{1}H NMR spectrum of CTX derivative **6**.

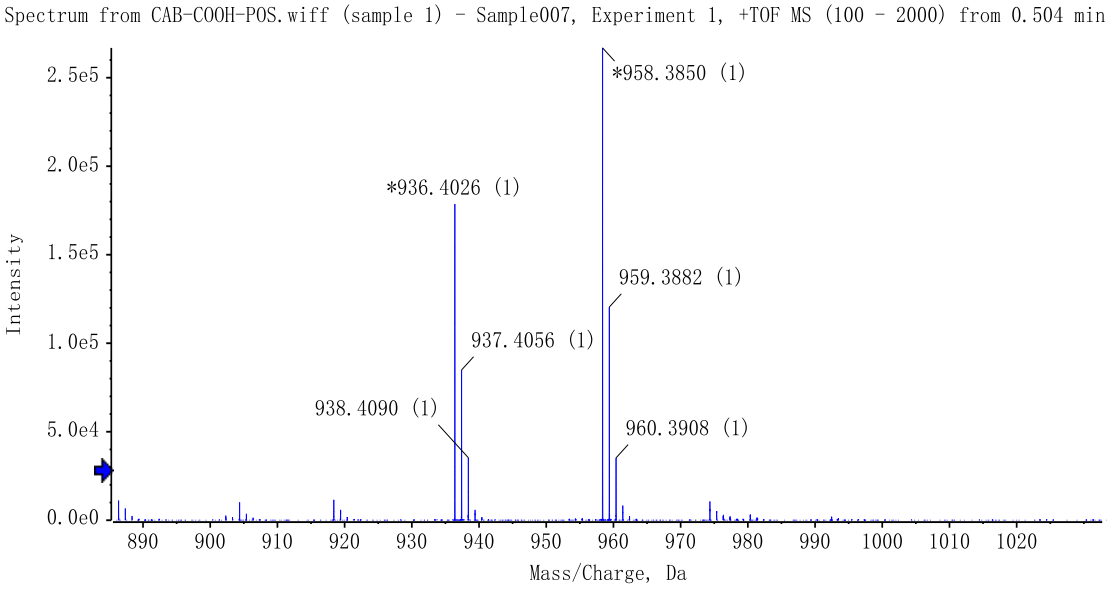
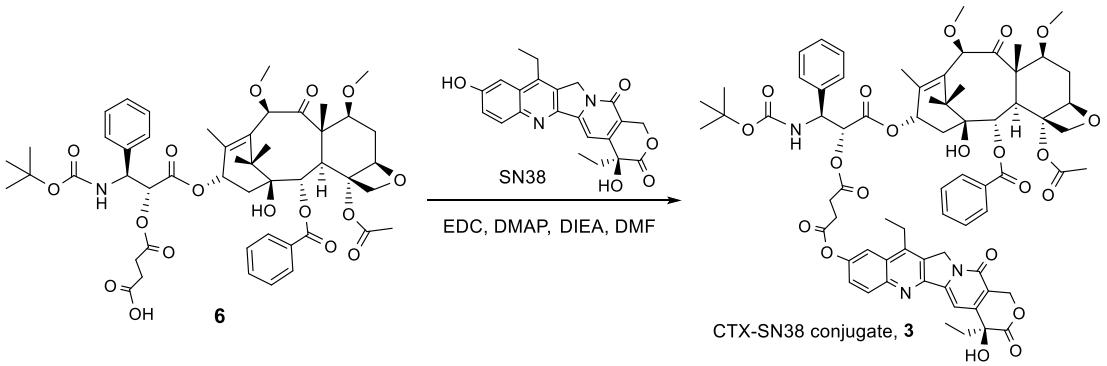


Figure S14. High-resolution mass spectrum of CTX derivative **6**.



To a solution of compound **6** (548 mg, 0.59 mmol) and SN38 (229 mg, 0.59 mmol) in 18 mL of anhydrous DMF were added EDC·HCl (125 mg, 0.65 mmol) and DMAP (79 mg, 0.65 mmol) and DIEA (146 μL, 0.89 mmol). The reaction mixture was stirred at 25 ºC overnight. After removing the solvent, DCM was added and washed with 5% citric acid, saturated $NaHCO_3$ and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum. The residue was purified by flash column chromatography on silica gel (DCM:MeOH=100:1) to give CTX-SN38 conjugate, compound **3** (321 mg, 42%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (d, 1H, *J* = 9.1 Hz), 8.11 (d, 2H, *J* = 7.4 Hz), 7.84 (d, 1H, *J* = 2.1 Hz), 7.65 (s, 1H), 7.60 (t, 1H, *J* = 7.3 Hz), 7.54 (dd, 1H, *J* = 9.2, 2.4 Hz), 7.49 (t, 2H, *J* = 7.5 Hz), 7.41–7.36 (m, 2H), 7.33–7.28 (m, 3H), 6.27 (s, 1H), 5.76 (d, 1H, *J* = 16.3 Hz), 5.65 (d, 1H, *J* = 7.1 Hz), 5.51 (s, 1H), 5.50 (s, 1H), 5.44–5.35 (m, 2H), 5.26 (s, 2H), 5.00 (d, 1H, *J* = 9.1 Hz), 4.81 (s, 1H), 4.31 (d, 1H, *J* = 8.5 Hz), 4.17 (d, 1H, *J* = 8.4 Hz), 3.90 (dd, 1H, *J* = 10.6, 6.5 Hz), 3.85 (d, 1H, *J* = 7.0 Hz), 3.74 (s, 1H), 3.42 (s, 3H), 3.30 (s, 3H), 3.15 (q, 2H, *J* = 7.6 Hz), 2.93 (dd, 4H, *J* = 17.6, 12.7 Hz), 2.86 (s, 1H), 2.71 (dd, 1H, *J* = 17.5, 12.2 Hz), 2.44 (s, 3H), 2.31 (s, 1H), 2.21 (d, 1H, *J* = 7.4 Hz), 2.00 (s, 3H), 1.90 (dt, 2H, *J* = 17.6, 7.1 Hz), 1.83–1.76 (m, 1H), 1.39 (t, 3H, *J* = 7.6 Hz), 1.33 (s, 9H), 1.25 (s, 3H), 1.21 (d, 6H, *J* = 6.3 Hz), 1.05 (t, 3H, *J* = 7.3 Hz).

^{13}C NMR (100 MHz, $CDCl_3$) δ 204.96, 173.89, 171.18, 170.37, 169.78, 168.20, 167.02, 157.64, 155.17, 152.01, 150.23, 149.43, 147.48, 146.81, 145.32, 139.33, 135.22, 133.58, 132.11, 130.16, 130.16, 129.29, 128.96, 128.96, 128.63, 128.63, 128.30, 127.41, 127.31, 127.31, 126.39, 126.39, 125.24, 118.67, 114.55, 98.12, 84.17, 82.54, 81.63, 80.69, 80.52, 78.86, 77.25, 74.86, 74.78, 72.79, 72.26, 66.33, 57.22, 57.07, 56.83, 53.44, 49.39, 47.37, 43.38, 34.99, 29.70, 28.75, 28.13, 28.13, 28.13, 26.71, 26.71, 26.71, 22.83, 22.83, 21.05, 14.45, 14.00, 10.39, 7.83.

HRMS: calcd for $[C_{71}H_{80}N_3O_{21}]^+$ $[M+H]^+$ = 1310.5284; obsd: 1310.5251.

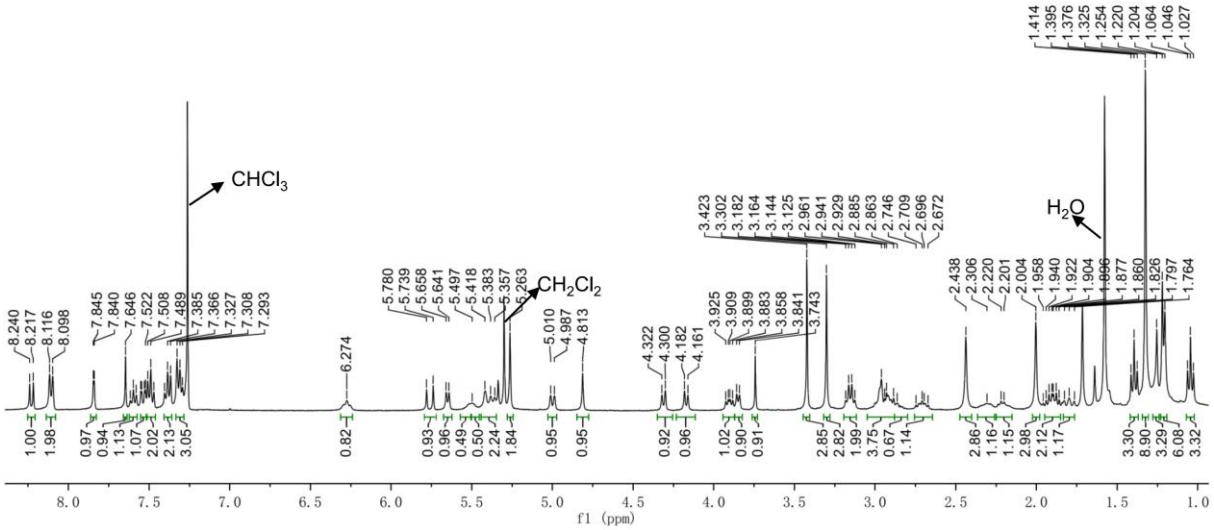


Figure S15. ^{1}H NMR spectrum of CTX-SN38 conjugate **3** in $CDCl_3$.

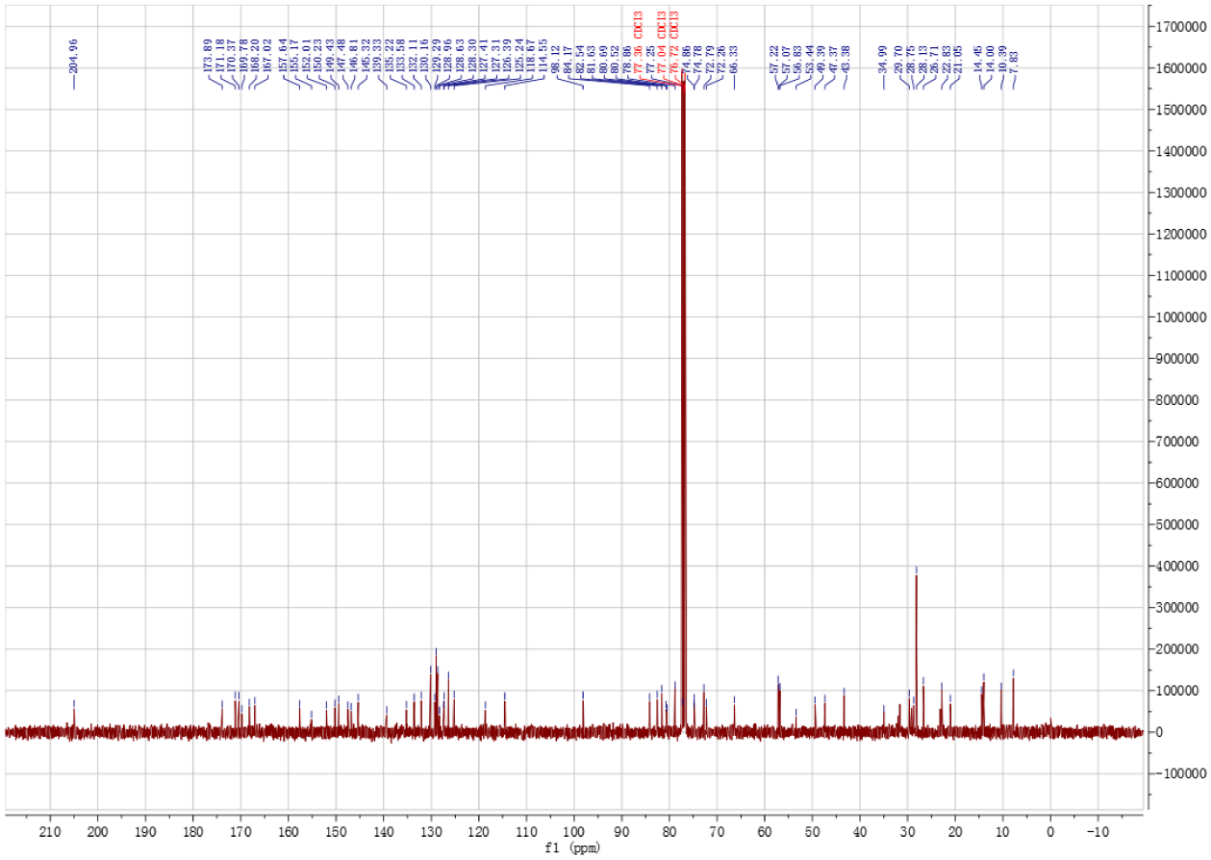


Figure S16. ^{13}C NMR spectrum of CTX-SN38 conjugate **3** in $CDCl_3$.

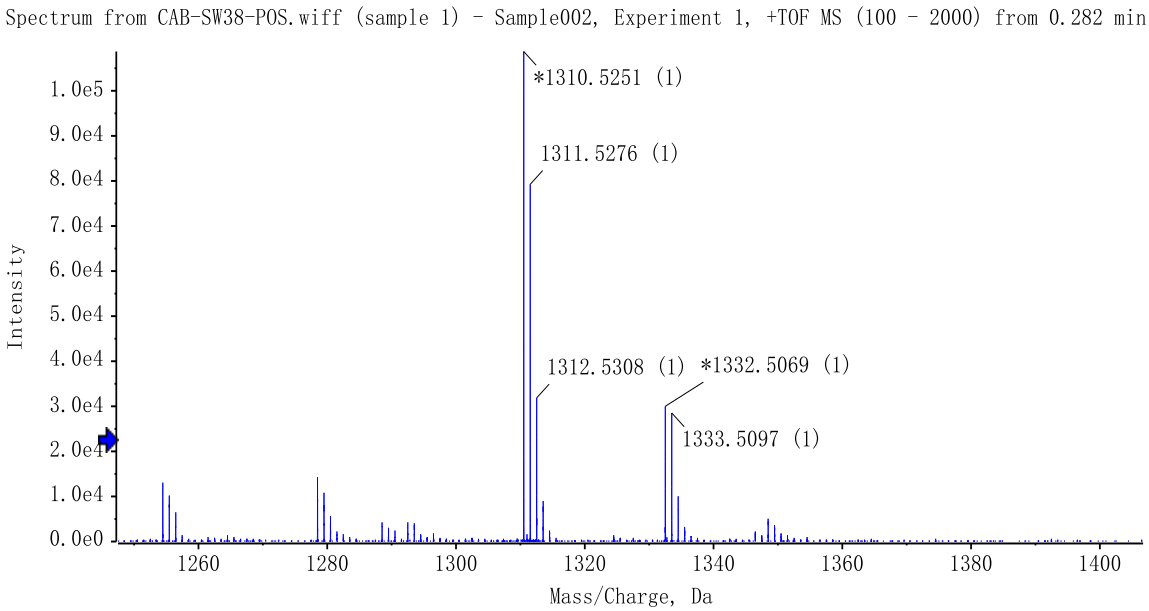


Figure S17. High-resolution mass spectrum of CTX-SN38 conjugate **3**.

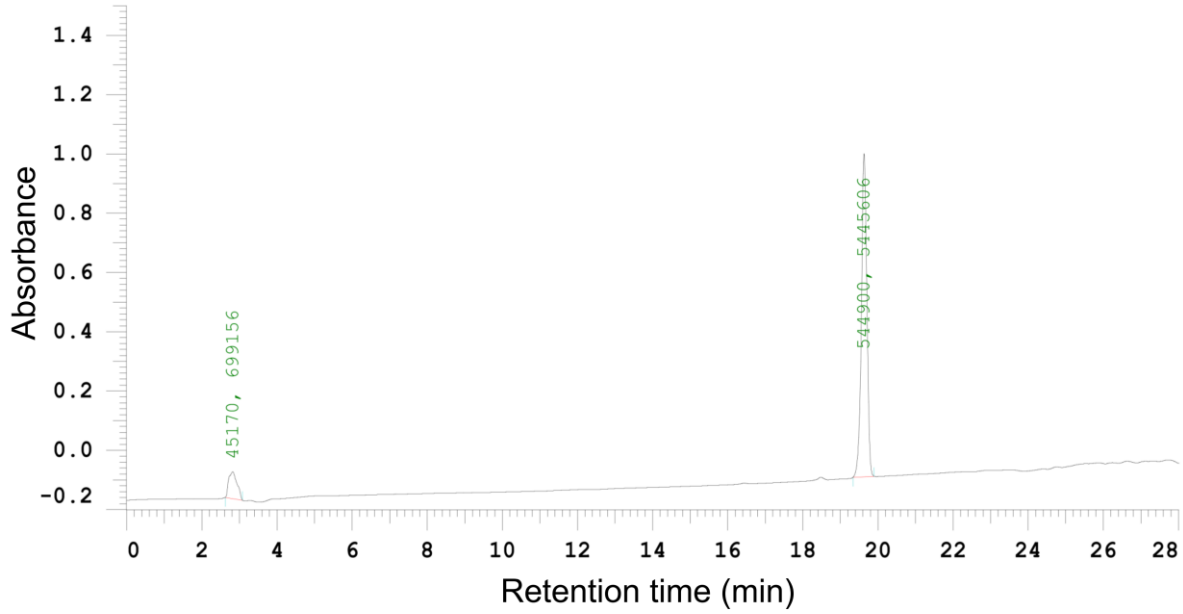
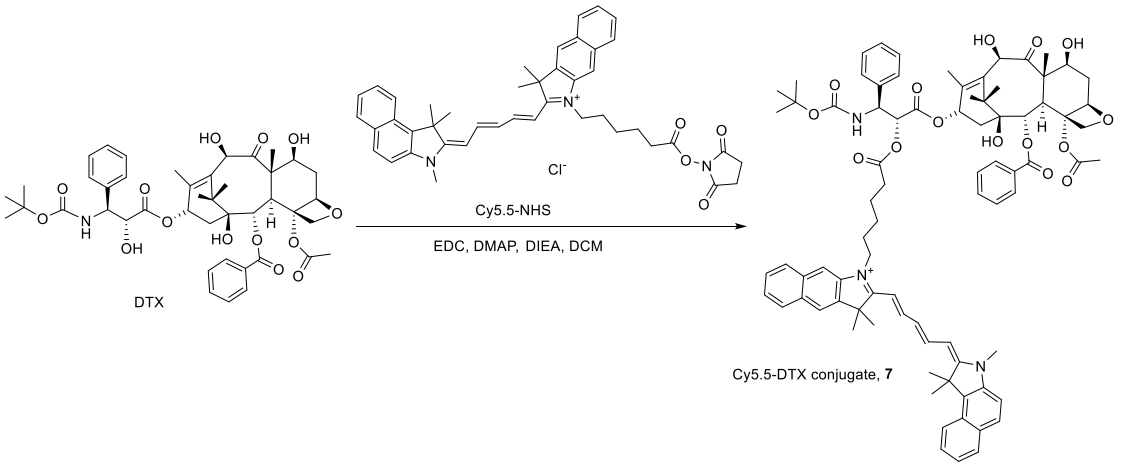


Figure S18. RP-HPLC chromatogram of CTX-SN38 conjugate **3**. The purified **3** was subjected to analytical HPLC. A gradient of 20-100% acetonitrile in water within 20 min was adopted as the mobile phase at a flow rate of 1 mL/min together with UV detection at a wavelength of 220 nm.

Synthesis of Cy5.5-DTX conjugate 7



To a solution of docetaxel (2.4 mg, 0.0023 mmol) and Cy 5.5-NHS (2 mg, 0.0023 mmol) in 1 mL of DCM were added EDC·HCl (0.48 mg, 0.0025 mmol), DMAP (0.31 mg, 0.0025 mmol) and DIEA (1.32 mg, 0.0025 mmol). The reaction mixture was stirred for 5 h. After removing the solvent, dichloromethane was added and washed with 0.1 N HCl and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum. The residue was purified by flash column chromatography on silica gel (DCM:MeOH=15:1) to give Cy 5.5-DTX conjugate **7** (3 mg, 93%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 (t, 4H, *J* = 6.7 Hz), 8.03 (s, 2H), 7.95 (t ,2H, *J* = 3.7 Hz), 7.93 (s, 1H), 7.62 (t, 3H, *J* = 7.7 Hz), 7.51 (dd, 2H, *J* = 8.1, 3.2 Hz), 7.48 (t, 2H, *J* = 5.5 Hz), 7.37 (t, 3H, *J* = 7.6 Hz), 7.31 (t, 3H, *J* = 8.8 Hz), 6.68 (s, 1H), 6.30 (d, 1H, *J* = 12.6 Hz), 6.24 (s, 1H), 6.20 (s, 1H), 5.67 (d, 1H, *J* = 7.0 Hz), 5.42 (s, 1H), 5.38–5.33 (m, 1H), 5.27 (s, 1H), 4.96 (d, 1H, *J* = 8.3 Hz), 4.31 (d, 2H, *J* = 8.2 Hz), 4.18 (d, 1H, *J* = 8.2 Hz), 4.09 (s, 2H), 3.91 (d, 1H, *J* = 6.2 Hz), 3.74 (s, 3H), 3.49 (s, 1H), 3.18–3.10 (m, 2H), 2.96 (s, 1H), 2.89 (s, 1H), 2.46 (s, 1H), 2.41 (s, 3H), 2.37–2.31 (m, 1H), 2.27–2.19 (m, 1H), 2.00 (s, 6H), 1.99 (s, 6H), 1.95 (s, 2H), 1.83 (s, 2H), 1.75 (s, 3H), 1.48 (s, 2H), 1.33 (s, 9H), 1.20 (s, 4H), 1.12 (s, 3H), 0.89 (d, 6H, *J* = 6.5 Hz).

^{13}C NMR (100 MHz, $CDCl_3$) δ 211.49, 173.81, 173.81, 172.36, 169.46, 168.52, 167.07, 152.73, 140.27, 139.51, 135.81, 133.86, 133.60, 131.82, 131.72, 130.52, 130.52, 130.16, 130.06, 130.05, 130.04, 130.04, 129.36, 128.86, 128.81, 128.81, 128.65, 128.65, 128.27, 128.17, 127.70, 127.68, 127.68, 126.78, 126.78, 126.67, 124.93, 122.27, 122.26, 122.22, 110.58, 110.42, 104.21, 84.64, 81.15, 80.20, 78.94, 77.25, 76.56, 75.16, 74.60, 74.25, 72.11, 71.19, 57.70, 53.44, 51.04, 46.59, 44.20, 43.14, 43.14, 36.89, 35.45, 33.27, 29.70, 28.21,

28.21, 28.21, 27.74, 27.74, 27.74, 27.55, 27.55, 27.55, 27.36, 26.46, 25.99, 24.34, 22.67, 22.67, 20.80, 14.57, 10.10.

HRMS: calcd for $[C_{83}H_{94}N_3O_{15}]^+$ $[M]^+$ = 1372.6679; obsd: 1372.6692.

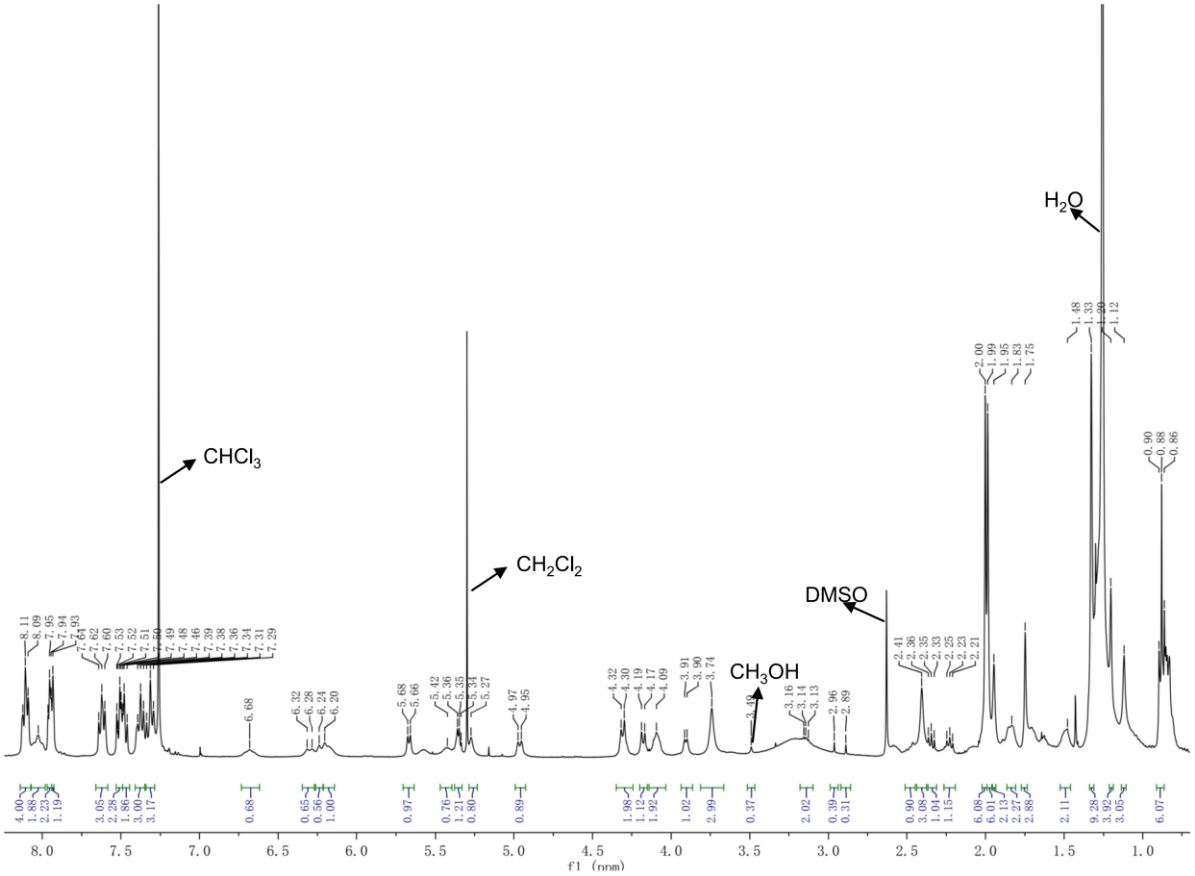


Figure S19. ^{1}H NMR spectrum of Cy5.5-DTX conjugate **7** in $CDCl_3$.

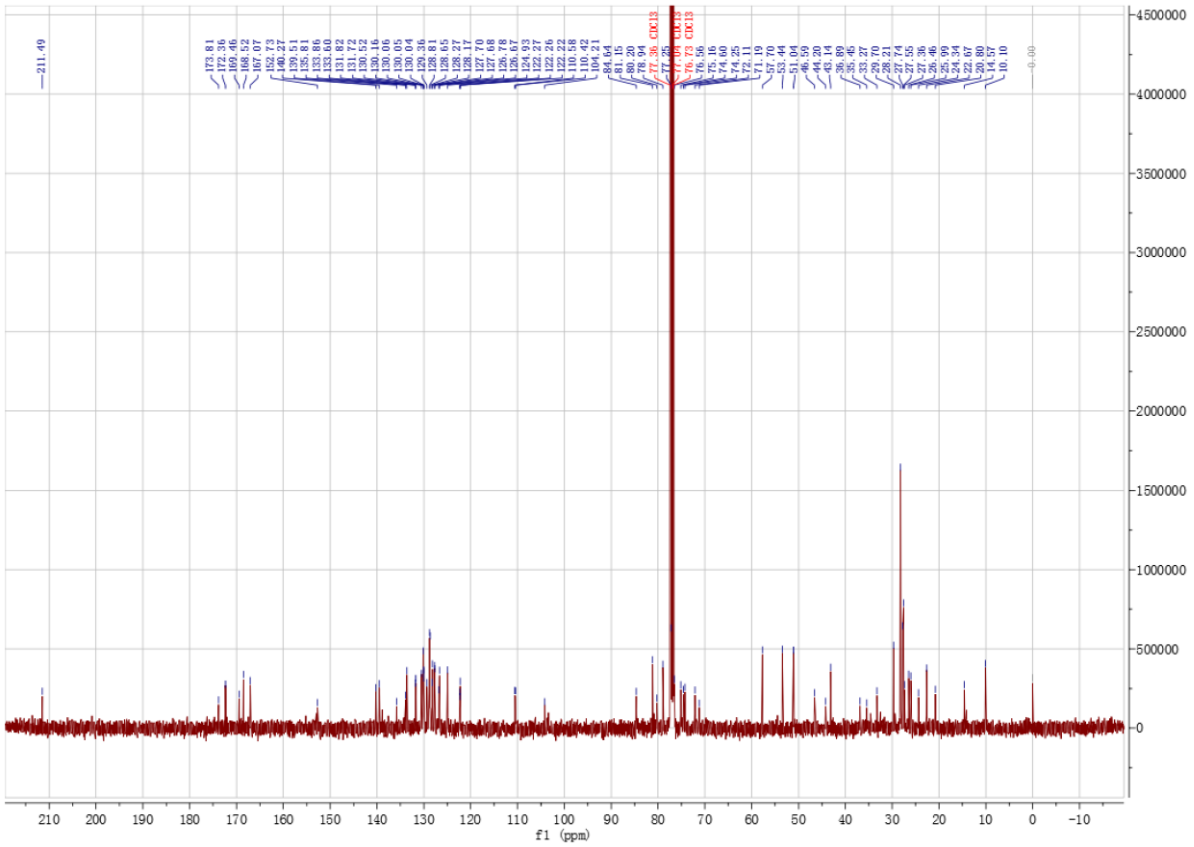


Figure S20. ^{13}C NMR spectrum of Cy5.5-DTX conjugate **7** in $CDCl_3$.

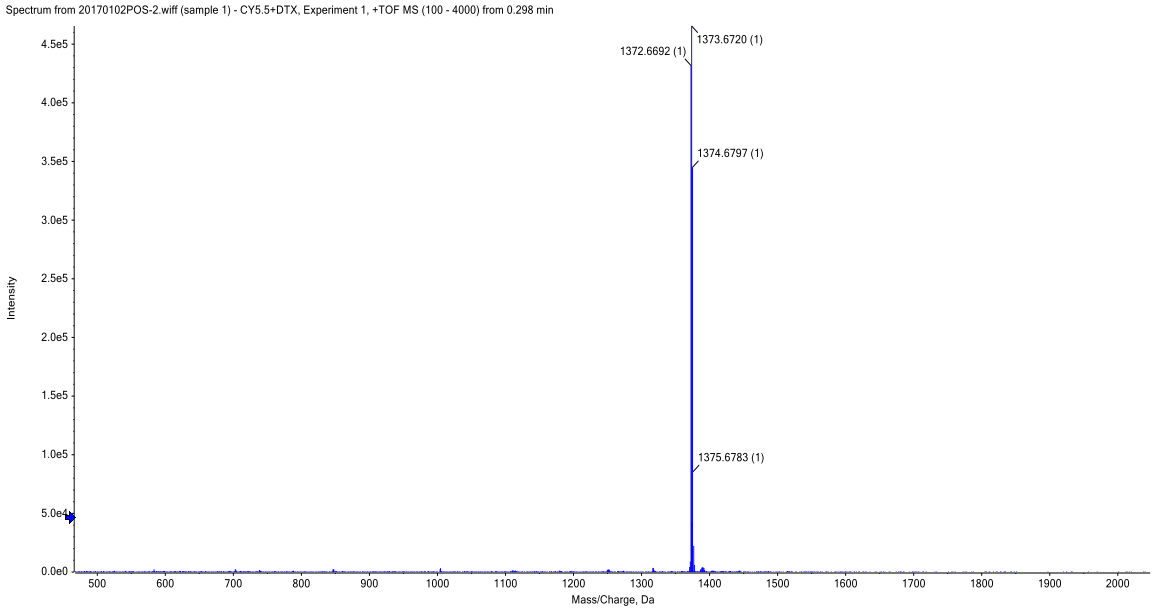


Figure S21. High-resolution mass spectrum of Cy5.5-DTX conjugate **7**.

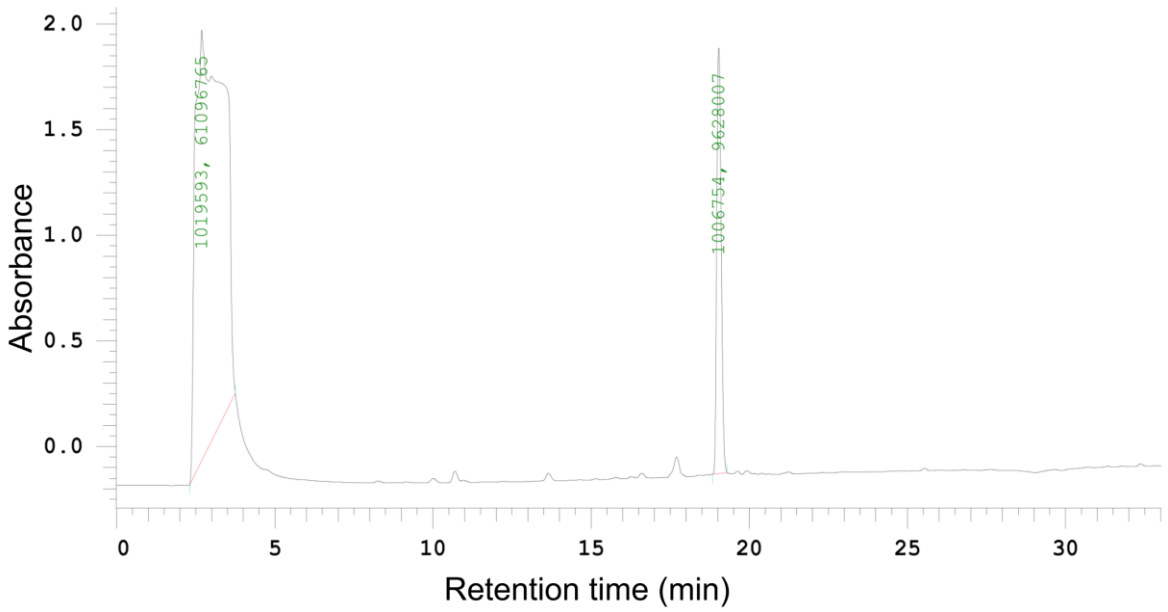


Figure S22. RP-HPLC chromatogram of Cy5.5-DTX conjugate **7**. The purified **7** was subjected to analytical HPLC. A gradient of 50-100% acetonitrile in water within 25 min was adopted as the mobile phase at a flow rate of 1 mL/min together with UV detection at a wavelength of 220 nm.

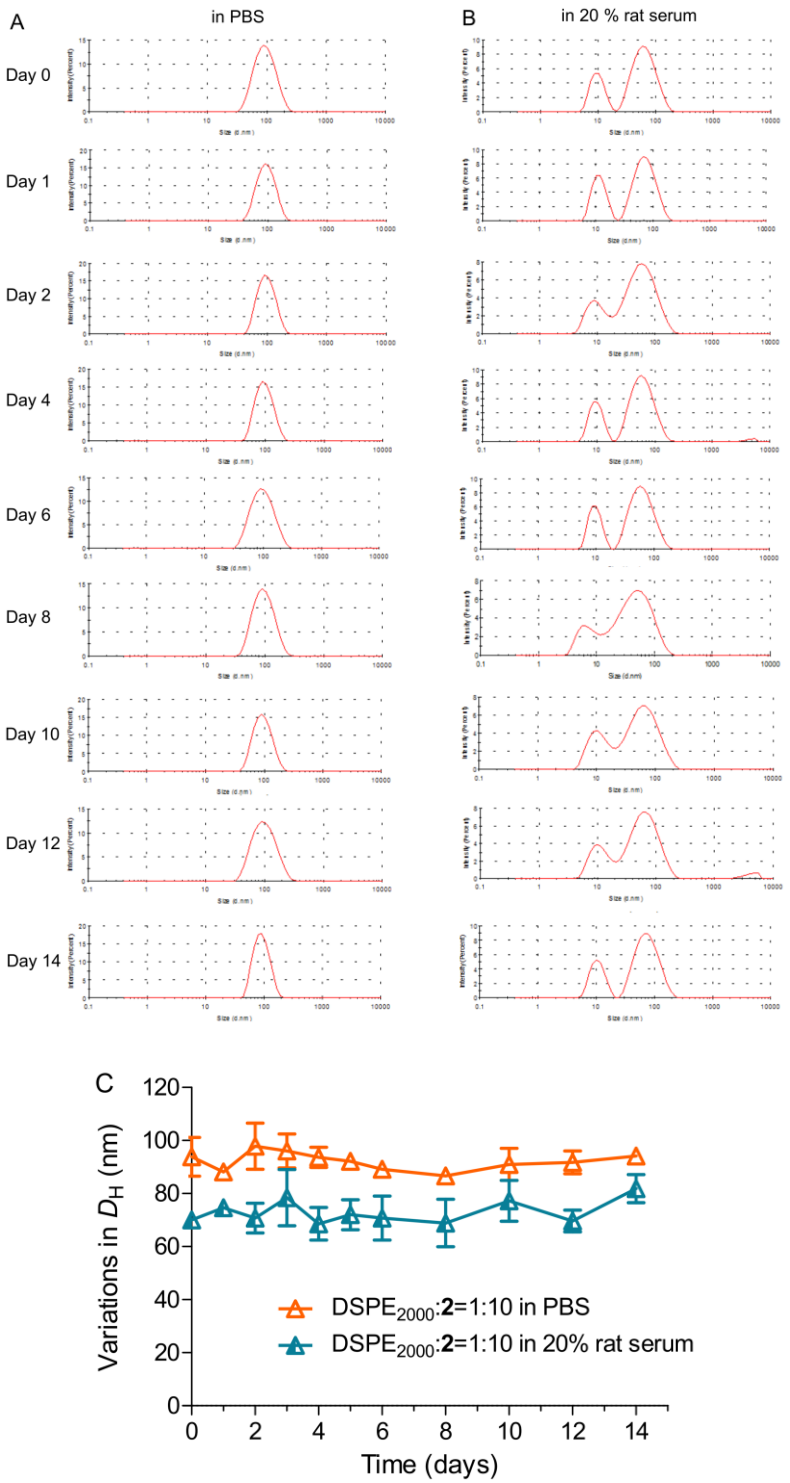


Figure S23. (**A** and **B**) hydrodynamic diameters (D_H) of **2**-NP ($DSPE_{2000}$:2=1:10) in PBS or 20% rat serum were measured by DLS analysis on day 0, 1, 2, 4, 8, 10, 12, 14. (**C**) Variations of D_H for **2**-NP in PBS and 20% rat serum over the long term incubation (at least up to 14 days) were presented.

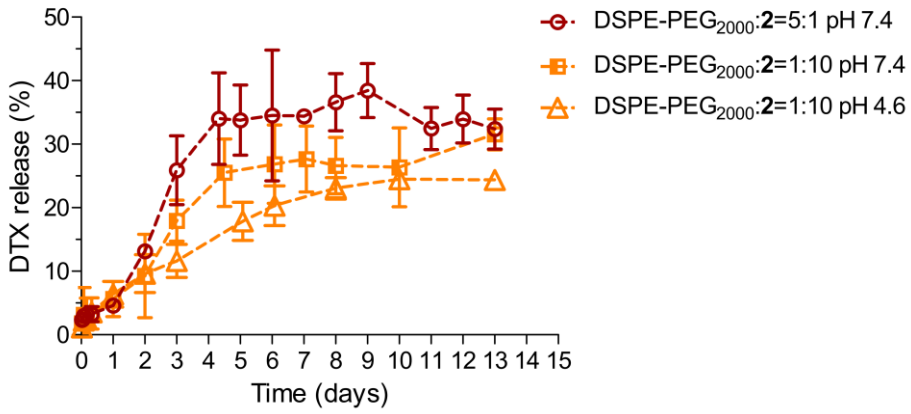


Figure S24. Drug release profiles of DTX from assembled NPs against PBS buffer. The released DTX was analyzed by HPLC.

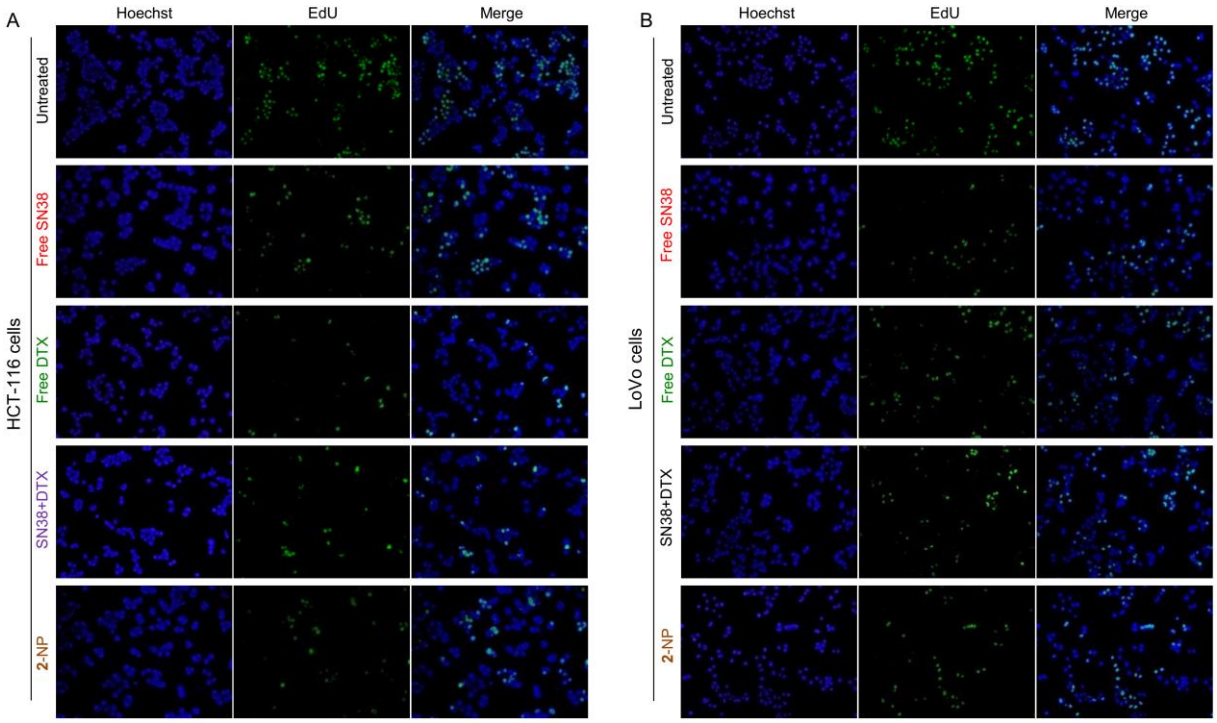
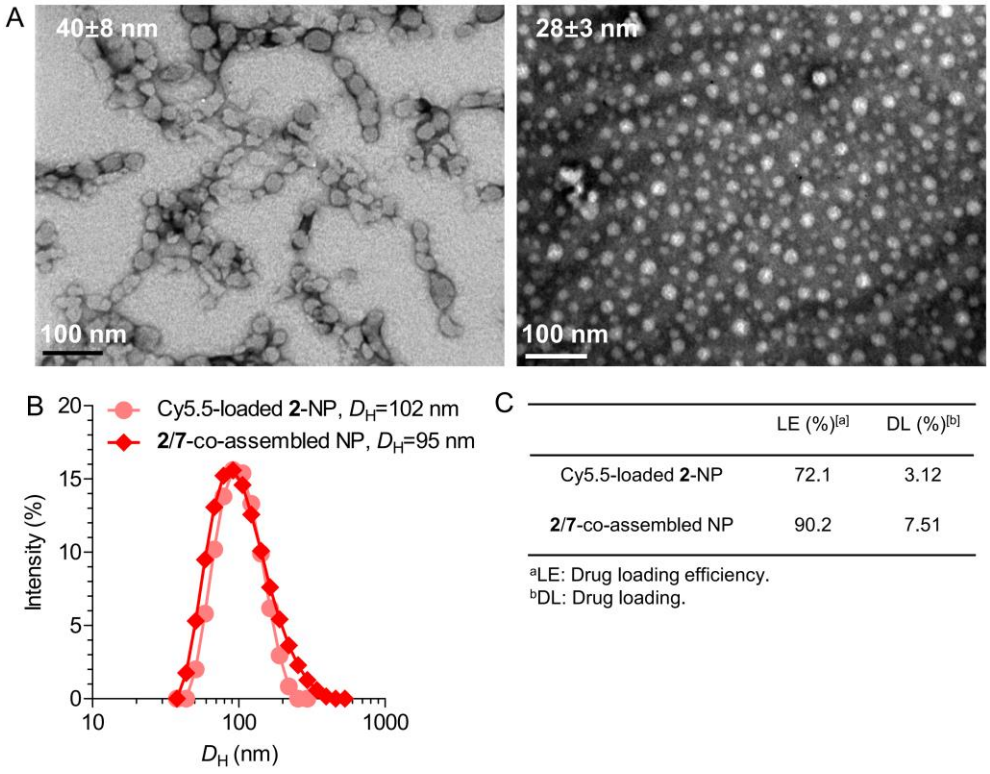


Figure S25. Effects of free SN38, free DTX, the [SN38+DTX] combination and **2**-NP on the proliferation of cancerous cells. The results of the EdU assay were analyzed by fluorescence microscopy (×200) in HCT-116 and LoVo cells incubated with various drugs (at 50 nM).



C

	LE (%)[a]	DL (%)[b]
Cy5.5-loaded **2**-NP	72.1	3.12
2/**7**-co-assembled NP	90.2	7.51

[a]LE: Drug loading efficiency.
[b]DL: Drug loading.

Figure S26. Characterization of Cy5.5-loaded **2**-NP and Cy5.5-DTX conjugate **7**-loaded **2**-NP (**2**/**7**-NP). (**A**) TEM-based morphology study of Cy5.5-loaded **2**-NP (left) and **2**/**7**-NP (right). Scale bars represent 100 nm. (**B**) Distribution of hydrodynamic diameter of Cy5.5-loaded **2**-NP and **2**/**7**-NP. (**C**) Drug loading and loading efficiency of Cy5.5 in both Cy5.5-loaded **2**-NP and **2**/**7**-NP.

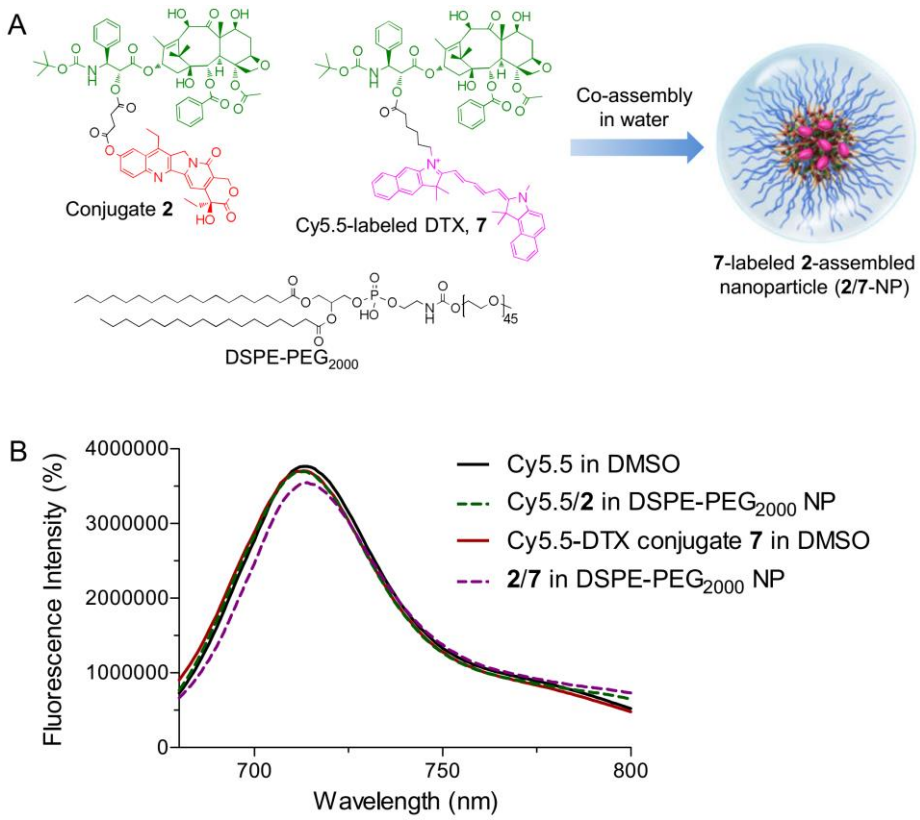


Figure S27. (**A**) Schematic illustration of the nano-assembly of **2**/**7**-NP. (**B**) The fluorescence intensity of Cy5.5 in four states: free Cy5.5 was dissolved in DMSO, Cy5.5 was incorporated into DSPE-PEG$_{2000}$ NP, Cy5.5-DTX conjugate **7** was dissolved in DMSO, or **7** was incorporated into DSPE-PEG$_{2000}$ NP.

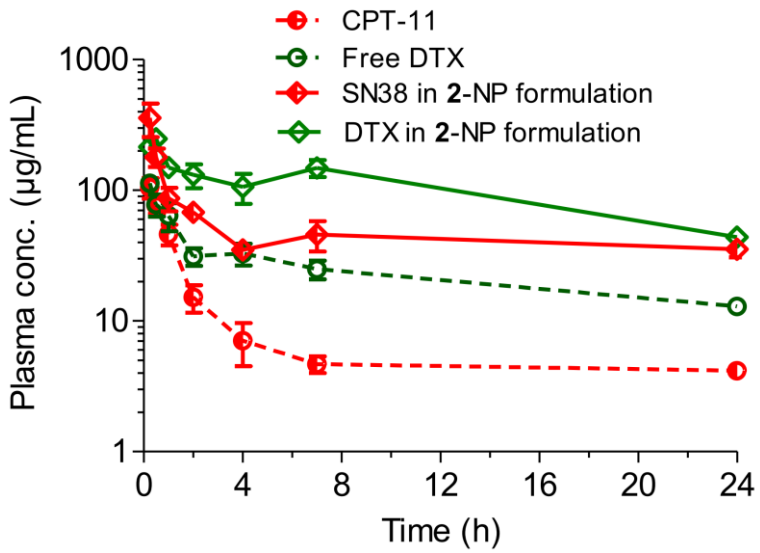


Figure S28. *In vivo* plasma concentration-time profile of CPT-11, free DTX, and **2**-NP following single intravenous injection of drug formulations. Sprague Dawley (SD) rats were injected with **2**-NP solutions at a dose of 30.6 mg/kg (SN38 equivalent dose of 10 mg/kg, DTX equivalent dose of 20.6 mg/kg). CPT-11 and free DTX were intravenously injected at doses of 15 mg/kg and 20.6 mg/kg, respectively. The total SN38 or DTX in the plasma was extracted and analyzed by HPLC using a C18 column (5 μm, 250 mm × 4.6 mm). A gradient of 20-80% acetonitrile in water within 25 min was adopted as the mobile phase at a flow rate of 1 mL/min together with UV detection at the wavelength of 220 nm for DTX and 378 nm for SN38. The data are presented as means ± SD (n = 5).

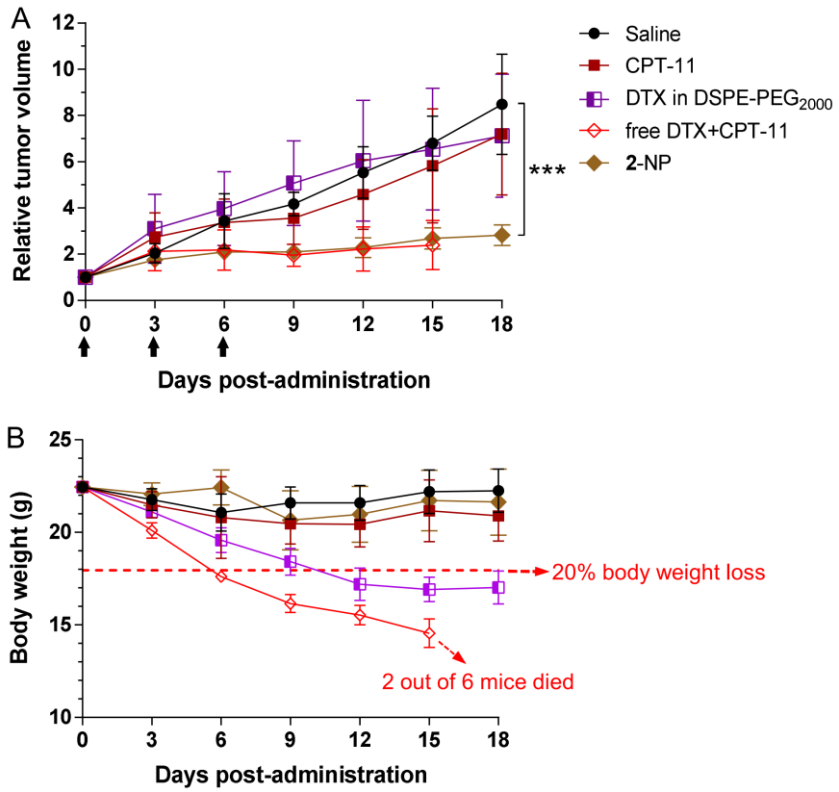


Figure S29. *In vivo* therapeutic effects of **2**-assembled NP (**2**-NP, SN38 equivalent dose of 5 mg/kg, DTX equivalent dose of 10.3 mg/kg) in comparison with controls, i.e., free CPT-11 (7.5 mg/kg), DTX formulated in DSPE-PEG_{2000} (the weight ratio of DSPE-PEG_{2000}/DTX = 20:1, DTX equivalent dose of 10.3 mg/kg), the combination of [DTX+CPT-11] (SN38 equivalent dose of 5 mg/kg, DTX equivalent dose of 10.3 mg/kg), and saline. HCT-116 tumor xenograft-bearing BALB/c nude mice (n = 6 in each group) were administered with drugs *via* IV injection at days 0, 3 and 6. (A) Tumor growth curve of different groups. (B) Body weight changes in mice receiving various treatments. Two out of six mice died in the group receiving the combination of [DTX+CPT-11] at day 15.

Table S1. In vitro combination index (CI) at different DTX/SN38 ratios for human colon HCT-116 cancer cells.

HCT-116 cell			
DTX:SN38[a]	DTX (nM)	SN38 (nM)	CI value[b]
1:1	1	1	0.71
	10	10	0.95
	100	100	0.85
	1000	1000	0.65
2:1	1	0.5	1.29
	10	5	1.64
	100	50	3.03
	1000	500	0.99
1:2	0.5	1	1.03
	5	10	1.97
	50	100	1.82
	500	1000	1.01
5:1	1	0.2	2.98
	10	2	5.35
	100	20	3.50
	1000	200	1.25
1:5	0.5	1	1.07
	5	10	1.72
	50	100	1.37
	500	1000	0.99
10:1	1	0.1	5.82
	10	1	7.16
	100	10	6.63
	1000	100	7.09
	5000	500	6.03
1:10	0.1	1	2.06
	1	10	3.41
	10	100	2.43
	100	1000	1.18

[a] Docetaxel (DTX) and SN38 were simultaneously exposed to cells for 24 h at indicated molar ratios.

[b] CI: combination index. When drug-drug interactions are synergistic, CI values are <0.9 (green); when additive, CI values are 0.9-1.1 (yellow); when antagonistic, CI values are >1.1 (red).

Table S2. In vitro combination index (CI) at different DTX/SN38 ratios for human colon LoVo cancer cells.

LoVo cell			
DTX:SN38[a]	DTX (nM)	SN38 (nM)	CI value[b]
1:1	1	1	0.37
	10	10	0.21
	100	100	0.37
	1000	1000	0.82
	5000	5000	0.73
2:1	1	0.5	0.60
	10	5	0.29
	100	50	0.42
	1000	500	2.20
	5000	2500	2.10
1:2	0.5	1	1.33
	5	10	0.27
	50	100	0.50
	500	1000	1.78
	2500	5000	1.36
1:5	1	0.2	0.49
	10	2	0.39
	100	20	0.42
	1000	200	1.69
	5000	1000	2.14
5:1	0.2	1	1.74
	2	10	0.23
	20	100	0.56
	200	1000	1.88
	1000	5000	1.42
10:1	1	0.1	0.47
	10	1	0.32
	100	10	0.44
	1000	100	2.25
	5000	500	2.49
1:10	0.1	1	3.57
	1	10	0.41
	10	100	0.86
	100	1000	1.90
	500	5000	1.60

[a] Docetaxel (DTX) and SN38 were simultaneously exposed to cells for 24 h at indicated molar ratios.

[b] CI: combination index. When drug-drug interactions are synergistic, CI values are <0.9 (green); when additive, CI values are 0.9-1.1 (yellow); when antagonistic, CI values are >1.1 (red).

Table 3. Pharmacokinetic parameters of **2**-NP solutions at a dose of 30.6 mg/kg (SN38 equivalent dose of 10 mg/kg, DTX equivalent dose of 20.6 mg/kg) as compared to free DTX that is formulated in polysorbate 80/ethanol, 1:1 (v/v) (20.6 mg/kg, DTX equivalent) and CPT-11 (15 mg/kg) *in vivo*.

PK parameter	CPT-11	Free DTX	SN38 in **2**-NP formulation	DTX in **2**-NP formulation
$t_{1/2}$ (h)	6.2	16.4	11.3	13.7
C_{max} (μg/mL)	105.0	113.1	357.5	247.0
$AUC_{(0\text{-}24\ h)}$ (μg·h/mL)	230.1	613.5	1258.8	2606.1
$AUC_{(0\text{-}inf)}$ (μg·h/mL)	267.6	920.1	1836.1	3471.2

*AUC indicates area under the concentration vs time curve; $t_{1/2}$, half-life of distribution phase; C_{max}, maximum concentration observed; The data are presented as the means ± SD (n = 5).

Characterization of prodrug-assembled nanoparticles (NPs)

Dynamic light scattering (DLS): The hydrodynamic diameters (D_H) and distribution of drug-assembled NPs (drug concentration at 0.5 mg/mL) were determined sung the Malvern Nano-ZS90 instrument (Malvern Instruments, Malvern, UK) at room temperature.

Transmission electron microscopy (TEM) analysis: TEM samples were prepared by dipping the solutions of NPs (drug concentration at 0.5 mg/mL) onto a 300-mesh copper grid coated with carbon. A few minutes after deposition, the solution was removed with filter paper, and the samples were dried at room temperature. Positive staining was performed using a 2 wt % aqueous uranyl acetate solution. TECNAL 10 (Philips) was used to obtain TEM images, operating at an acceleration voltage of 80 kV.

Quantification of drug encapsulation efficiency (EE) and drug loading (DL): After the preparation of prodrug conjugate-assembled NPs, the solutions were centrifuged at 6,000 *g* for 5 min to remove precipitates. To determine the EE values, precipitates were recovered and dissolved in DMSO, and the amounts of prodrugs in precipitates were analyzed by UV-vis spectroscopy at 378 nm. On the other hand, to quantify drug loading capacity, the supernatant was ultracentrifuged at 100,000 *g* for 30 min (Optima^{TM} L-100 XP Ultracentrifuge, Beckman) to obtain prodrug-formulated NPs, and then freeze-dried. The freeze-dried samples were weighed and dissolved in acetonitrile and sonicated for 10 min. Thereafter, the drug amounts in samples were measured by UV-vis spectroscopy at 378 nm, with pure conjugates as the standard. The EE and DL values were calculated using the following equations (1) and (2):

$$EE\ (\%) = W_{drug\ in\ NPs}/W_{initial\ drug\ added} \times 100 \quad (1)$$

$$DL\ (\%) = W_{drug}/(W_{drug} + W_{DSPE\text{-}PEG2k}) \times 100 \quad (2)$$

Stability measurement of prodrug-assembled NPs: The freshly prepared NP solutions were diluted with DI water, making the final drug concentration at 0.1 mg/mL. The NP solutions were stored at room temperature to test their stability. At pre-determined time intervals, the hydrodynamic diameters were characterized by DLS measurement.

In vitro drug release kinetics: The dialysis method was used to assess the drug release kinetics from **2**-NP against phosphate buffered saline. Briefly, 5 mL of NP solutions containing a drug concentration of 0.1 mg/mL were placed into dialysis tubes with a molecular weight cutoff of 7 kDa and dialyzed against 25 mL of PBS (0.2% Tween 80) in a thermocontrolled shaker with a stirring speed of 150 rpm at 37°C. At pre-determined time points, 2 mL of samples were withdrawn and 2 mL of fresh PBS media was supplemented.

The samples were subjected to analytical RP-HPLC using a C18 column. A gradient of 20-80% acetonitrile in water was used. For SN38 and DTX analysis, UV detection at 378 nm and 220 nm, respectively, was performed.

Cell-based assays to assess synergistic effects

Cytotoxicity assay: The cytotoxicity of various free drugs and drug formulations was measured by MTS assay. Briefly, HCT-116 and LoVo cells were seeded in a 96-well culture plate at 1×10^4 and 8×10^3 cells per well, respectively. The plates were incubated at 37°C, and cells were allowed to attach for 24 h. Fresh media containing drugs were then supplemented, and the cells were further incubated for another 24 h. The untreated cells were included as the control. At the end of the treatment, 10 μL of MTS (Promega, Madison, WI, USA) was added into each well and incubated for 30 min at 37°C. The absorbance at 490 nm was measured using a model ELX800 Micro Plate Reader (Bio-Tek Instruments, Inc, Winooski, USA) to calculate the cellular proliferation. The cell viability was calculated using the formula: cell viability = (A sample/A control) × 100%, where A represent the absorbance at 490 nm. Dose-response curves were generated from the means of three independent experiments.

Calculation of combination index (CI): To evaluate the combinational effect of DTX and SN38 in different ratios, MTS assay was performed. Briefly, HCT-116 and LoVo cells were seeded in a 96-well culture plate at 1×10^4 and 8×10^3 cells per well, respectively, and incubated at 37 °C for 24 h. Subsequently, the adherent cells were treated with single DTX or SN38 or the combination of DTX and SN38 in different molar concentration ratios (i.e., 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10, see Table S1 and S2). After 24-h incubation, the cell viability was determined by MTS assay. The cell inhibition rate was calculated by the formula: Inhibition rate = (1 - cell viability) × 100%. The cell inhibition rate of different drug ratios were input into CompuSyn software (ComboSyn Inc, Paramus, NJ, USA) and then combination indices (CIs) were calculated [1, 2]. When DTX-SN38 interactions are synergistic, CI values are <0.9 (green); when additive, CI values are 0.9-1.1 (yellow); when antagonistic, CI values are >1.1 (red). The results were shown in Table S1 and S2.

EdU assay for quantifying cell proliferation: A Click-iT® EdU Alexa Fluor® 488 Assay Kit (Invitrogen) was used to determine DNA synthesis according to the manufacturer's protocol. Briefly, cells (3×10^4 cells/well) were seeded and grown in 48-well plates and exposed to free SN38 (50 nM), free DTX (50 nM) or **2**-NP (50 nM) for 24 h. The untreated cells were included as the control. A volume of 100 μL of EdU (10 μM) was added to each well, and the

cells were incubated for an additional 4 h at 37°C. Thereafter, the cells were fixed with 4% paraformaldehyde in PBS for 30 min and permeabilized with 0.5% Triton X-100 for 20 min at room temperature. After washing with PBS, 100 μL of pre-allocated Alexa Fluor 488 staining action liquid was added to each well, and the cells were incubated for 30 min in the dark at room temperature. After removing the reaction solution, the cells were subsequently stained with 100 μL Hoechst 33342 (5 μg/mL) for 30 min. Finally, the cells were visualized under a confocal laser scanning microscope (CLSM). The cell proliferation ratio was expressed as the ratio of EdU-positive cells (green) to total Hoechst-positive cells (blue).

Flow cytometry to determine cell cycle distribution and apoptosis: HCT-116 cells were seeded into 6-well plates, incubated at 37°C, and allowed to attach for 24 h. Then, fresh media containing free SN38 (50 nM), free DTX (50 nM) or **2**-NP (50 nM) were added and further incubated for another 24 h. The untreated cells were included as the control. After drug treatment, the cells were centrifuged at 1000 rpm for 5 min and washed with cold PBS. The cells were fixed with 75% ethanol at 4°C overnight. The cells were then collected and washed twice with PBS. Thereafter, the cells were stained with a solution containing propidium iodide (PI) (50 μg/mL) and incubated in the dark for 30 min. Cell cycle distribution was then analyzed with a BD FACSCanto™ II flow cytometer.

The cell apoptotic rate was determined by flow cytometry analysis with the fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (Multi Sciences, China). HCT-116 cells were collected by trypsinization, washed twice and resuspended in 500 μL 1 × binding buffer with 5 μL of FITC Annexin V and 10 μL of PI. After incubation for 15 min, the samples were subjected to analysis by flow cytometry. The results were analyzed with the BD FACSCalibur™ system.

Western blot: For western blotting, HCT-116 cells were treated with free SN38 (50 nM), free DTX (50 nM) or **2**-NP (50 nM) for 24 h. The cells without drug treatment were used as a control. After three washes, the cells were collected and lysed with RIPA lysis buffer (25 mM Tris, pH 7.6, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% deoxycholic acid) containing 1% protease inhibitor cocktail set III (Novagen) at 4°C. After centrifugation at 12,000 rpm at 4°C for 10 min, the supernatant was collected, and the protein content in each sample was measured with the BCA Protein Assay Kit. Equal amounts of protein from each sample were resolved by 10% SDS-PAGE and electrotransferred onto an Immun-Blot polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% non-fat milk-TBS-Tween solution for 2 h at room temperature and incubated overnight with primary antibodies. The

membranes were washed and incubated with appropriate secondary antibodies for 2 h at room temperature. Specific protein bands were visualized using EZ-ECL Kit HRP. The bands were digitally photographed. GAPDH was used as an internal standard to normalize protein expression. The primary antibodies used were as follows: rabbit anti-cleaved PARP, cleaved caspase-3 and cdc25C (Cell Signaling Technology); mouse anti-p21 (Cell Signaling Technology); and rabbit anti-CDK1 (Beyotime Biotechnology).

In vivo NIR imaging of 2-assembled nanomedicine

Assembly of Cy5.5-loaded nanomedicine (2/7-NP): Cy5.5-labeled DTX (compound **7**) was first synthesized in a one-step reaction. The ^{1}H NMR and high-resolution mass spectra, as well as the synthetic details, are available in the supporting information.

To prepare Cy5.5-loaded **2**-NP, free Cy5.5 was mixed with **2** and DSPE-PEG_{2000} in DMSO at 1:10:1 (weight ratio; compound **2** was at 40 mg/mL), and this mixture (100 μL) was then rapidly injected into DI water (1 mL) under ultrasonication. To prepare **2**/**7**-NP, compound **7** was mixed with **2** and DSPE-PEG_{2000} in DMSO at 1:10:1 (weight ratio; compound **2** was at 40 mg/mL). Prior to systemic injection, the solutions were dialyzed against DI water to remove the organic solvent and the concentration of Cy5.5 in each formulation was determined by UV-vis spectroscopy (ε = 209,000 $M^{-1}cm^{-1}$ at 673 nm provided by Lumiprobe).

Nanodrug tracking and distribution in vivo by NIR imaging: HCT-116 (human colon cancer cells) tumors were generated by subcutaneous injection of tumor cells into the right flank of 5-week-old BALB/c nude mice. After 10 days, the animals were randomly separated into three experimental groups (n = 6 in each group). The mice were intravenously (IV) injected with free Cy5.5, free Cy5.5-loaded **2**-NP and Cy5.5-DTX conjugate **7**-loaded **2**-NP (20 μg per mouse at a Cy5.5-equivalent dose). *In vivo* fluorescence imaging was conducted at the indicated time points using an *in vivo* imaging system (Clairvivo OPT, SHIMADZU Corporation, Kyoto, Japan). The wavelengths used for excitation and emission for Cy5.5 were 673 and 707 nm, respectively. The mice were sacrificed at 24 h after IV injection, with their major organs (i.e., tumor, liver, heart, lung, spleen, and kidneys) collected and imaged.
In vivo pharmacokinetic properties of 2-NP and free drugs: SD rats (n = 5 for each group) were used to assess the blood circulation of **2**-NP compared with free drugs. The animals were intravenously administered with **2**-NP solutions at a dose of 30.6 mg/kg (SN38 equivalent dose of 10 mg/kg, DTX equivalent dose of 20.6 mg/kg). Free DTX that is formulated in polysorbate 80/ethanol, 1:1 (v/v) (20.6 mg/kg, DTX equivalent) and CPT-11 (15 mg/kg) were intravenously injected as references. Blood samples were collected in microtubes, and plasma was harvested at 15 min, 30 min, 1 h, 2 h, 4 h, 7 h, and 24 h post-administration. The plasma samples were stored at -20 °C until analysis. The concentrations of total drugs including free

drugs and conjugates in the plasma samples were measured by HPLC. The data were fitted to a non-compartment pharmacokinetic model.

Histological analysis: The mice in each group were sacrificed on day 8 after three injections. The tumor tissues and normal organs were fixed with 4% paraformaldehyde in PBS and embedded in paraffin before making tissue sections. Four-micrometer sections were prepared and stained with hematoxylin and eosin (H&E). The tumor tissues were also stained and processed for the terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay. The numbers of apoptotic cells in tumor tissues were quantified in three random fields for each group.

References

1. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol. Rev. 2006; 58: 621-81.
2. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul. 1984; 22: 27-55.