Supporting Information

Maksimenko et al. 10.1073/pnas.1313459110

SI Results and Discussion

The formulation of hydrophobic drugs avoiding toxic excipients represents a real pharmaceutical challenge. In this study we have shown that the conjugation of squalene to doxorubicin resulted in the spontaneous formation of nanoassemblies in water with improved anticancer activity. To generalize the "squalenoylation" platform to another hydrophobic anticancer drug, the squalenoyl-paclitaxel (SQ-PTX) prodrug was synthesized, as described in *SI Materials and Methods*.

The SQ-PTX prodrug was synthesized and formulated as nanoassemblies (NAs) in water, using simple nanoprecipitation technique without the aid of surfactants. The resulting suspension contained nanoassemblies of 240-nm mean diameter, with narrow particle size distribution (PSD < 0.2), as measured by quasielastic light scattering (QELS, nanosizer). These NA suspensions were found to be physically stable concerning particle size, at standard concentration of 2 or 5 mg/mL.

SQ-PTX NAs were then evaluated for cytotoxic activity in vitro against five human and two murine cancer cell lines (human lung cell line A549, human hormone-dependent breast cell lines MCF-7, human pancreatic carcinoma cell lines MiaPaCa-2 or Panc1, human intestinal cell line LS174-T, and murine resistant leukemia L1210-10K or wild-type leukemia L1210 cell lines) using free PTX as control. The cell viability was checked by a proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Results showing the concentrations required to inhibit cell growth by 50% (IC_{50} values) are presented in Table S8. In all cases, because of to their prodrug nature, SQ-PTX NAs showed lower activity than paclitaxel. According to the measured in vitro anticancer activity of SQ-PTX NAs, a human lung carcinoma xenograft model developed by injection of A549 cells in the flank of athymic nude mice was selected for in vivo evaluation.

The systemic toxicity of SQ-PTX NAs was first investigated, comparatively to PTX-Cremophor EL, by determining the maximum tolerated dose (MTD) after five consecutive intravenous injections into female nude mice (Fig. S8). In the PTX-Cremophor EL group, mice receiving doses up to 10 mg/kg per injection (at a PTX equivalent dose) survived, but all mice in the 20- and 30-mg/kg per injection groups died. For SQ-PTX NAs, neither mortality nor toxicity was observed at dose levels tested (Fig. S8). To further define MTD, the systemic toxicity was investigated by monitoring the body weight loss for a period of 16 d (Fig. S8). Using a 10% weight loss as a threshold value for animal health status, we therefore concluded that the MTD for PTX-Cremophor EL was 10 mg/kg per injection (PTX equivalent dose) and that the MTD of SQ-PTX NAs was more than 35 mg/kg per injection (PTX equivalent dose) (Fig. S8). To be noted that the maximum injected dose of SQ-PTX NAs was limited by the maximum concentration of nanoassemblies in the suspension and by the maximum volume able to be injected, both corresponding to a PTX equivalent dose of 35 mg/kg. Whatever the dosing protocol, SQ-PTX NAs appeared dramatically less toxic (more than three times) than PTX-Cremophor (Fig. S8).

The antitumor efficacy of SQ-PTX prodrug NAs has been then investigated on the human lung (A549) carcinoma. After tumors had grown to 80–100 mm^3, the animals were divided into four groups ($n = 8$) in such a manner as to minimize weight and tumor size differences among the groups. Based on the MTD previously determined, the following treatments and doses were administered by intravenous injections in the lateral tail vein for 5 consecutive days: (*i*) saline 0.9%, (*ii*) squalenic acid (SQCOOH) NAs 100 mg/kg, (*iii*) PTX-Cremophor EL at a PTX equivalent dose of 10 mg/kg (MTD), or (*iv*) SQ-PTX NAs at a PTX equivalent dose of 35 mg/kg. The mice were monitored regularly for changes in tumor size and weight. As indicated in Fig. S9*A*, the growth of A549 tumors were not affected by the treatment with the control squalenic acid nanoparticles (SQCOOH NAs), compared with saline-treated tumors. In contrast, the treatment with SQ-PTX NAs reduced the tumor volume by 50% at day 30 ($P < 0.01$), whereas mice treated with PTX-Cremophor EL showed tumor inhibition of 46% compared to untreated controls.

Of note, a significant weight loss was observed only in PTX-Cremophor EL–treated mice (5–10%), which was the expression of drug's toxicity.

SI Materials and Methods

Reagents. Doxorubicin and daunorubicin hydrochloride were purchased from APAC Pharmaceutical. Paclitaxel was purchased from Indena. Myocet was purchased from Cephalon Europe. Caelyx was purchased from Janssen-Cilag. Squalene, MTT, and sodium acetate were purchased from Sigma-Aldrich. Cell culture reagents were purchased from Invitrogen-Life Technologies. Tetrahydrouridine was purchased from Merck Chemicals. All solvents were purchased from Carlo Erba; TLC plates and silica gels for chromatography were purchased from Merck. Water used for the experiments was deionized and filtered through membrane filter (Milli-Q Academic). The Annexin V-FITC apoptosis assay kit and cell culture reagents were purchased from Invitrogen-Life Technologies.

Synthesis of 1,1′,2-Tris-norsqualenoyl Acid: (4e,8e,12e,16e)-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentenoic Acid. ***First step, synthesis of 2-Hydroxy-3-bromosqualene.*** To a solution of squalene (4.290 g, 10.4 mmol) in tetrahydrofuran (THF) (30 mL), H_2O (5 mL) is added and then THF drop-wise to obtain a clear solution under argon atmosphere. *N*-bromosuccinimide (2.231 g, 12.5 mmol) is added portion-wise and the reaction mixture is stirred at room temperature for 3 h. The solvent is removed under reduced pressure, and brine (100 mL) is added and extracted with ethyl acetate (5×20 mL). The organic layers are dried over anhydrous sodium sulfate and the solvent is removed under reduced pressure. The crude is purified by flash chromatography (methylene chloride: hexane 1:1) to obtain 2-hydroxy-3-bromosqualene as a pale-yellow oil (1.652 g, Yield: 31%). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ(ppm) = 5.10–5.25 (m, 5H); 4.01 (dd, $J = 11.3$ Hz, $J = 1.9$ Hz, 1H); 2.32–2.35 (m, 1H), 1.95–2.17 (m, 18H); 1.77–1.88 (m, 1H); 1.70 (s, 3H); 1.63 (bs, 15H); 1.37 (s, 3H); 1.36 (s, 3H). APCI-MS: *m/z* 507.3 $[M]^+$.
Second step, synthesis of 2,3-oxidosqualene. To a solution of 2-hydroxy-3-bromosqualene (1.652 g, 3.2 mmol) in methanol (60 mL), K_2CO_3 (0.898 g, 6.5 mmol) is added and the reaction mixture is stirred at room temperature for 2 h, then concentrated under reduced pressure. H_2O (120 mL) is added and extracted with ethyl acetate (4×30 mL). The organic layers are dried over anhydrous sodium sulfate, and the solvent is removed under reduced pressure to obtain 2,3-oxidosqualene as a yellow oil (1.360 g, Yield: 98%) without any further purification. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ(ppm) = 5.06–5.17 (m, 5H); 2.69 (t, $J = 6.1$ Hz, 1H); 1.95–2.19 (m, 20H); 1.68 (s, 3H); 1.60 (s, 3H); 1.58 (bs, 12H); 1.28 (s, 3H); 1.24 (s, 3H). APCI-MS: *m/z* 427.3 $[M+1]^+$.
Third step, synthesis of 1,1′,2-Tris-norsqualene aldehyde. To a solution of periodic acid (1.379 g, 6.0 mmol) in H_2O (5 mL), a solution of 2,3-oxidosqualene (1.434 g, 3.4 mmol) in dioxane (12 mL) is added and the reaction mixture is stirred at room temperature for 2 h. Water (150 mL) is added and extracted with ethyl acetate (3×40 mL). The organic layers are washed with brine (100 mL)

and H_2O (100 mL), then dried over anhydrous sodium sulfate and concentrated under reduced pressure to obtain 1,1′,2-Tris-norsqualene aldehyde as a pale yellow oil (1.111 g, Yield: 86%) without any further purification. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ(ppm) = 9.73 (s, 1H); 5.06–5.13 (m, 5H); 2.49 (t, *J* = 7.1 Hz, 2H); 2.30 (t, *J* = 7.1 Hz, 2H); 1.97–2.10 (m, 16H); 1.67 (s, 3H); 1.55 (bs, 15H). APCI-MS: *m/z* 385.3 $[M+1]^+$.

Fourth step, synthesis of 1,1′,2-Tris-norsqualenoyl acid: (4e,8e,12e,16e)-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentenoic acid. Sulfuric acid (11.6 mL) is added to distilled water (110 mL) under magnetic stirring; next, sodium dichromate dehydrate (6.35 g, 0.021 mol) is slowly dissolved in the previously prepared acidic solution to obtain chromic acid. All these procedures are carried out at 0 °C, using an ice-bath. 1,1′,2-Tris-norsqualene aldehyde (7.94 g, 0.021 mol) is dissolved in diethyl ether (160 mL) at 0 °C under magnetic stirring. Then, solution containing chromic acid is added to aldehyde drop-wise, and the mixture reacts for 2 h at 0 °C, monitoring product formation by TLC analysis (petroleum ether/diethyl ether/methanol, 70:23:7). The reaction mixture is washed with brine (3 × 1000 mL) until neutral pH, then dried over anhydrous sodium sulfate and evaporated under vacuo to give the crude product. The pure product is obtained by purification with flash chromatography (petroleum ether/diethyl ether, 95:5). 2.86 g (0.0071 mol) of 1,1′,2-Tris-norsqualenoyl acid are obtained (35% yield) as a colorless oil. 1H-NMR ($CDCl_3$, 300 MHz): δ(ppm)= 9.73 (s, 1H); 5.06–5.13 (m, 5H); 2.38 (t, *J* = 6.1 Hz, 2H); 2.26 (t, *J* = 8.2 Hz, 2H); 1.97–2.10 (m, 16H); 1.67 (s, 3H); 1.55 (bs, 15H). APCI-MS: *m/z* 401.33 $[M+1]^+$.

Synthesis of Doxorubicin-14-Squalenate. Trimethylorthoformate (0.20 mL, 1.83 mmol) was added to a solution of daunorubicin hydrochloride (Fig. S1) (0.20 g, 0.35 mmol) dissolved in methanol/1,4-dioxane [1:2 (vol/vol), 12 mL]. The reaction mixture was then stirred at room temperature for 20 min. To this mixture was further added a $Br_2/CHCl_3$ [1:9 (wt/vol), 0.68 mL, 0.43 mmol] solution. After stirring for 40 min at 30 °C, the resulting solution was poured into dry ether (200 mL) and the solid residue was filtered and washed with ether (50 mL × 3). The solid was recrystallized from acetone/ether [1:1 (vol/vol), 10 mL], filtered off, washed with ether, and dried over P_2O_5 to give 14-bromo-daunorubicin (0.19 g, 84%) as a red solid with melting point 176–177 °C. The 14-Bromo-daunorubicin (415.6 mg, 0.625 mmol) and 1,1′,2-Tris-norsqualenoic acid (500 mg, 1.25 mmol) were dissolved in acetone (50 mL) under inert Argon atmosphere. Potassium carbonate (260 mg, 1.875 mmol) was then added and the reaction mixture was stirred at room temperature for 48 h (dark). The solvent was evaporated and the crude product was purified by silica gel flash column chromatography (95:5, CH_2Cl_2–MeOH) to give a red powder (Fig. S1) (249.5 mg, 43%). The target compound dissolved in anhydrous THF (240 mg in 2 mL) was then converted to hydrochloride salt by adding an anhydrous, titrated 4 M solution of HCl in dioxane (1.2 eq., 0.075 mL) and stirring at −20 °C for 2 h. The solvents were then removed and the red solid product (Fig. S1) was further purified by washing with diisopropylether. The yield of doxorubicin-14 squalenate hydrochloride (SQ-Dox) was 171.9 mg (70%). The purity of SQ-Dox was checked by SiO_2 TLC eluted CH_2Cl_2: MeOH:HCOOH:H_2O (88:15:2:1, Rf 0.5) and by HPLC-MS. 1H NMR (methanol-d_4): 8.02 (d, 1H, H-3), 7.87 (d, 1H, H-1), 7.70 (t, 1H,, H-2), 5.46 (s, 1H, H-10), 5.3–5.25 (m, 2H, H-14a, H-14b) and 5.20 [s, 5H, C(sq-H)], 5.19 (s, 1H, H-7), 4.15 (q, 1H, H-50), 4.01 (s, 3H, OCH3), 3.74 (m, 2H, H-30, H-40), 3.24 (d, 1H, H-10), 3.00 (d, 1H, H-10), 2.43 (m, 1H, H-8), 2.29 and 2.35 (s 4H, CH2 SQ), 2.13 (m, 1H, H-8), 2.03 (m, 16H, CH2 SQ), 1.97 (m, 1H, H-20), 1.82 (m, 1H, H-20), 1.71 [m, 18H, C(sq)-CH3], 1.29 (d, 3H, CH3); HPLC: Waters XTerra RP-18 column eluted with water, methanol, (starting 50:50, and then after 7-min gradient up to 100% methanol, 15 min) plus formic acid 0.05%. Elution time was 28.95 min. The elution was monitored at 234 and 480 nm using a Waters 2996 Photodiode Array detector. ESI MS (Waters micromass) *m/z* calculated for $[C_{54}H_{71}NO_{12} + H]^+$: 927.14. Found 927.2 (MH+). Elemental analysis calculated: C 67.38%, H 7.54%, Cl 3.68%, N 1.46%. Found: C 67.42%, H 7.61%, Cl 3.67%, N 1.42%.

Synthesis of 2′-Trisnorsqualenoyl-Paclitaxel Ester (SQ-PTX). Paclitaxel (Indena) (1.2 g, 1.4 mmol), dissolved in 30 mL of dichloromethane (DCM), was reacted with EDAC [*N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide] (0.6 equivalent), in the presence of DMAP (4-dimethylamino pyridine) (0.2 equivalent) and 1,1′,2-trisnorsqualenoic acid (0.6 equivalent) previously dissolved in DCM at room temperature (Fig. S7). After 3 h, the reaction was stopped with water and extracted with brine. The crude mixture was purified by chromatography on SiO_2 eluted with a gradient (from 95:5–80:20) of DCM/ethyl acetate to give the 2′-trisnorsqualenoyl-paclitaxel ester (Yield: 65%). TLC control DCM/ethanol (97:3, Rf 0.55). The purity of compound was checked by HPLC on a RP-18 reverse-phase column (LiChrospher 100 RP 18e 5 μm; Merck) eluted with an acetonitrile/water mixture (40:60 and, after 5 min, gradient up to 100% acetonitrile, 20 min), elution time 19.23 min. Purity by HPLC was above 92%. Characterization: 1H-NMR (300 MHz, $CDCl_3$): 8.13 (d, 2H, C23, C27ArH), 7.75 (d, 2H, C39, C43 ArH), 7.62 (t, 1H, C25 ArH), 7.53–7.49 (band, 3H, C24, C26, C41 ArH), 7.43–7.35 (band, 7H, C33, C34, C35, C36, C37, C40, C42 ArH), 6.91 (d,1H, 4′NH), 6.35 [s, 1H, C(10)-H], 6.24 [m, 1H, C(13)-H], 5.99 [dd, 1H, C (3′)-H], 5.69 [d, 1H, C(2)-H], 5.53 [d, 1H, C(2')-H], 5.20 [m, 5H, C(SQ-H)], 4.97 [d, 1H, C(5)-H], 4.44 [m, 1H, C(7)-H], 4.33 [d, 1H, C(20)-Ha], 4.20 [d, 1H, C(20)-Hb], 3.85 [d, 1H, C (3)-H], 2.55 [m 1H, C(6)-Ha], 2.49 [s, 3H, C(29)-H], 2.45 (m, 2H, CH2-CH2-CO SQ), 2.30 (t, 2H, CH2-CH2-CO SQ), 2.23 [s, 3H, C(31)-H], 2.09 [s, 3H, C(18)-H], 2.00 (m, 16H, CH2 SQ), 1.97 [m, 1H, C(6)-Hb], 1.70 [m, 1H, C(14)-Ha], 1.67 [s, 3H, C(19)-H], 1.61 [m, 18H, C(SQ)-CH3], 1.25 [m, 1H, C(14)-Hb], 1.21 [s, 3H, C(16)-H], 1.13 [s, 3H, C(17)-H]. Electrospray ionization-MS calculated for C74H93NO15: 1235.65. Found 1237.24 (MH+). Elemental Analysis calculated: C 71.88%, H 7.58%, N 1.13%. Found: C 71.99%, H 7.63%, N 1.19%.

Preparation and Stability Studies of SQ-Dox NAs. To evaluate the optimal conditions for the preparation of stable nanoassemblies, the influence of different conditions were tested by modifying the volume of the solvent (0.12, 0.25, 0.5, 0.75, 1, and 1.5 mL THF), the volume of water (0.5, 0.75, 1, 1.5, and 2 mL) and the SQ-Dox NAs final concentration (0.5, 1, 2, 4, and 8 mg/mL). Furthermore, maintaining the SQ-Dox NAs concentration at 2 mg/mL, the ionic strength (1, 10, and 100 mM NaCl), the nature of buffer salts (acetate, citrate, phosphate, borate, and carbonate) and different pH values (4.5, 7.4, 8.0, and 10.2) were also tested (Tables S1–S4). The typical procedure for the preparation of the NAs, as described in the main text, was applied. The NA preparation was sensitive to dilution (increasing water fraction), to ionic strength but also to the type of counter ions (PBS and carbonate versus acetate or borate) (Tables S1–S4).

The stability of the NAs (0.2 mg/mL) was investigated in water and in different buffers [acetate (pH 4.5; 100 mM), citrate (pH 4.5; 100 mM), phosphate (pH 7.4; 100 mM), borate (pH 8.0; 10 mM), borate (pH 9.0; 100 mM) and carbonate (pH 10.2; 100 mM)] at 20 °C by determination of the mean particle size using a nanosizer (Table S5). The release profiles of doxorubicin from SQ-Dox NAs under different pH values (4.5, 7.4, 8.0, and 9.0) was performed by HPLC analysis as described in *Drug Release from SQ-Dox NAs* (Fig. S3). Briefly, SQ-Dox NAs (150 μM) were incubated at 37 °C in different buffers [acetate (pH 4.5; 100 mM), phosphate (pH 7.4; 100 mM), borate (pH 8.0; 10 mM) and borate (pH 9.0, 100 mM)] for 24 h. Aliquots of the incubation

medium were removed at different time intervals (0.5, 1, 2, 4, 6, 8, and 24 h) and ultracentrifuged at 10,000 × *g* during 15 min. The released doxorubicin was then quantified in the supernatant using a reverse-phase HPLC apparatus equipped with a C18 column and using trichloroacetic acid, acetonitrile, and H_2O [0.8:37:63 (wt/vol/vol), pH 3.2] as eluant solution. The eluting fractions containing doxorubicin were monitored at 480 nm using a Waters 2996 Photodiode Array detector.

Preparation of SQ-PTX NAs. A solution of SQ-PTX (1 mg) in 0.5 mL of ethanol was added drop-wise, under stirring (500 rpm) into 1 mL of 5% aqueous dextrose solution. Nanoprecipitation of the nanoassemblies occurred spontaneously. Ethanol was completely evaporated using a Rotavapor at 37 °C under vacuum to obtain an aqueous suspension of pure SQ-PTX NAs (final concentration 1 mg/mL). The mean diameter of the nanoassemblies was 240 nm, as determined at 20 °C by QELS with a nanosizer (Zêtasizer Nano ZS Malvern; Malvern Instruments). Because higher concentration of active drug was required for in vivo experiments, attempts were made to prepare SQ-PTX NA suspensions up to 5 mg/mL^{-1} by nanoprecipitation in 5% aqueous dextrose solution.

Physico-Chemical Characterization of SQ-Dox NAs. The size and the charge of the nanoassemblies were determined at 20 °C by QELS using a nanosizer (Coulter N4MD; Coulter Electronics) after 1:10 dilution in distilled water. All nanoparticle suspensions were maintained at 5 °C in the dark before use. The resulting suspensions of SQ-Dox gave very stable nanoassemblies of 130 nm in diameter with narrow PSD (PSD < 0.2). The morphology of SQ-Dox NAs was examined using transmission electron or cryogenic transmission electron microscopy (Cryo-TEM). For the observation by TEM, one drop (3 µL) of the SQ-Dox NA suspension (2 mg/mL) was deposited onto Formvar carbon-coated 400-mesh nickel electron microscope grid. Samples were stained with 1% phosphotungstic acid and observed using a Philips EM208 electron microscope. For cryo-TEM, one drop (5 µL) of the SQ-Dox NAs suspension (2 mg/mL) was deposited onto a perforated carbon film mounted on a 200-mesh electron microscopy grid. Most of the drop was removed with a blotting filter paper and the residual thin films remaining within the holes were vitrified after immersion in liquid ethane. The specimen was then transferred using liquid nitrogen to a cryo-specimen holder and observed using a JEOL FEG-2010 electron microscope.

Drug Release from SQ-Dox NAs. To determine the kinetics of doxorubicin release, 135 µL of SQ-Dox NAs (4.3 mg/mL) were incubated at 37 °C in 4 mL of PBS solution (100 mM, pH 7.4) containing (or not) 10% FCS or in 4 mL of borate solution (10 mM, pH 8.0) containing (or not) 25 U/mL esterases (SQ-Dox nanoassemblies concentration was 150 µM). Aliquots of the incubation medium were removed at different time intervals: 1, 2, 4, and 24 h for PBS solution containing (or not) 10% FCS; 30, 60, and 120 min for BSB solution containing (or not) 25 U/mL esterases and ultracentrifuged at 10,000 × *g* for 15 min. The released drug was then quantified in the supernatant using a reverse-phase HPLC apparatus equipped with a C18 column and using trichloroacetic acid, acetonitrile, and H_2O [0.8:37:63 (wt/vol/vol) pH 3.2] as eluant solution. The eluting fractions containing doxorubicin were monitored at 480 nm using a Waters 2996 Photodiode Array detector.

Cell Proliferation and Apoptosis Assays. MTT was used for cytotoxicity assays. Briefly, 2000 of MiaPaCa-2 cells per well were incubated for 24 h, in 200 µL medium containing 10% FCS in 96-well plates. The cells were then exposed for 72 h to series concentrations of SQ-Dox NAs, squalenic acid nanoassemblies, or free doxorubicin. After drug exposition, the medium was removed and 100 µL of MTT solution (0.5 mg/mL in DMEM containing 10% FCS) was added to each well. The plates were incubated for 2 h at 37 °C and 100 µL of 20% SDS solution was then added to each well for 24 h at 37 °C. Absorbance was measured at 570 nm using a plate reader (Perkin-Elmer). All experiments were set up in quadruplicate to determine the mean and the SD values.

Apoptosis induction was determined using Dead Cell Apoptosis Kit (Invitrogen), caspase-3, and poly(ADP ribose) polymerase (PARP) activity measurement. An early indicator of apoptosis is the rapid translocation and accumulation of the phospholipid phosphatidylserine from the cytoplasmic interface of the cell membrane to the extracellular surface. This event can be detected by Annexin-V staining. Thus, after 72 h of treatment with SQ-Dox NAs or free doxorubicin, cells were washed and resuspended in Annexin-V binding buffer. FITC-conjugated Annexin-V (1 µg/mL) was added to cells and incubated for 1 h at 37 °C in the dark. Propodium iodide (200 µM) was then added to the cells and incubated for 15 min at 0 °C in the dark. Analysis was done by fluorescence-flow cytometry and apoptosis data expressed as the percentage of Annexin-V and propodium iodide-positive cells. Results were the representative of three independent experiments with triplicate samples for each condition.

For caspase-3 activity measurement, cells were washed with PBS and lysed in lysis solution. The activity levels were determined using specific caspase colorimetric assay kits (Roche). PARP cleavage was evaluated by Western blot analysis as described in Western blot section, using specific monoclonal antibody.

In Vitro Anticancer Activity of SQ-PTX NAs. A549 (lung carcinoma cell line), MCF-7 (breast adenocarcinoma cell line), Panc1, and MiaPaCa-2 (human pancreatic cancer cell lines) cells were obtained from the American Type Culture Collection. LS174-T (human colon carcinoma cell line) was obtained from the LCS Collection (Germany). Murine leukemia cell line L1210 wild-type and L1210-10K were kindly provided by Lars Petter Jordheim (Université Claude Bernard Lyon I, Lyon, France). All cell lines were maintained as recommended. Briefly, A549 cells were maintained in F12-K medium. L1210 wild-type and L1210-10K cells were cultured in RPMI medium 1640. LS174-T cells were maintained in MEM medium. MiaPaCa-2 and Panc1 cells were grown in DMEM. MCF7 cells were cultured in a mixture of DMEM/Ham's F-12 (1:1) media. All media were supplemented with 10% heat-inactivated FCS (56 °C, 30 min), penicillin (100 U/mL^{-1}), streptomycin (100 $\mu g/mL^{-1}$), and L-glutamine (2 mM). Medium for MiaPaCa-2 cell line was supplemented additionally with 2.5% heat-inactivated horse serum (Gibco) (56 °C, 30 min). Cells were maintained in a humid atmosphere at 37 °C with 5% CO_2. The cytotoxic activity of SQ-PTX NAs toward these cell lines was determined using the MTT test, measuring mitochondrial dehydrogenase activity. The cells in exponential growth phase were seeded into 96-well plates (5×10^3 per well) and were preincubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. After overnight incubation, the cells were then exposed to a series of concentrations of free PTX or SQ-PTX NAs for 72 h. After drug exposure, the medium was removed and 100 µL of MTT solution (0.5 mg/mL^{-1} in DMEM containing 10% FCS) was added to each well. The plates were incubated for 2 h at 37 °C and 100 µL of 20% SDS solution was then added to each well for 24 h at 37 °C. Absorbance was measured at 570 nm using a plate reader (Metertech Σ 960, Fisher Bioblock). The percentage of surviving cells was calculated as the absorbance ratio of treated to untreated cells. The inhibitory concentration 50% (IC_{50}) of the treatments was determined from the dose–response curve. To avoid the differences in the data because of difference in cell passage number and other conditions of each individual experiment done at different times, the data plotted are an average of three different experiments performed simultaneously on the same day using different well plates.

Modified Boyden Chamber Invasion/Migration Assays. Invasion of MiaPaCa-2 tumor cells was evaluated using a Matrigel-coated modified Boyden chamber (Transwell; Sigma) according to the manufacturer's advice. Boyden chambers with 8-μm polycarbonate membranes coated with 4 mg/mL growth factor-reduced Matrigel were used for migration or invasion assay. Briefly, 3×10^4 of MiaPaCa-2 cells per well were seeded into the upper well of the chamber and incubated in 300 μL medium containing 10% FCS in 96-well plates for 24 h. The cells were treated for 72 h with free doxorubicin or SQ-Dox NAs at IC_{50} concentration (i.e., 90 nM and 180 nM, respectively). After treatment, cells on the upper surface of the well were removed. The cells on the lower surface were fixed in 3% paraformaldehyde (PFA) and stained with DAPI at 200 μM. Then, the transmigrated cells were counted using a Leica microscope at 10× magnification. For each experiment, five random high power fields were counted.

Preparation of Tumors for Histology and Immunohistochemistry Evaluations. Following the completion of a treatment schedule using free doxorubicin or SQ-Dox NAs, two mice from each group (i.e., untreated and treated) were killed, and their tumors were excised and fixed overnight in FineFix at 4 °C. The next day, the tumors were washed by placing in ethanol and toluene, and then fixed in paraffin. For immunohistochemical studies, 5-μm-sized paraffin-embedded tumor sections were cut using a microtome blade, and fixed on the glass slide for overnight at 56 °C.

Hematoxylin-Eosin-Safran Staining. The tumor sections were analyzed by optical microscopy using the Hematoxylin-Eosin-Safran (HES) staining technique. Briefly, the paraffin-embedded tumor sections were deparaffinized by dipping in toluene and ethanol, and washed with purified water. Then, the tumor sections were stained using HES by successive washings in water or ethanol and finally with toluene. Subsequently, the sections were mounted for observation under a microscope (Leica) at 100× magnification.

Proliferation Index Measurement in Tumor Cells. K_i-67 staining was performed for proliferation study. Purified mouse anti-human K_i-67 antibody (BD Pharmingen) was used for the demonstration of the K_i-67 antigen. Paraffin-embedded tumor sections prepared from the untreated or treated tumors collected from mice, and human tonsil tissue section (a positive control for K_i-67 antigen) were deparaffinized by washing the sections with toluene, ethanol, and purified water. Demasking of epitopes was performed in citrate buffer at 85 °C to 90 °C. Then, the hydrophobic markings were performed around the tumor sections. The endogenous peroxidases were blocked by incubating the tissue sections using 0.3% H_2O_2 solution. To prevent the nonselective binding of antibody, the tissue sections were incubated with horse normal serum (Vectastain). Next, the tissue sections were incubated with anti-human K_i-67 antibody for 1 h, followed by incubation with antimouse IgG biotinylated antibody. To ensure specific staining of the K_i-67^+ cells, the tissue sections were also treated separately with either the first or second antibody. The tissue sections were washed with PBS and incubated with avidin-biotin mixture for 30 min. Later, the tissue sections were incubated with diaminobenzidene in dark for 10 min, washed with PBS, colored with hematoxylin, and mounted for observation under microscope. The labeled cells were counted under 40× magnification on five different slides of each tumor sample. The significance of the difference between the treatment groups was determined by the two-tailed Student's *t* test.

Caspase-3 Index Measurement in Tumor Cells. Caspase-3 staining was performed for apoptosis study. Sections (5 μm) from tumors were deparaffinized and rehydrated. Tissues were then denatured for 4 min in a microwave oven [in a citrate buffer, 0.01 mol/L (pH 6.0)]. The sections were incubated overnight at 4 °C with an antibody to anti–active caspase-3 (Roche Diagnostics), followed by incubation with antimouse IgG biotinylated antibody. To ensure specific staining of the caspase-3^+ cells, the tissue sections were also treated separately with either first antibody or second antibody. The tissue sections were washed with PBS and incubated with avidin-biotin mixture for 30 min. Later, the tissue sections were incubated with diaminobenzidene in dark for 10 min, washed with PBS, colored with hematoxylin, and mounted for observation under microscope. The labeled cells were counted under 40× magnification, of five different slides of each tumor sample. The significance of the difference between the treatment groups was determined by the two-tailed Student's *t* test.

Antitumor Evaluation of SQ-PTX NAs in the A549 Human Lung Carcinoma Subcutaneous Model. Six- to 8-wk-old female athymic nude mice (weighing 18–20 g) were purchased from Harlan Laboratory. The systemic toxicity of SQ-PTX NAs was first investigated, compared to PTX, by determining the MTD after repeated intravenous injection into female nude mice. All drugs were administrated on 5 consecutive days by intravenous injection in the lateral tail vein (10 μL/g of body weight). Three groups of nude mice ($n = 4$) received PTX-Cremophor EL at a PTX equivalent dose of 10, 20, and 30 mg/kg and three groups of nude mice received SQ-PTX NAs at a PTX equivalent dose of 10, 20, and 35 mg/kg. It is noteworthy that the dose of SQ-PTX NAs was limited by the maximum concentration of the NAs in the suspension (5 mg/mL^{-1}) and by injection volume (10 μL/g of body weight). The body-weight change was monitored for a period of 16 d. The physical states of mice were monitored for a period of 30 d. The MTD was estimated, based on the threshold at which all animals survived with a body-weight loss less than 10%.

The antitumor efficacy of SQ-PTX NAs was further evaluated, comparatively to free PTX, on A549-bearing mice. Next, 200 μL of the A549 cell suspension, equivalent to 1×10^6 cells, were injected subcutaneously into nude mice toward the upper portion of the right flan, to develop a solid tumor model. Tumors were allowed to grow until a volume ~90 mm^3 before initiating the treatment. Tumor length and width were measured with calipers, and the tumor volume was calculated using the following equation: Tumor volume (V) = length × $width^2/2$. Tumor-bearing nude mice were randomly divided into four groups of eight each and injected for 5 consecutive days with either: (*i*) saline 0.9%, (*ii*) SQCOOH NAs 100 mg/kg, (*iii*) PTX-Cremophor EL at a PTX equivalent dose of 10 mg/kg (MTD), or (*iv*) SQ-PTX NAs at a PTX equivalent dose of 35 mg/kg by intravenous injection in the lateral tail vein. The injected volume was 10 μL/g of body weight. The mice were monitored regularly for changes in tumor size and weight.

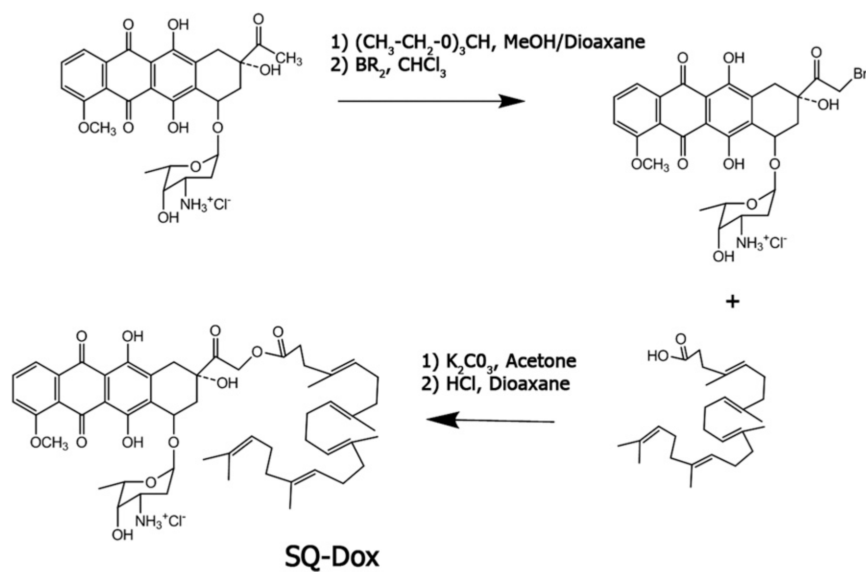

Fig. S1. Scheme of SQ-Dox bioconjugate synthesis.

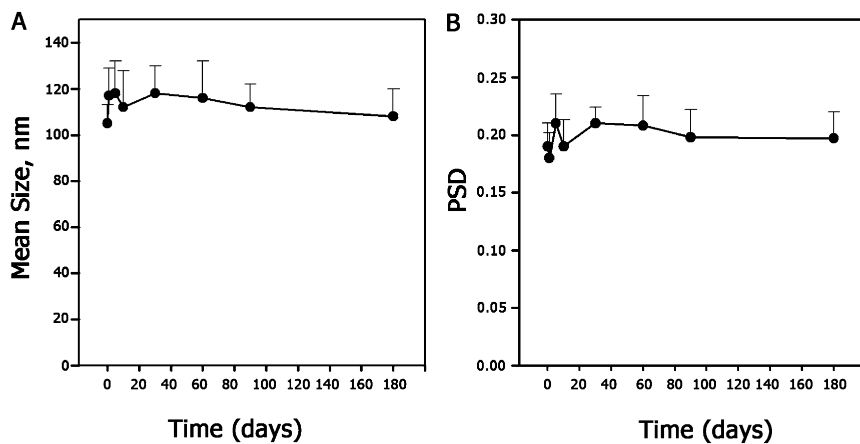

Fig. S2. Influence of storage on mean size (*A*) and PSD (*B*) of SQ-Dox NAs. Nanoassemblies were stored at refrigerator temperature for 180 d. At different time intervals (0, 1, 5, 10, 30, 60, 90, and 180 d), the average size and PSD were determined. Samples were estimated in triplicates. The values are the mean ± SD.

Fig. S3. The release profiles of doxorubicin from SQ-Dox NAs under different pH values (4.5, 7.4, 8.0, and 9.0). SQ-Dox NAs (150 μM) were incubated at 37 °C in different buffers [acetate (pH 4.5; 100 mM), phosphate (pH 7.4; 100 mM), borate (pH 8.0; 10 mM) and borate (pH 9.0, 100 mM)] for 24 h. Aliquots of the incubation medium were removed at different time intervals (0.5, 1, 2, 4, 6, 8, and 24 h) and ultracentrifuged at 10,000 × *g* for 15 min. The released doxorubicin was then quantified in the supernatant using a reverse-phase HPLC apparatus equipped with a C18 column, and using trichloroacetic acid, acetonitrile, and H_2O [0.8:37:63 (wt/vol/vol), pH 3.2] as eluant solution. The eluting fractions containing doxorubicin were monitored at 480 nm using a Waters 2996 Photodiode Array detector.

Fig. S4. MiaPaCa-2 cell-line sensitivity to SQ-Dox nanoassemblies. (*A*) MTT assay. Tumor cells were submitted for 72 h to increasing concentrations of free doxorubicin or SQ-Dox NAs. (*B*) Inhibition of cell migration and invasion by SQ-Dox NAs or free doxorubicin. Invasion of MiaPaCa-2 tumor cells was evaluated using a Matrigel coated modified Boyden chamber. MiaPaCa-2 cells were seeded into the upper well of the chamber and treated for 72 h with either free doxorubicin or SQ-Dox NAs at IC_{50} concentrations (90 nM and 180 nM, respectively). The transmigrated cells into lower well were stained with DAPI at 200 μM and analyzed by photography using a fluorescence microscopy at 10× magnification (Leica). Control: Parental untreated MiaPaCa-2 cells; SQ-Dox NAs: SQ-Dox NA-treated MiaPaCa-2 cells; Doxorubicin: doxorubicin-treated MiaPaCa-2 cells. (*C*) The migration or invasion cells were counted in five random fields of vision. Values were the number of cells relative to that of parental MiaPaCa-2 cells, and expressed as mean ± SD for three independent experiments ($*P < 0.05$, $**P < 0.01$).

Fig. S5. Toxicological data obtained after 11-wk treatment of spontaneously hypertensive (SH) rats by saline 0.9%, free doxorubicin (1 mg·kg·wk) or SQ-Dox NAs (1 mg·kg·wk or 2 mg·kg·wk, equivalent doxorubicin). (*A*) Survival curve. (*B*) Weekly food consumption. (*C*) Body weight. (*D*) Heart weight. For more experimental details, see *Materials and Methods*.

Fig. S6. (*A*) Immunohistochemical staining of tumor tissues derived from injected murine lung M109 cancer cells. Paraffin sections from tumor biopsies treated by SQ-Dox NAs (5 × 15 mg/kg, equivalent doxorubicin, MTD) (*A*), free doxorubicin (5 × 3 mg/kg, MTD) (*B*), SQCOOH NAs (5 × 100 mg/kg) (*C*), and saline 0.9% (*D*) were submitted to HES staining and imaged by microscopy (Leica) at 10× magnification. For more experimental details, see *SI Materials and Methods*.

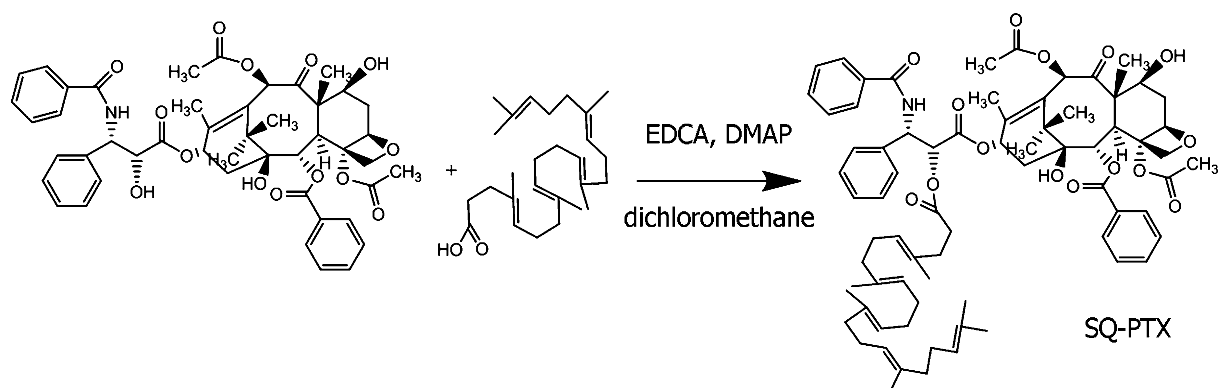

Fig. S7. Scheme of SQ-PTX bioconjugate synthesis.

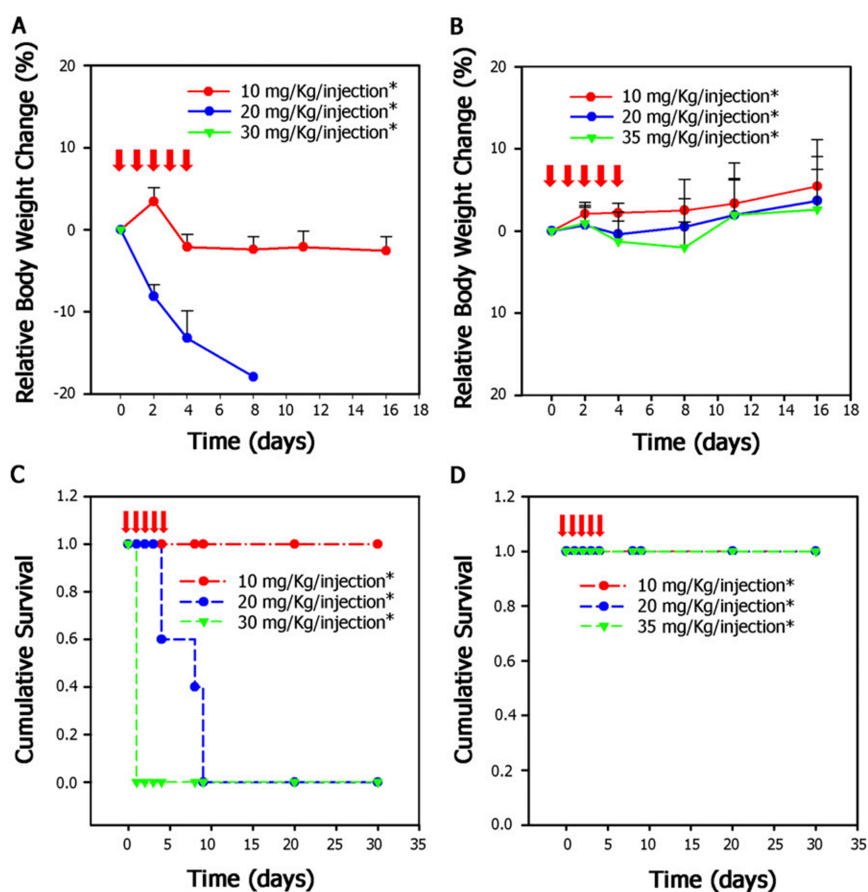

Fig. S8. Body-weight changes (*A* and *B*) and survival rate (*C* and *D*) observed after intravenous administration of PTX-Cremophor EL (*A* and *C*) and SQ-PTX nanoassemblies (*B* and *D*) into female nude mice. The mice were injected for 5 consecutative days. For more experimental details, see *SI Materials and Methods*. *PTX equivalent dose. Red arrows correspond to the days of treatment.

Fig. S9. Tumor growth inhibition by SQ-PTX NAs of mice bearing human lung (A549) tumor. Tumor volume (*A*) and body weight (*B*) were regularly measured during the experimental period. The mice were treated for 5 consecutative days with PTX-Cremophor EL (10 mg/kg per injection equivalent PTX, MTD), SQ-PTX NAs (35 mg/kg per injection equivalent PTX), saline (NaCl 0.9%), or SQCOOH NAs (100 mg/kg per injection) ($n = 8$, $^{**}P < 0.01$). For more experimental details, see *SI Materials and Methods*. Red arrows correspond to the days of treatment.

Table S1. Formulation studies for SQ-Dox NA preparation: Size measurement of NAs prepared modifying the volume of solvent (THF) and water

Volume (mL)	Size (nm)
THF	
0.12	130.3 ± 7.30
0.25	158.0 ± 4.10
0.50	138.3 ± 7.90
0.75	142.8 ± 12.5
1.00	133.7 ± 3.20
1.50	130.3 ± 7.30
Water	
0.50	120.9 ± 3.60
0.75	154.6 ± 11.8
1.00	135.4 ± 5.90
1.50	204.8 ± 10.5
2.00	304.7 ± 13.5

Table S2. Formulation studies for SQ-Dox NA preparation: Size measurement of NAs prepared modifying the final SQ-Dox NAs concentration

Final concentration of SQ-Dox NA (mg/mL)	Size (nm)
0.5	288.1 ± 15.6
1.0	154.3 ± 8.10
2.0	132.2 ± 5.20
4.0	129.7 ± 2.30
8.0	149.6 ± 3.50

Table S3. Formulation studies for SQ-Dox NA preparation: Size measurement of NAs prepared modifying the ionic strength

NaCl (mM)	Size (nm)
1	119 ± 6
10	155 ± 2
100	455 ± 25

Table S4. Formulation studies for SQ-Dox NA preparation: Size measurement of NAs prepared using different buffers instead of water

Buffer	1 mM	10 mM	100 mM
Acetate pH 4.5	102.8 ± 5.60	125.3 ± 8.10	254.3 ± 12.6
Citrate pH 4.5	142.3 ± 9.60	322.8 ± 29.7	Precipitation
Phosphate pH 7.4	123.9 ± 2.30	141.3 ± 8.20	321.5 ± 11.4
Carbonate pH 10.2	126.7 ± 7.20	680.5 ± 18.9	Precipitation
Borate pH 10.2	720.5 ± 21.6	Precipitation	Precipitation

Table S5. SQ-Dox NA stability (as size measure) after incubation in different buffers

Buffer	0 h	24 h	48 h
Acetate pH 4.5*	133.8 ± 5.60	141.0 ± 9.10	211.0 ± 11.1
Citrate pH 4.5*	380.4 ± 19.1	Precipitation	
Phosphate pH 7.4*	115.2 ± 5.10	151.3 ± 6.40	523.0 ± 21.2
Borate pH 8.0[†]	112.5 ± 7.18	132.4 ± 10.6	257.0 ± 11.2
Borate pH 9.0*	220.0 ± 11.6	212.5 ± 8.60	470.1 ± 11.7
Carbonate pH 10.2*	526.3 ± 23.2	Precipitation	
Water	102.8 ± 3.80	125.3 ± 8.10	120.9 ± 8.70

*100 mM.
[†]10 mM.

Table S6. Maximum tolerated dose of free doxorubicin, Myocet, Caelyx, and SQ-Dox NAs after single (1×) or repeated injections (5×)

	Mouse Mmodel	
Treatment	CD2F1	Nude (nu/nu)
Doxorubicin (mg/kg i.v. 1×)	6	8
Doxorubicin (mg/kg i.v. 5×)	3 (15*)	3 (15*)
SQ-Dox NAs (Dox-eq. mg/kg i.v. 1×)	> 20	> 20
SQ-Dox NAs (Dox-eq. mg/kg i.v. 5×)	15 (75*)	15 (75*)
Myocet (Dox-eq. mg/kg i.v. 1×)	—	8
Myocet (Dox-eq. mg/kg i.v. 5×)	—	3 (15*)
Caelyx (Dox-eq. mg/kg i.v. 1×)	—	15
Caelyx (Dox-eq. mg/kg i.v. 5×)	—	5 (25*)

*Total dose of treatment.

Table S7. Mass balance of doxorubicin biodistribution for free doxorubicin or SQ-Dox NAs

	Doxorubicin		SQ-Dox NAs	
Tissue	2 h (% of ID)*	24 h (% of ID)	2 h (% of ID)	24 h (% of ID)
Tumor	0.26 ± 0.13	0.14 ± 0.06	0.09 ± 0.01	0.47 ± 0.14
Heart	1.5 ± 0.2	0.37 ± 0.12	0.14 ± 0.02	0.15 ± 0.03
Lung	2.9 ± 0.3	0.9 ± 0.3	0.6 ± 0.3	1.4 ± 0.2
Spleen	3.5 ± 0.4	1.2 ± 0.4	1.5 ± 0.3	2.7 ± 0.4
Kidney	16.8 ± 12.7	1.7 ± 0.6	0.5 ± 0.3	3.9 ± 0.5
Liver	38.1 ± 1.1	14.2 ± 1.4	15.7 ± 1.7	37.6 ± 3.1
Total[†]	63.6 ± 14.83	17.61 ± 2.88	18.53 ± 2.63	46.12 ± 4.37

*Percentage of initial dose recovered (8 mg/kg equivalent doxorubicin for SQ-Dox NAs or 8 mg/kg for free doxorubicin) in each tissue and overall. Mean ± 95% confidence interval (n = 4).
[†]The total recovered dose in the assayed tissues, which does not include drug in other tissues or eliminated from the body.

Table S8. In vitro cytotoxicity of nanoassemblies of squalenoyl prodrug of PTX against various human cancer cell lines

Compound	IC_{50} (nM)						
	L1210	L1210-10K	Panc1	MCF7	A549	MiaPaCa-2	LST174-T
PTX-SQ NAs	400	230	240	160	145	250	900
PTX	10	7	5	5	3	3	5