Supporting Information for

Therapeutic Modalities of Squalenoyl Nanocomposites in Colon Cancer: An Ongoing Search for Improved Efficacy

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EXPERIMENTAL SECTION

Synthesis of 4-(N)-Trisnorsqualenoyl-Gemcitabine (SQ-gem). To a mixture of *tris*norsqualenoic acid (0.50 g, 1.2 mmol) and triethylamine (0.15 g, 1.4 mmol) in anhydrous THF (5 mL) cooled at 0 °C was added dropwise ethylchloroformate (0.135 g, 1.2 mmol). The mixture was stirred at 0 °C for 15 min and a solution of gemcitabine hydrochloride (0.37 g, 1.2 mmol) and triethylamine (0.15 g, 1.4 mmol) in anhydrous DMF (5 ml) was added dropwise. The reaction mixture was stirred for 72 h at room temperature and then concentrated *in vacuo*. Aqueous sodium hydrogen carbonate was added and the mixture was extracted with ethyl acetate (3 x 50 ml). The combined extracts were washed with water, dried over MgSO4, and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel eluting with 1 to 5% methanol in dichloromethane to give pure 4-*N*-*tris*norsqualenoyl-gemcitabine as an amorphous white solid (0.46 g, 57%). $[\alpha]_D$ = 3.1 (c = 0.95, CH2Cl2); IR (neat, cm^{-1}) 3500-3150, 2950, 2921, 2856, 1709, 1656, 1635, 1557, 1490, 1435, 1384, 1319, 1275, 1197, 1130, 1071; 1H NMR (300 MHz, CDCl3) δ: 9.15 (broad s, 1 H, NHCO), 8.16 (d, 1 H, *J* = 7.5 Hz, H6), 7.47 (d, 1 H, *J* = 7.5 Hz, H5), 6.18 (t, 1 H, *J* = 7.0 Hz, H1'), 5.22-5.15 (m, 5 H, *H*C=C(CH3)), 4.49-4.43 (m, 1 H, H3'), 4.10-4.01 (m, 2 H, H4' and H5'), 3.92 (d, 1H , *J* = 10.5 Hz, H5'), 2.55 (m, 2 H, NHCOCH2), 2.38-2.28 (m, 2 H, NHCOCH2CH2), 2.13-1.91 (m, 16 H, CH2), 1.69 (s, 3H, C=C(CH3)2), 1.61 (s, 15 H, C=C(C*H*3)); 13C NMR (75 MHz, CDCl3) δ: 173.7 (*C*ONH), 163.0 (HN*C*=N), 155.8 (N*C*ON), 145.4 (CH, C6), 135.1 (C), 134.9 (2 C), 132.7 (C), 131.2 (C), 125.7 (CH), 124.4 (2 CH), 124.2 (2 CH), 122.4 (CF2), 97.7 (CH, C5), 81.7 (2 CH), 69.2 (CH), 59.9 (CH2), 39.7 (2 CH2), 39.5 (CH2), 36.5 (CH2), 34.3 (CH2), 28.3 (2 CH2), 26.8 (CH2), 26.7 (CH2), 26.6 (CH2), 25.6 (CH3), 17.6 (CH3), 16.0 (3 CH3), 15.8 (CH3); MS (CI, isobutane): *m/z* (%): 646 (100%); Anal. Calcd for C36H53N3O5: C, 66.95, H, 8.27, N, 6.51. Found: C, 66.76, H, 8.40, N, 6.39.

Synthesis of 2-Methoxy-5-(1-(3,4,5-Trimethoxyphenyl)Vinyl)Phenol (isoCA-4). IsoCA-4 was synthesized as indicated in Figure S1.

Procedure for the Synthesis of 4-Methyl-N'-[1-(3,4,5-Trimethoxyphenyl)Ethylidene]-Benzenesulfonohydrazide (1, Figure S1). To a solution of 3',4',5'-trimethoxyacetophenone (0.37 g, 1.75 mmol) in EtOH (20 mL) was added *p*-toluenesulfonyl hydrazide (0.39 g, 2.1 mmol). The resulting solution was stirred at reflux for 4 h. After cooling to room temperature and crystallization in ethanol, the product was obtained as white needles, yield 79%. Mp: 167-168 °C. TLC: R*f* 0.42 (Cyclohexane/EtOAc: 70/30). IR (neat, cm^{-1}): 3356, 3192, 2981, 2353, 2195, 2181, 2147, 1577, 1509, 1449, 1408, 1350, 1324, 1230, 1163, 1124, 1055, 1003. 1H NMR (300 MHz, CDCl3): 7.92 (d, 2H, *J* = 8.2 Hz), 7.74 (s, 1H), 7.31 (d, 2H, *J* = 8.2 Hz), 6.86 (s, 2H), 3.86 (s, 6H), 3.85 (s, 3H), 2.41 (s, 3H), 2.14 (s, 3H). 13C NMR (75 MHz, CDCl3): 152.9 (2C), 152.5, 144.2, 139.6, 135.4, 132.8, 129.5 (2C), 128.2 (2C), 103.8 (2C), 60.9, 56.1 (2C), 21.6, 13.6. MS (APCI) (M + H)+ *m/z* 379.

Procedure for the Synthesis of 2-Methoxy-5-[1-(3,4,5-Trimethoxyphenyl)Vinyl]Phenol (isoCA-4). Tosylhyrazone (1) (190 mg, 0.5 mmol), $PdCl_2(MeCN)_2$ (7 mg, 0.025 mmol, 5 mol %), diphenylphosphinopropane (dppp) (20 mg, 0.05 mmol, 10 mol %), and 5 ml of dioxane were mixed under argon for 5 minutes at rt. Cs_2CO_3 (490 mg, 1.5 mmol) was then added, the reaction mixture is stirred for 1 minute and arylhalide (2) (153 mg, 0.42 mmol) is added. The mixture was stirred at 90 °C until the reaction completed by TLC analysis (4 h). The crude reaction mixture was allowed to cool to room temperature. EtOAc was added to the mixture, which was filtered through celite. After concentration, the residue was dissolved in 14 mL of EtOH and 2 mL of DCM, *p*-toluene sulfonic acid (433 mg, 2.52 mmol) was added. The temperature was raised to 60 °C for 2.5 h and then the mixture was cooled to rt. Water (10 mL) was added and the aqueous phase was extracted with AcOEt (3 X 15 mL). The organic phase was dried over $MgSO_4$, then concentrated. The crude residue was then purified on silica

gel to give the desired phenol product.

IsoCA-4 (2-Methoxy-5-[1-(3,4,5-Trimethoxyphenyl)Vinyl]Phenol). White solid, yield 76% (101 mg, 0.32 mmol). Mp: 109-110 °C. TLC: R*f* 0.21 (Cyclohexane/EtOAc: 80/20). IR (neat, cm^{-1}): 3417, 2937, 2837, 1579, 1506, 1460, 1411, 1346, 1281, 1254, 1124, 1005. ^{1}H NMR (300 MHz, $CDCl_3$): 6.97 (d, 1H, *J* = 2.1 Hz), 6.82 (m, 2H), 6.55 (s, 2H), 5.61 (bs, 1H), 5.37 (d, 1H, *J* = 1.5 Hz), 5.30 (d, 1H, *J* = 1.5 Hz), 3.91 (s, 3H), 3.87 (s, 3H), 3.81 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): 152.8 (2C), 149.5, 148.4 (2C), 145.2, 137.8, 137.4, 134.7, 120.2, 114.4, 112.8, 110.1, 105.8 (2C), 60.9, 56.1 (2C), 55.9. MS (ESI+) $(M + H)^+$ *m/z* 317.24. Anal. ($C_{18}H_{20}O_5$) C, H.

Analytical Methods

Preparation and Stability Studies of Squalenoyl Nanocomposites. To evaluate the optimal conditions for the preparation of SQ-gem/isoCA-4 NAs, the influence of the drugs molar ratio (isoCA-4:SQ-gem) was tested (Tables S1). The typical procedure for the preparation of the NAs, as described in the main text, was applied. Small sizes were obtained until a molar ratio isoCA-4:SQ-gem of 1. Increasing this ratio resulted into big aggregates, probably due to the precipitation of the water non soluble isoCA-4. The optimal conditions for the preparation of SQCOOH/isoCA-4 NAs were obtained in a similar procedure (Table S2). Small sizes were prepared only until a molar ratio isoCA-4:SQCOOH NAs of 1:2.

The stability of the SQ-gem/isoCA-4 NAs suspension after dilution in 1, 5, 10 and 50 L was also studied. The presence of the nanoparticles was detected by quasi-elastic light scattering (QELS) with a nanosizer (Zêtasizer Nano ZS Malvern; Malvern Instruments SA, Orsay, France). In spite of the huge dilution (up to 50,000 times), the presence of nanoparticles was quite clearly detected in the diluted suspension. Additionally, it was observed that the size of the nanoparticles was not changed after any dilution (see Table S3).

Cholesterol BODIPY Dyes Release. The release profiles of cholesterol BODIPY dyes from squalenoyl nanocomposites after their incubation in PBS solution containing 10% (vol/vol) FCS was performed by fluorometric analysis at 515 and 560 nm in order to detect both fluorescent markers using a spectrofluorometer (Perkin Elmer, Model LS50B, USA) (Figure S6). Briefly, SQCOOH/isoCA-4/BChol-green/BChol-red (10 µM isoCA-4), SQ-gem/BChol-green/BChol-red (10 µM SQ-gem) and SQ-gem/isoCA-4/BChol-green/BChol-red (10 µM SQ-gem/isoCA-4) NAs were incubated at 37 °C in PBS solution containing 10% (vol/vol) FCS for 24 h. Aliquots of the incubation medium were removed at different time intervals (*i.e.*, 1, 4, 8 and 24 h) and ultracentrifuged at 15,000 × g during 30 min. The released cholesterol BODIPY dyes were then measured in the supernatant using a spectrofluorometer and quantified by using the following equation: % (released cholesterol BODIPY dye) = [fluorescence of released cholesterol BODIPY dye/ total fluorescence of cholesterol BODIPY dyes] x 100%.

Drug Release. To determine the kinetics of gemcitabine release from nanoassemblies, 250 µL of SQ-gem or SQ-gem/isoCA-4 (5 µmol.mL^{-1}) NAs were added to 2.25 mL of heat-inactivated FCS (56 °C, 30 min) supplemented with 200 µg mL^{-1} tetrahydrouridine (THU). Individual vials were used for each time point. The reaction mixture was incubated at 37 °C, and aliquots (300 µL) of incubation medium were removed at predetermined time points (*i.e.*, 0, 0.5, 1, 2, 4, 8 and 24 h).

For sample preparation at 48 h, 100 µL of SQ-gem or SQ-gem/isoCA-4 (5 µmol mL^{-1}) NAs were added to 400 µL of heat-inactivated FCS supplemented with 200 µg mL^{-1} tetrahydrouridine (THU). 500 µL of new heat-inactivated fetal calf serum were added at 24 h and 300 µL of incubation medium were removed at 48 h.

For sample preparation at 72 h, 150 µL of SQ-gem or SQ-gem/isoCA-4 (5 µmol mL^{-1}) NAs were added to 350 µL of heat-inactivated FCS supplemented with 200 µg mL^{-1}

tetrahydrouridine (THU). 500 µL of new heat-inactivated fetal calf serum were added at 24 h and 48 h, and 300 µL of incubation medium were removed at 72 h.

All samples were centrifuged at 15,000 x g during 30 min. Then, a 100 µL aliquot of the supernatants was used for sample treatment and drug quantification in the same conditions as for plasma samples in pharmacokinetic studies (see bioanalysis section below).

Quantification of Gemcitabine, SQ-gem and isoCA-4 in Biological Samples. *Pharmacokinetic and Tumor Distribution*: gemcitabine, SQ-gem and isoCA-4 were quantified in mice plasma and tumor by UPLC-MS/MS. 2'-deoxycytidine and colchicine (Sigma, France) were used as internal standards (IS).

For *in vivo* plasma quantitation, 1 mL of acetonitrile/methanol (vol/vol) containing IS (2'deoxycytidine 0.3 µg/mL and colchicine 0.1 µg/mL) was added to 100 µL of plasma sample. Mixture was vortex-mixed and then centrifuged at 20,000 x g for 15 min at +4 °C. Supernatant was transferred to a fresh tube and evaporated to dryness at 40 °C with a nitrogen stream in a Turbovap LV evaporator (Biotage, UK). Extract was then resuspended with 200 µL of a mixture of mobile phase (A [0.2% formic acid in water]/B [0.2% formic acid in methanol]; 75/25; v/v). Debris were removed by spinning at 20,000 x g for 5 min at +4 °C and supernatant was transferred to an injection vial for LC-MS/MS analysis.

For *in vivo* tumor measurement, tissue samples (around 1 g) were homogenized in 2 ml of water containing tetrahydrouridine (12 µg/mL), using an homogenizer Ultra-Turrax IKA T25 (Imlab, France). 500 µL of acetonitrile containing IS (2'deoxycytidine 0.6 µg/mL and colchicine 0.2 µg/mL) were added to 100 µL of homogenate sample. Cellular debris were removed by spinning at 20,000 x g for 15 min at +4 °C and transferring the supernatant to a fresh tube. Then, samples were evaporated to dryness at 40 °C with a nitrogen stream in a Turbovap LV evaporator (Biotage, UK) and resuspended in 200 µL of mobile phase (A/B;

75/25; v/v). Debris were removed by spinning at 20,000 x g for 5 min at +4 °C and supernatant was transferred to an injection vial for LC-MS/MS analysis.

Bioanalysis: For both plasma and tumor extracts, 10 µL aliquot was injected into the chromatographic system. LC-MS/MS conditions were the same for both matrices. For the three compounds (*i.e.*, gemcitabine, SQ-gem and isoCA-4) to be quantified, linear regression with 1/X2 weighing were used for calibrations which ranged from 10 to 1,700 ng/mL and from 30 to 5,000 ng/g for plasma and tumor tissue, respectively. The extract samples maintained at + 4 °C in the autosampler were chromatographically separated using a Waters ACQUITY UPLC® System with a Acquity UPLC BEH Amide 1.7 µm, 2.1x150 mm column and a reversed phase gradient over a run time of 5.5 minutes. Initial conditions consisted of mobile phase A (0.2% formic acid in water) and mobile phase B (0.2% formic acid in methanol) with a column temperature of 50 °C and a flow rate of 0.300 mL/min. The gradient conditions ramped from 5% B to 95% B between 1.0 and 2.5 min, then maintained up to 3.5 min, ramped to 5% in 0.01 min and then maintained up to 5.5 min for re-equilibration. The MS analysis was performed on a Waters Quattro Premier™ TQ Mass Spectrometer operated in positive ion electrospray MRM mode. Briefly, main tune parameters were as follows: capillary voltage was setup at 3 kV, source temperature was 120 °C, desolvation temperature was 350 °C, cone gas flow was 100 L/Hr and desolvation gas flow was 800 L/Hr. The analytes and the IS were specifically monitored using a pair value cone voltage (V) / collision energy (eV) set at 50/25, 25/15, 55/25, 60/28 and 90/45 for gemcitabine, 2'-deoxycytidine, isoCA-4, colchicine and SQ-gem, respectively. Monitored MRM transitions were m/z 264.11/111.95, 228.23/111.97, 317.39/149.05, 400.23/310.32 and 646.5/112.14 for the above list of compounds, respectively. Transitions were alternatively monitored with dwell time comprised between 0.05 and 0.2 s while quadrupole resolution in both Q1 and Q3 was set at 0.7 FWMH. Under these UPLC-MS/MS conditions gemcitabine and 2'deoxycytidine

displayed a mean retention time of 1.3 min, while colchicine, isoCA-4, and SQ-gem showed a mean retention time of 2.97, 3.07 and 3.37 min, respectively.

Cell Proliferation Assays. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was used to test the cytotoxicity of SQ-gem/isoCA-4 NAs in comparison to free gemcitabine, free isoCA-4, SQCOOH/isoCA-4 NAs, SQ-gem NAs, a mixture of gemcitabine with isoCA-4 or SQCOOH/isoCA-4 NAs, and mixture of SQ-gem NAs with isoCA-4 or SQCOOH/isoCA-4 NAs. Briefly, $5x10^3$ of LS174-T cells and $1x10^4$ of HUVECs per well were incubated in 200 µL medium containing 10% (vol/vol) FCS in 96-well plates for 24 hours. The cells were then incubated with increasing concentrations of tested compounds for 3, 6, 24 and 72 hours before being treated with MTT. Then, the medium was removed and 200 µL of the fresh medium for further incubation up to 72 hours, or 100 µL of MTT solution (0.5 mg mL^{-1} in DMEM containing 10% (vol/vol) FCS) were added to each well. The plates were incubated for 2 hours at 37 °C and 100 µL of 20% SDS solution was then added to each well for 24 hours at 37 °C. Absorbance was measured at 570 nm using a plate reader (Metertech Σ 960, Fisher Bioblock, Illkirch, France). The percentage of surviving cells was calculated as the absorbance ratio of treated to untreated cells. The inhibitory concentrations 50% (IC_{50}) were determined from the dose-response curve. All experiments were set up in quadruplicate to determine means and SDs. No cytotoxic effects were observed for control SQCOOH NAs.

To investigate the cytotoxicity (on LS174-T cancer cells) of squalene-based nanoassemblies activated (or not) by cathepsins B and D, SQ-gem or SQ-gem/isoCA-4 (100 nM) NAs were incubated for 24 h at 37 °C in PBS solution (10 mM, pH 8.0) containing (or not) 5, 10 or 25 U/mL cathepsins B and D. $5x10^4$ of LS174-T cells per well were preincubated in 1 mL medium containing 10% (vol/vol) FCS in 12-well plates for 24 hours. The LS174-T cells were then treated with prepared solutions (10 nM) for 6 hours. Free gemcitabine (10 nM)

or its mixture with free isoCA-4 (10 nM) was used as controls. Then, the cells were incubated in a new medium without any drug up to 72 h, with further analysis by MTT assay. All experiments were set up in quadruplicate to determine means and SDs. As clearly shown in Figure S5, increasing cathepsins concentration, also increased the anticancer activity of squalene-based nanoassemblies.

To evaluate the *in vitro* cytotoxicity towards both human colon cancer cells (LS174-T) and HUVECs exposed to exactly the same concentrations of drugs than those found in the tumor nodules *in vivo*, at 8 h post administration, 10^5 of LS174-T cells and of HUVECs per well were incubated in 1 mL medium containing 10% (vol/vol) FCS in 12-well plates for 24 hours. The cells were then treated with drug mixtures (*i.e.*, 91 ng/mL [0.35 µM] Gem + 28,884 ng/mL [44.7 µM] SQ-gem + 227 ng/mL [0.72 µM] isoCA-4 *versus* 68 ng/mL [0.23 µM] Gem + 3,325 ng/mL [10.5 µM] isoCA-4, corresponding to drugs concentration after administration of SQ-gem/isoCA-4 NAs and Gem+isoCA-4 at 8 h post administration, respectively) for 6 hours. After exposure to the drugs, the cells were washed by PBS and then incubated in a new medium without any drug up to 72 h for further analysis by MTT assay.

Apoptosis Assay by Annexin V Staining. To assess apoptosis, cells were stained with an Annexin V-FITC apoptosis assay kit (Invitrogen, France) following the protocol provided by the manufacturer. 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 hours of treatment with free gemcitabine, the mixture of free gemcitabine with free isoCA-4, SQ-gem NAs or SQ-gem/isoCA-4 NAs, all at IC_{50} concentration (IC_{50} was 6, 32, 4 and 6 nM for SQ-gem/isoCA-4 NAs, SQ-gem NAs, gemcitabine and gemcitabine+isoCA-4, respectively), LS174-T cells were washed and resuspended in 100 µL 1 x Annexin V binding buffer. Subsequently, 5 µL of FITC-conjugated Annexin V and 1 µL

of propidium iodide (PI) solution (100 μg mL^{-1}) were added to each cell suspension. After 15 min incubation in the dark at room temperature, stained cells were analyzed by flow cytometry (Accuri 6; Accuri, Ann Arbor, MI) using a BD Accuri CFlow Plus software. All the samples were assayed in triplicate and apoptotic fraction was calculated as follows: %=[(apoptotic cell number)/(total cell number)]×100%.

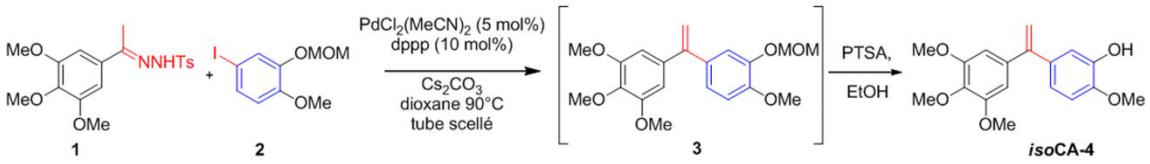

Figure S1. A Pd-catalyzed cross-coupling reaction of *N*-tosylhydrazone (1) with arylhalide (2) gives rise to 1,1-diarylethylene (isoCA-4).

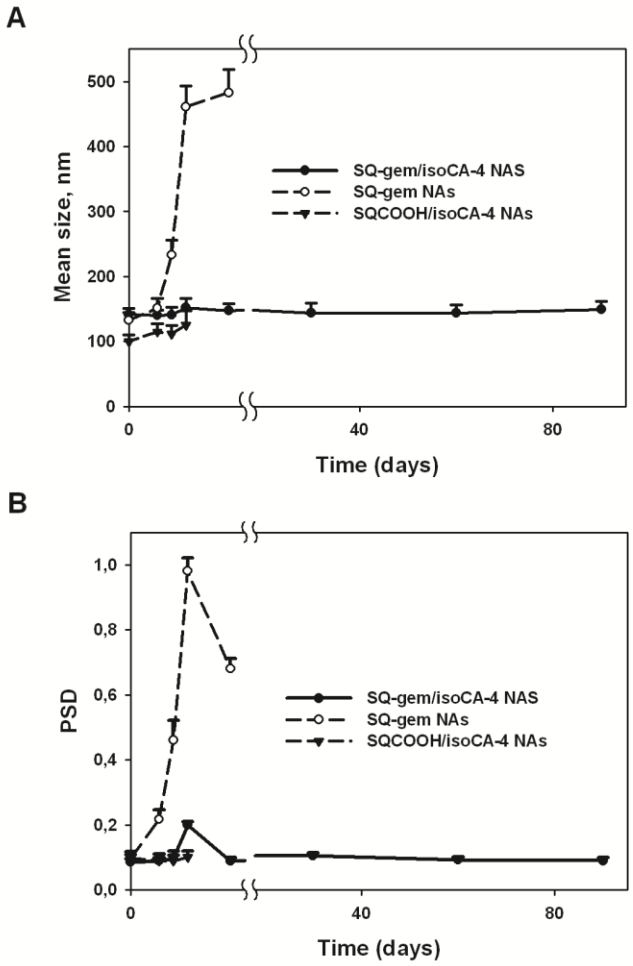

Figure S2. Influence of duration of storage on mean size (A) and particle size distribution (PSD) (B) of squalene-based nanocomposites (*i.e.*, SQCOOH/isoCA-4, SQ-gem and SQ-gem/isoCA-4 NAs). NAs were stored at refrigerator temperature for 90 days. At different time intervals (*i.e.*, 0, 2, 3, 4, 7, 15, 30, 60 and 90 days), the average size and PSD were determined. Samples were estimated in triplicates. Statistical analysis of the data was performed using the Student's *t*–test. A probability, *P*, of less than 0.005 ($P<0.005$) was considered significant in this study. As SQ-gem or SQCOOH/isoCA-4 NAs stability was less than 7 or 4 days of storage, respectively, only SQ-gem/isoCA-4 NAs were analyzed for particle size and PSD at days 15, 30, 60 and 90. These results clearly suggest that isoCA-4 contributed to stabilize the SQ-gem-based nanocomposites and prevented agglomeration during 90 days of storage (4 °C).

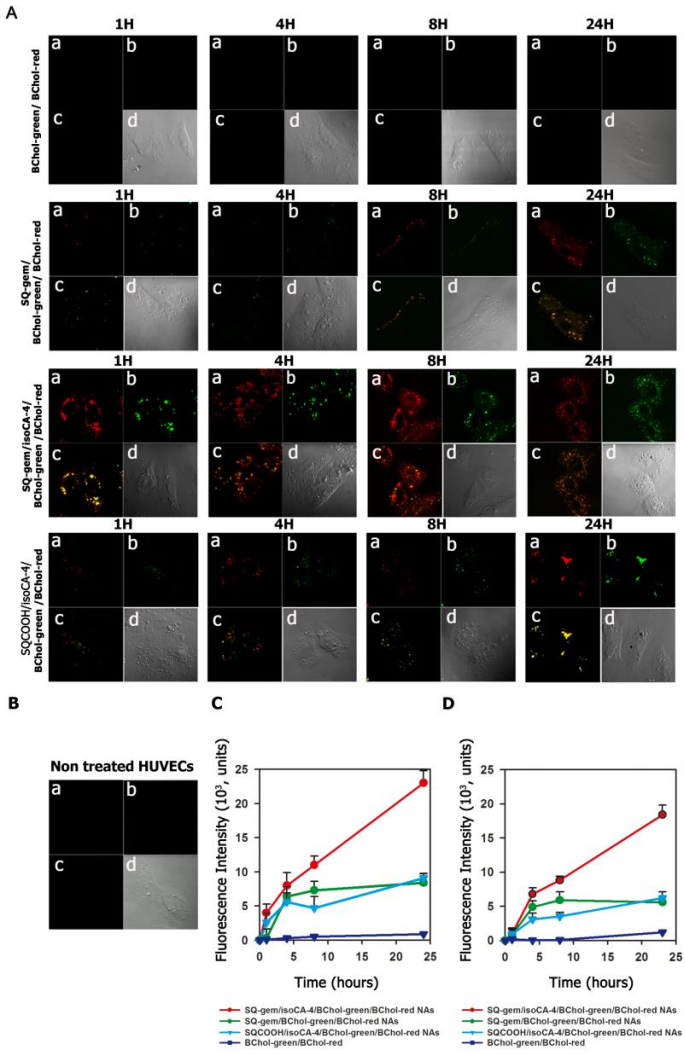

Figure S3. Cell internalisation of squalene-based nanocomposites in HUVECs (A, C and D). (A) Intracellular localization of SQ-gem/BChol-green/BChol-red NAs, SQ-gem/isoCA-4/BChol-green/BChol-red NAs and SQCOOH/isoCA-4/BChol-green/BChol-red NAs, as imaged by confocal microscopy (in each picture, a-BChol-red fluorescence in square top left, b - BChol-green fluorescence in square top right, c – merge red and green fluorescences in square bottom left and d - contrast phase in square bottom right). The mixture of free cholesterol BODIPY dyes (BChol-green with BChol-red) was used as negative control. The original magnification was ×63. (B) Non treated cells. (C, D) Time course of nanocomposites accumulation into HUVECs exposed to 10 µM of fluorescently labeled SQCOOH/isoCA-4 NAs, SQ-gem NAs or SQ-gem/isoCA-4 NAs as measured by flow cytometry. The mixture of free cholesterol BODIPY dyes (BChol-green with BChol-red) was used as negative control. After incubation at different time intervals (*i.e.*, 1, 4, 8 and 24 h), the cells were trypsined and then analyzed on a FACScan flow cytometer using BD Accuri CFlow Plus software (Accuri Cytometers Ltd., UK), with argon laser as light source of 488 nm wavelength. The cells containing fluorescent nanoassemblies were selected as fluorescent positive cells on cytogram, in a total of 10,000 cells. The fluorescence of cholesterol analogues BChol-green and BChol-red was detected at 515 nm (C) and 560 nm (D), respectively. The intracellular detection of both cholesterol analogues (*i.e.*, BChol-green and BChol-red) confirms NAs uptake. Data represent the mean ± SD from triplicate independent experiments.

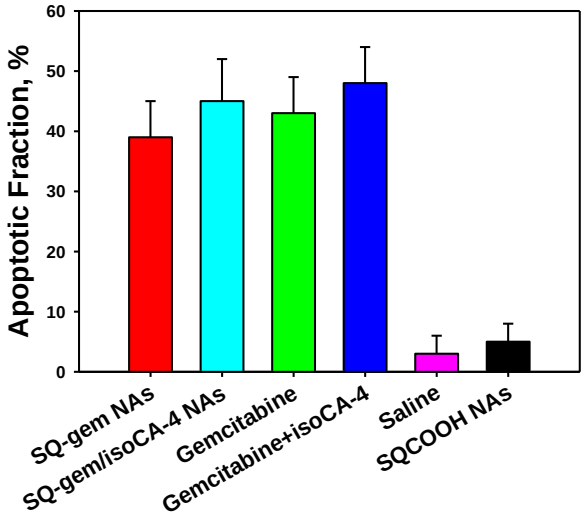

Figure S4. The apoptotic fraction (%) following the treatment of LS174-T cells with free gemcitabine, a mixture of free gemcitabine with free isoCA-4, SQ-gem NAs or SQ-gem/isoCA-4 NAs. The cancer cells were treated with NAs or free drugs at IC_{50} (IC_{50} was 6, 32, 4 and 6 nM for SQ-gem/isoCA-4 NAs, SQ-gem NAs, gemcitabine and gemcitabine+isoCA-4, respectively). Data represent the mean ± SD from triplicate independent experiments (P<0.001 for all squalene-based nanocomposites and free drugs *versus* non treated cells).

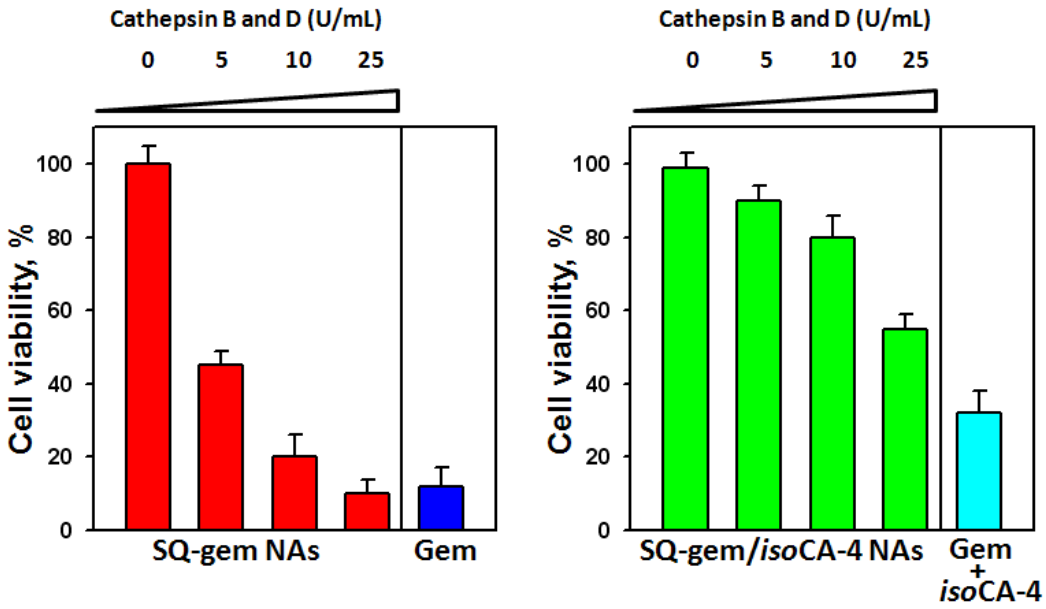

Figure S5. Sensitivity of LS174-T cancer cells to squalene-based nanocomposites pre-treated with cathepsins B and D. SQ-gem or SQ-gem/isoCA-4 (100 nM) NAs were incubated for 24 h at 37 °C in PBS solution (10 mM, pH 8.0) containing (or not) 5, 10 or 25 U/mL cathepsins B and D. The LS174-T cells were then treated with prepared solutions (10 nM) for 6 hours. Free gemcitabine (10 nM) or its mixture with free isoCA-4 (10 nM) was used as controls. Then, the cells were incubated in a new medium without any drug up to 72 h, with further analysis by MTT assay. All experiments were set up in quadruplicate to determine means and SDs.

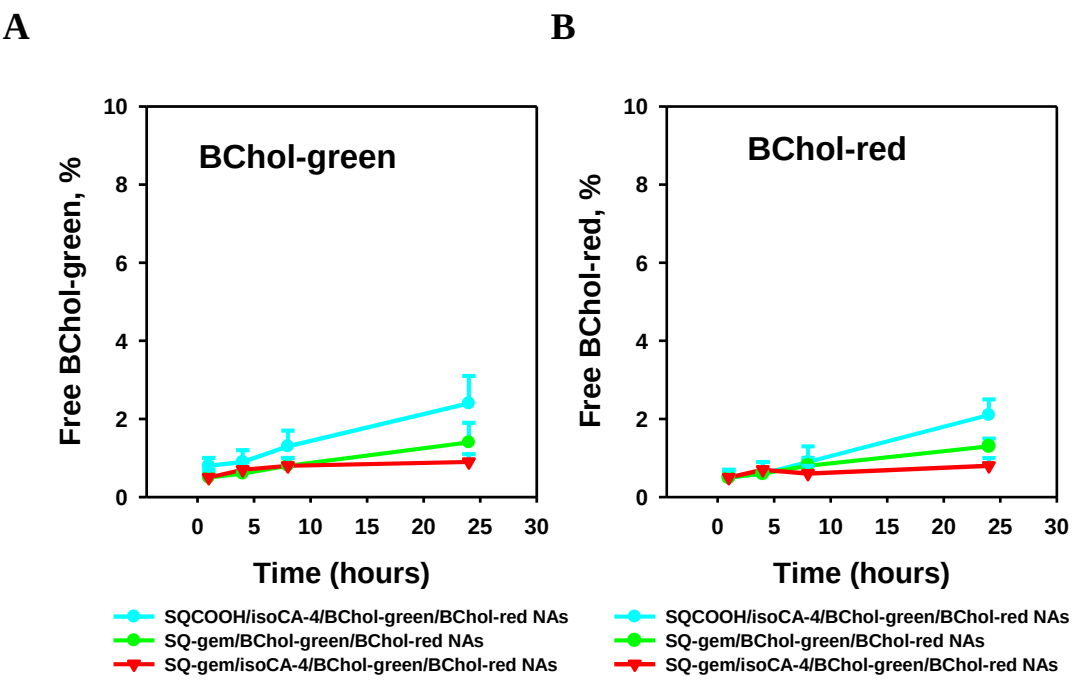

Figure S6. The release profiles of cholesterol BODIPY dyes (*i.e.*, BChol-green [A] and BChol-red [B]) from squalenoyl nanocomposites at 37 °C in PBS solution containing 10% (vol/vol) FCS. SQCOOH/isoCA-4/BChol-green/BChol-red (10 µM isoCA-4), SQ-gem/BChol-green/BChol-red (10 µM SQ-gem) and SQ-gem/isoCA-4/BChol-green/BChol-red (10 µM SQ-gem/isoCA-4) NAs were incubated at 37 °C in PBS solution containing 10% (vol/vol) FCS for 24 h. Aliquots of the incubation medium were removed at different time intervals (*i.e.*, 1, 4, 8 and 24 h) and ultracentrifuged at 15,000 × g during 30 min. The released cholesterol BODIPY dyes were then measured at wavelengths 515 nm (A) and 560 nm (B) nm in the supernatant using a spectrofluorometer (Perkin Elmer, Model LS50B, USA) and quantified by using the following equation: % (released cholesterol BODIPY dye) = [fluorescence of released cholesterol BODIPY dye/ total fluorescence of cholesterol BODIPY dyes] x 100%. Data represent the mean ± SD from triplicate independent experiments.

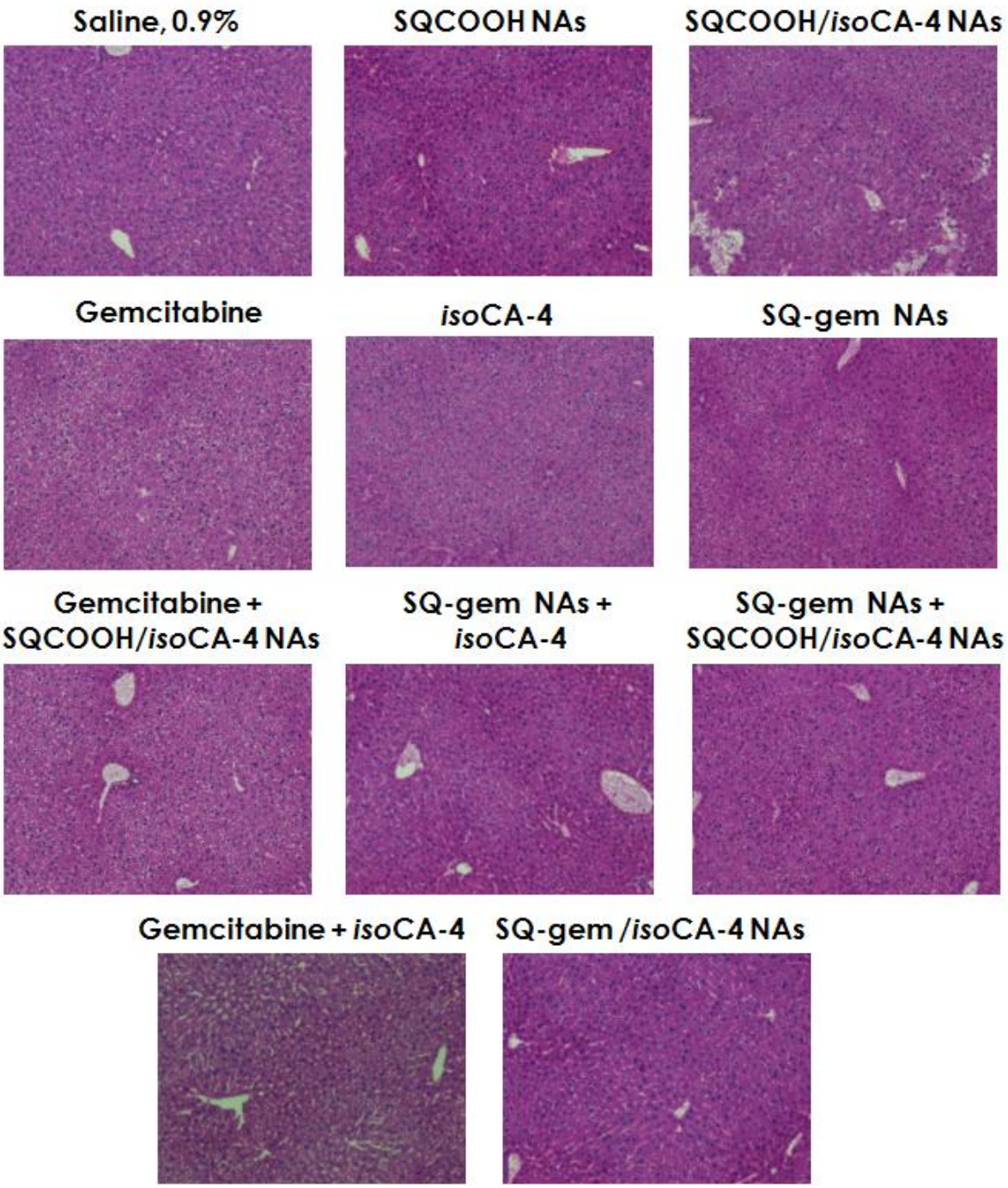

Figure S7. Histology of liver tissue in nude mice transplanted with human colon tumors and injected with squalenoyl nanocomposites and free drugs. Human colon tumor cells LS174-T were grafted into the nude mice by subcutaneous injection. The liver of nude mice was excised on day 22 and analyzed by hematoxylin and eosin (H&E) staining. The original magnification was ×10.

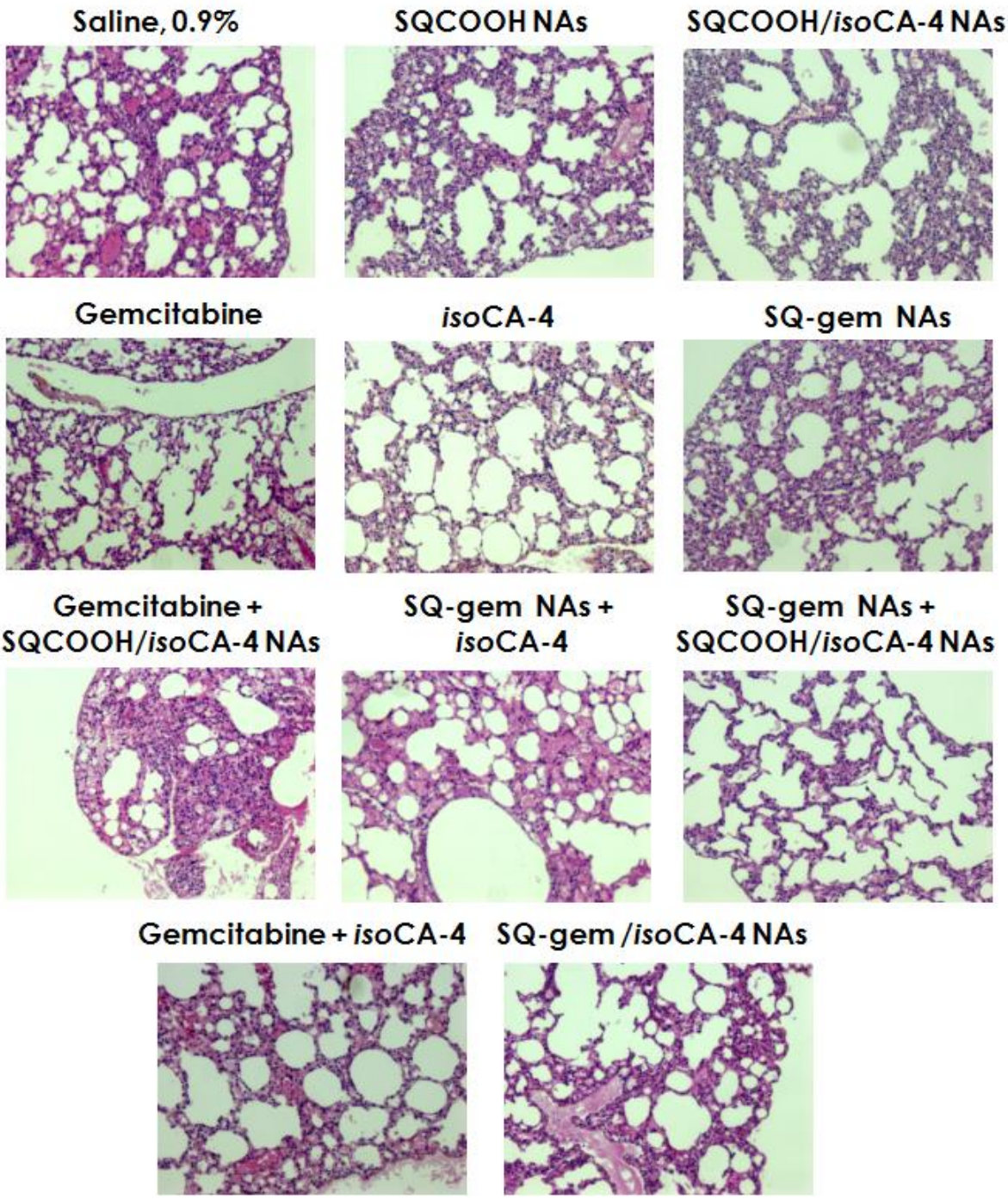

Figure S8. Histology of lungs tissue in nude mice transplanted with human colon tumors and injected with squalenoyl nanocomposites and free drugs. Human colon tumor cells LS174-T were grafted into the nude mice by subcutaneous injection. The lungs of nude mice were excised on day 22 and analyzed by hematoxylin and eosin (H&E) staining. The original magnification was ×10.

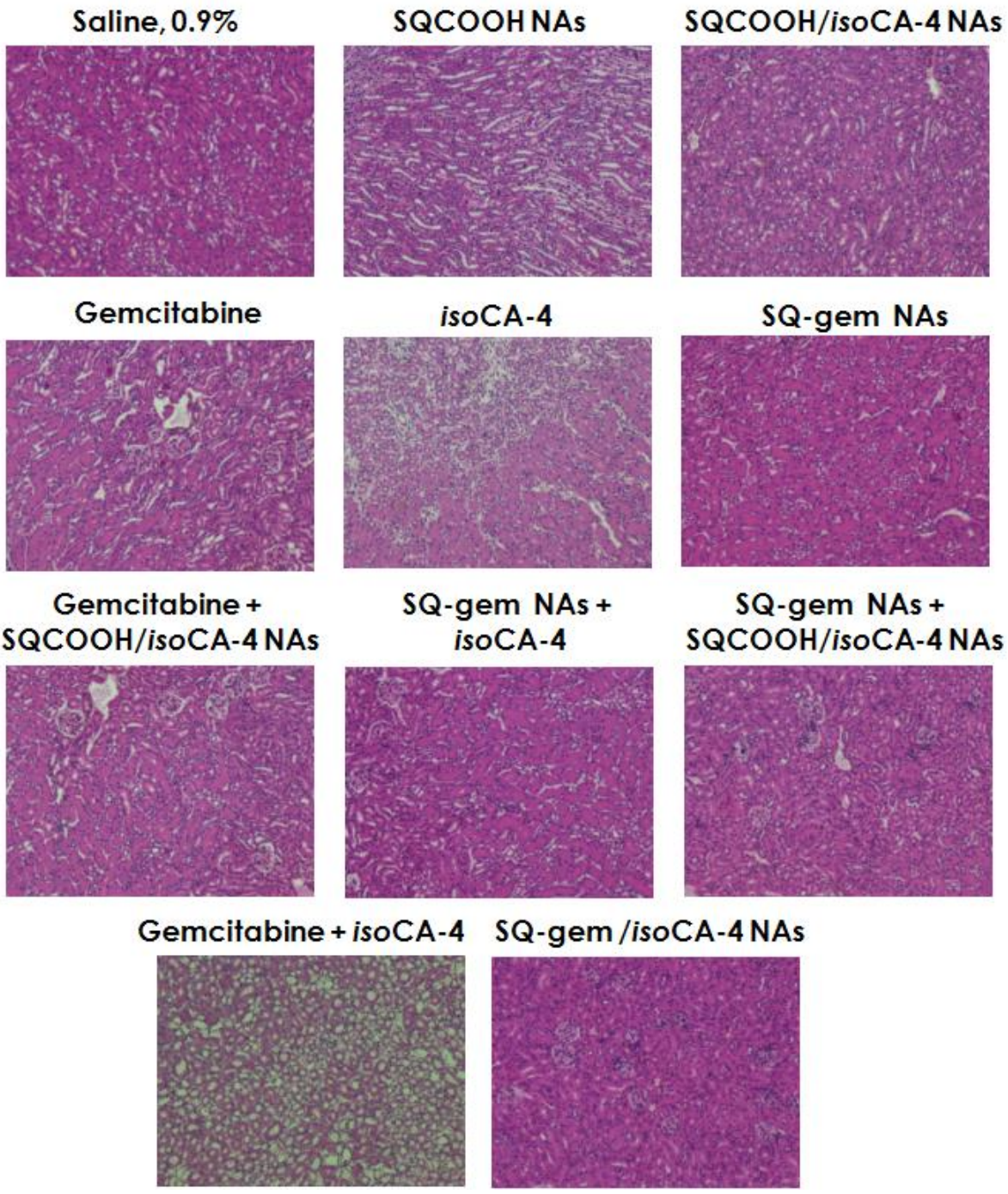

Figure S9. Histology of kidneys tissue in nude mice transplanted with human colon tumors and injected with squalenoyl nanocomposites and free drugs. Human colon tumor cells LS174-T were grafted into the nude mice by subcutaneous injection. The kidneys of nude mice were excised on day 22 and analyzed by hematoxylin and eosin (H&E) staining. The original magnification was ×10.

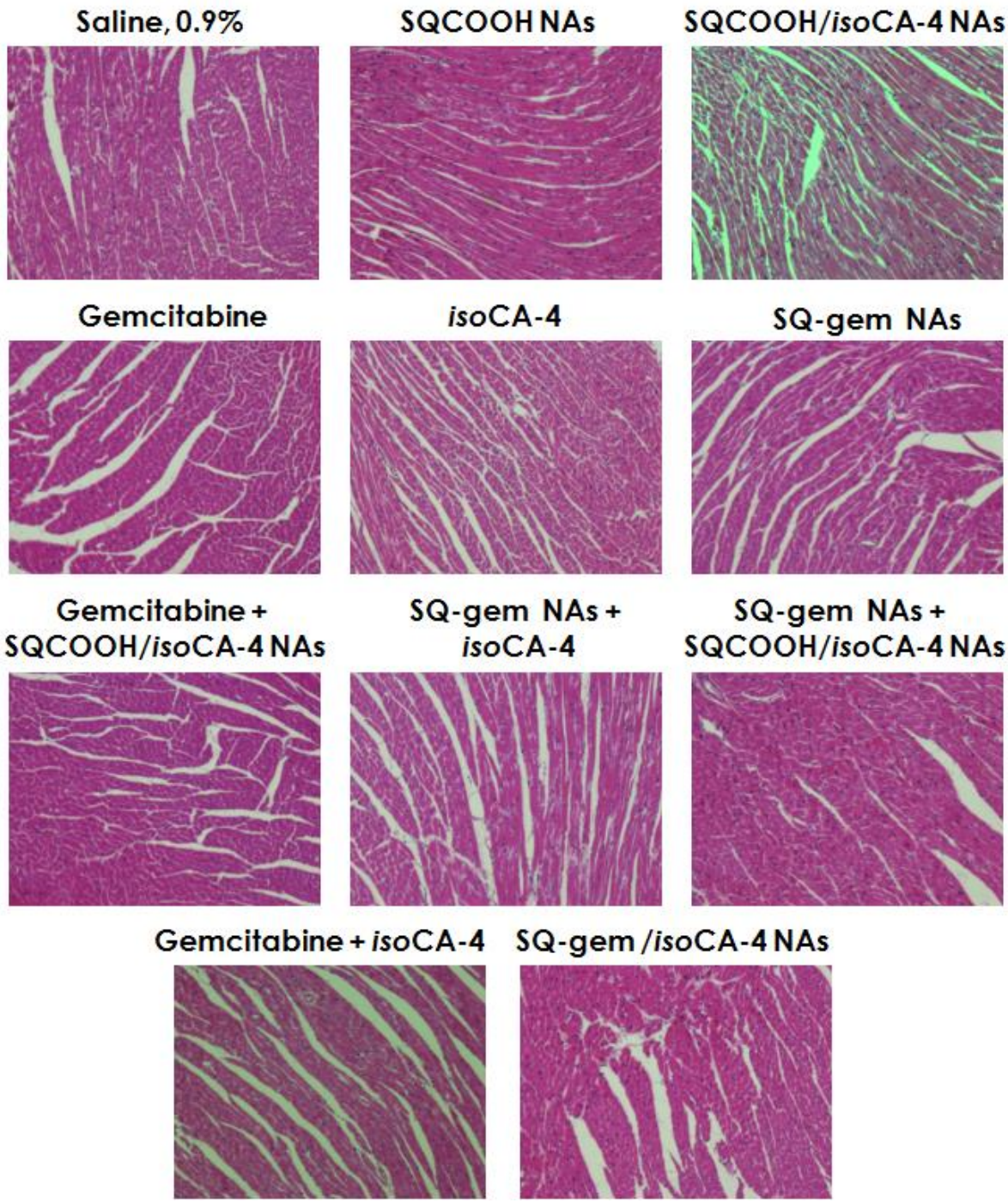

Figure S10. Histology of the heart tissue in nude mice transplanted with human colon tumors and injected with squalenoyl nanocomposites and free drugs. Human colon tumor cells LS174-T were grafted into the nude mice by subcutaneous injection. The heart of nude mice was excised on day 22 and analyzed by hematoxylin and eosin (H&E) staining. The original magnification was ×10.

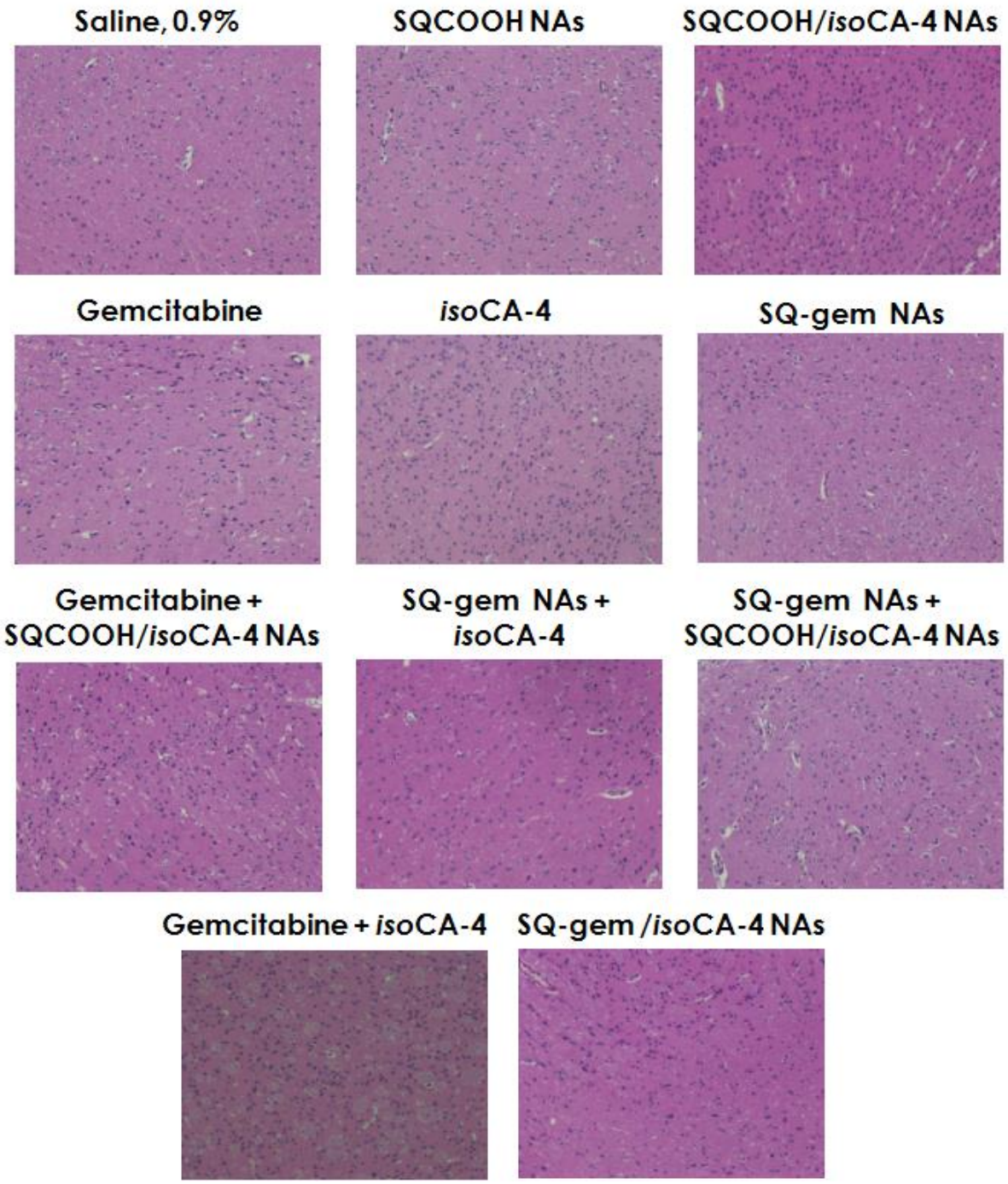

Figure S11. Histology of brain tissue in nude mice transplanted with human colon tumors and injected with squalenoyl nanocomposites and free drugs. Human colon tumor cells LS174-T were grafted into the nude mice by subcutaneous injection. The brain of nude mice was excised on day 22 and analyzed by hematoxylin and eosin (H&E) staining. The original magnification was ×10.

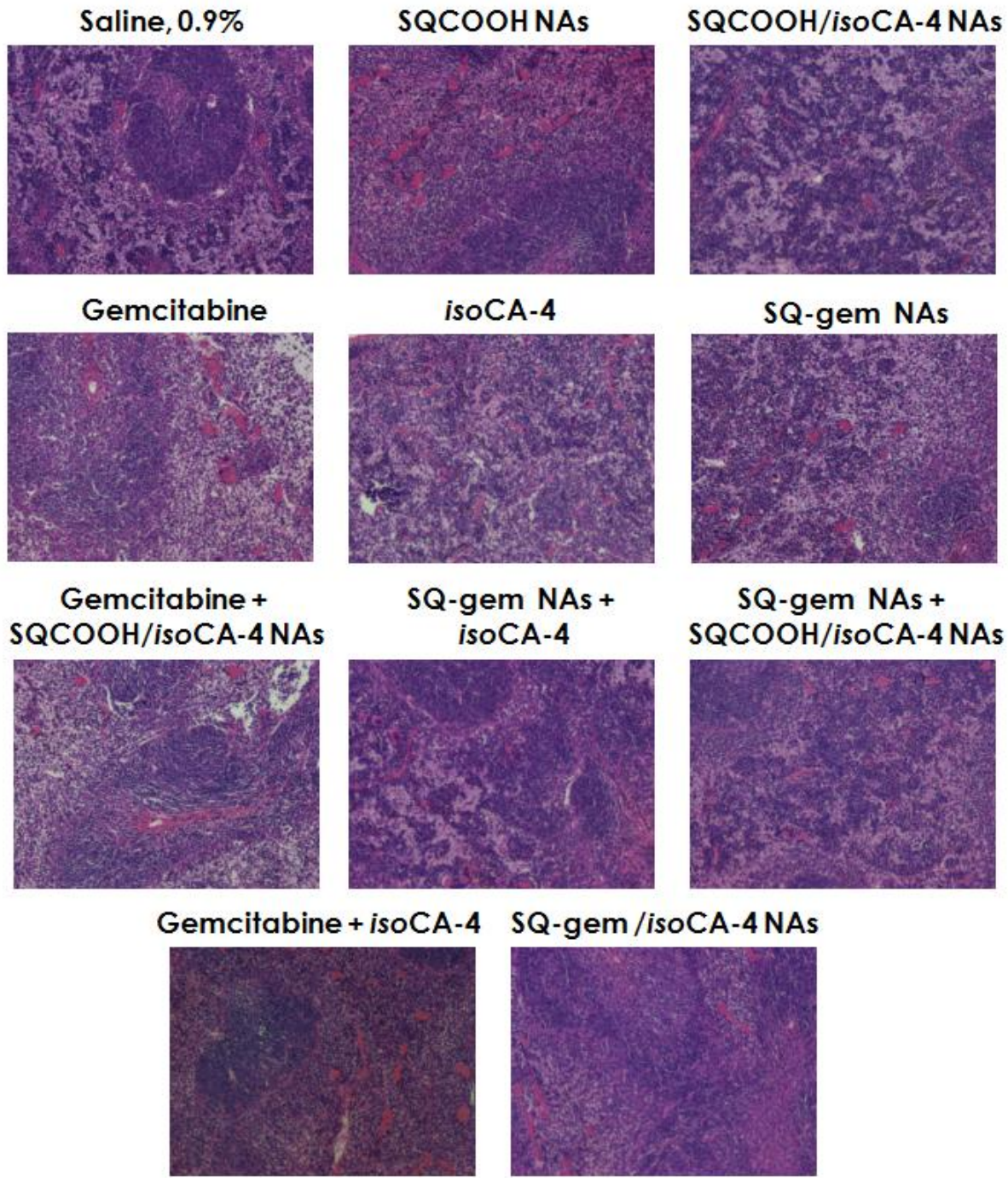

Figure S12. Histology of spleen tissue in nude mice transplanted with human colon tumors and injected with squalenoyl nanocomposites and free drugs. Human colon tumor cells LS174-T were grafted into the nude mice by subcutaneous injection. The spleen of nude mice was excised on day 22 and analyzed by hematoxylin and eosin (H&E) staining. The original magnification was ×10.

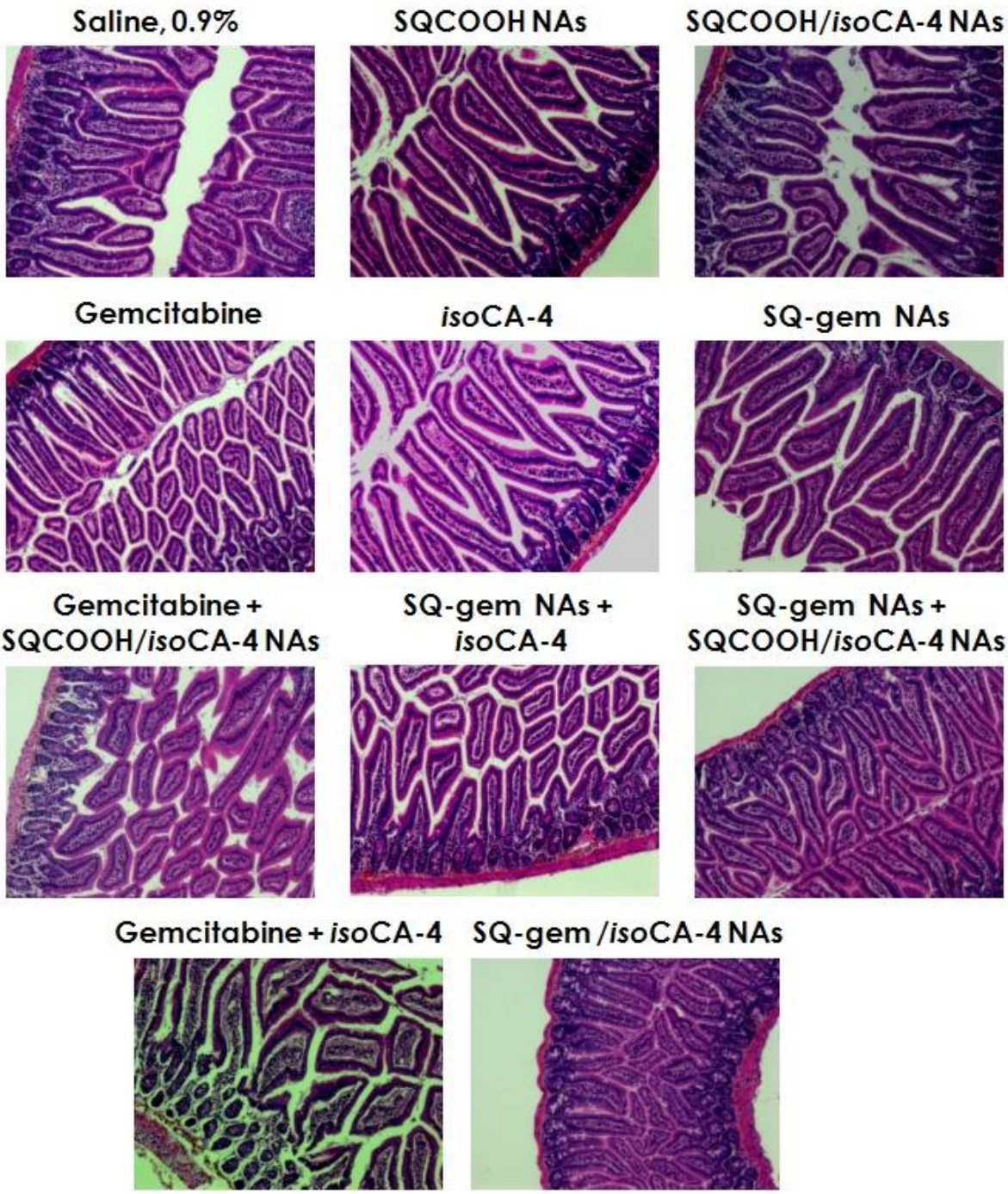

Figure S13. Histology of small intestine tissue in nude mice transplanted with human colon tumors and injected with squalenoyl nanocomposites and free drugs. Human colon tumor cells LS174-T were grafted into the nude mice by subcutaneous injection. The small intestine of nude mice was excised on day 22 and analyzed by hematoxylin and eosin (H&E) staining. The original magnification was ×10.

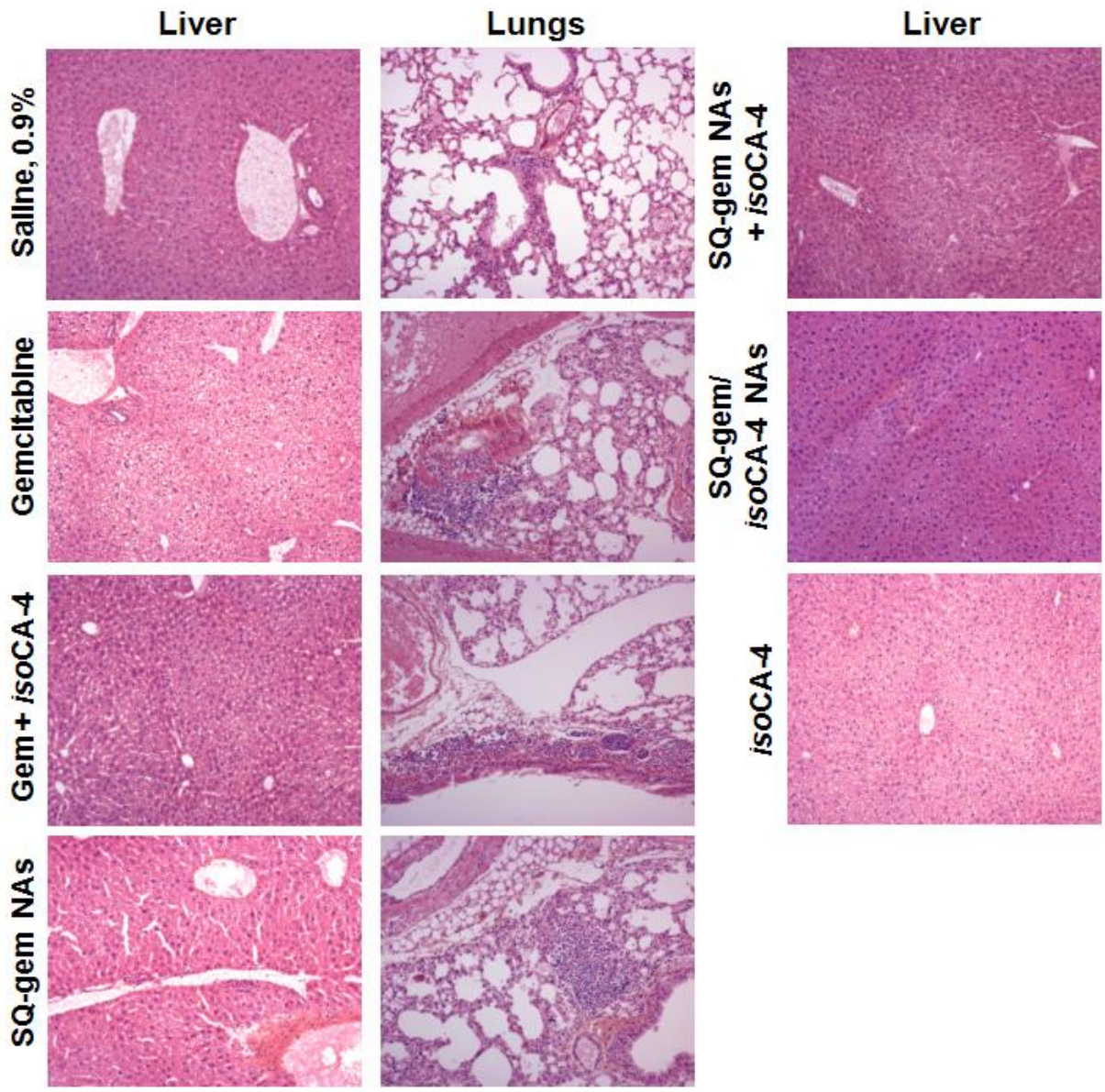

Figure S14. Slight and occasional histological changes of liver and lungs in nude mice transplanted with human colon tumors and injected with free gemcitabine, its mixture with free isoCA-4, free isoCA-4 and SQ-gem NAs with (or without) free isoCA-4. No special toxicity except of hepatocyte regeneration was observed with SQ-gem/isoCA-4 nanoassemblies. Human colon tumor cells LS174-T were grafted into the nude mice by subcutaneous injection. The liver and lungs of nude mice were excised on day 22 and analyzed on paraffin embedded sections stained with hematoxylin and eosin (H&E). The original magnification was ×100.

Table S1. Size of SQ-gem/isoCA-4 NAs, as a function of the molar ratio of SQ-gem and isoCA-4.

	Molar Ratio (SQ-gem:isoCA-4)				
	4:1	2:1	1:1	1:1.5	1:2
Size (nm)	173 ± 4	165 ± 5	142 ± 6	Precipitation	Precipitation

Table S2. Size of SQCOOH/isoCA-4 NAs, as a function of the molar ratio of SQCOOH and isoCA-4.

	Molar Ratio (SQCOOH:isoCA-4)				
	10:1	2:1	1:1	1:2	1:5
Size (nm)	124 ± 8	100 ± 7	Precipitation	Precipitation	Precipitation

Table S3. SQ-gem/isoCA-4 NA size after dilution in water.

	0.001 L	1 L	5 L	10 L	50 L
Size	147	149	139	136	142
PSD	0.09	0.45	0.16	0.22	0.40

Table S4. Sensitivity of the LS174-T tumor cell line to squalene-based nanocomposites (IC_{50}, μM).

	LS174-T						
	3 h	3=>72 h*	6 h	6=>72 h*	24 h	24=>72 h*	72 h
Gemcitabine	> 25	0.250	> 25	0.100	> 25	0.005	0.004
isoCA-4	> 25	> 25	> 25	> 25	> 25	0.016	0.006
Gemcitabine + isoCA-4	> 25	0.016	> 25	0.006	> 25	0.006	0.006
SQ-gem NAs	> 25	> 25	> 25	0.250	> 25	0.016	0.032
SQ-gem/isoCA-4 NAs	> 25	> 25	> 25	0.250	> 25	0.012	0.006
SQ-gem NAs + isoCA-4	> 25	> 25	> 25	0.360	> 25	0.012	0.060
SQCOOH/isoCA-4 NAs	> 25	23	> 25	18	> 25	0.270	0.200
SQCOOH/isoCA-4 NAs + Gemcitabine	> 25	0.360	> 25	0.220	> 25	0.120	0.080
SQCOOH/isoCA-4 NAs + SQ-gem NAs	> 25	>25	> 25	0.120	> 25	0.105	0.020
SQCOOH NAs	> 25	> 25	> 25	> 25	> 25	> 25	> 25

* The cells were exposed to tested compounds for 3, 6, 24 and 72 h. After exposure to the drug the cells were washed by PBS and were then analyzed by measuring the half maximal inhibitory concentration of cell proliferation or were incubated in a new medium without any drug up to 72 h with further analysis by MTT assay.

Table S5. Sensitivity of HUVECs to squalene-based nanocomposites (IC_{50}, μM).

	HUVECs						
	3 h	3=>72 h*	6 h	6=>72 h*	24 h	24=>72 h*	72 h
Gemcitabine	> 25	0.400	> 25	0.250	> 25	0.016	0.005
isoCA-4	> 25	0.100	> 25	0.070	> 25	0.040	0.005
Gemcitabine + isoCA-4	> 25	0.032	> 25	0.025	> 25	0.025	0.005
SQ-gem NAs	> 25	0.175	> 25	0.175	> 25	0.060	0.057
SQ-gem/isoCA-4 NAs	> 25	0.025	> 25	0.025	> 25	0.025	0.005
SQ-gem NAs + isoCA-4	> 25	0.020	> 25	0.012	> 25	0.012	0.012
SQCOOH/isoCA-4 NAs	> 25	0.012	> 25	0.012	> 25	0.012	0.012
SQCOOH/isoCA-4 NAs + Gemcitabine	> 25	0.080	> 25	0.060	> 25	0.012	0.012
SQCOOH/isoCA-4 NAs + SQ-gem NAs	> 25	0.120	> 25	0.016	> 25	0.016	0.016
SQCOOH NAs	> 25	> 25	> 25	> 25	> 25	> 25	> 25

* The cells were exposed to tested compounds for 3, 6, 24 and 72 h. After exposure to the drug the cells were washed by PBS and were then analyzed by measuring the half maximal inhibitory concentration of cell proliferation or were incubated in a new medium without any drug up to 72 h with further analysis by MTT assay.

Table S6. Stereometric analysis of morphological changes in biopsies of heart, brain, small intestine, spleen, liver, kidneys and lungs.

Treatment	Heart	Brain	Small intestine	Kidneys	Spleen
SQ-gem/isoCA-4 NAs	no toxicity	no toxicity	no toxicity	no toxicity	no toxicity
SQ-gem NAs + isoCA-4	no toxicity	no toxicity	no toxicity	no toxicity	no toxicity
SQ-gem NAs + SQCOOH/isoCA-4 NAs	no toxicity	no toxicity	no toxicity	no toxicity	no toxicity
SQ-gem NAs	no toxicity	no toxicity	no toxicity	no toxicity	no toxicity
Gemcitabine + isoCA-4	no toxicity	no toxicity	no toxicity	no toxicity	no toxicity
Gemcitabine + SQCOOH/isoCA-4 NAs	no toxicity	no toxicity	no toxicity	no toxicity	no toxicity
Gemcitabine	no toxicity	no toxicity	no toxicity	no toxicity	no toxicity
isoCA-4	no toxicity	no toxicity	no toxicity	no toxicity	no toxicity
SQCOOH/isoCA-4 NAs	no toxicity	no toxicity	no toxicity	no toxicity	no toxicity
SQCOOH NAs	no toxicity	no toxicity	no toxicity	no toxicity	no toxicity
Saline	no toxicity	no toxicity	no toxicity	no toxicity	no toxicity

Treatment	Liver	Lungs
SQ-gem/isoCA-4 NAs	Regeneration of hepatocytes	no toxicity
SQ-gem NAs + isoCA-4	Slight lymphoid infiltration	no toxicity
SQ-gem NAs + SQCOOH/isoCA-4 NAs	no toxicity	no toxicity
SQ-gem NAs	Slight lymphoid infiltration	Slight lymphoid infiltration
Gemcitabine + isoCA-4	Regeneration of hepatocytes	Slight lymphoid infiltration
Gemcitabine + SQCOOH/isoCA-4 NAs	no toxicity	no toxicity
Gemcitabine	Slight lymphoid infiltration; Regeneration of hepatocytes	Slight lymphoid infiltration
isoCA-4	Rare necrotic hepatocytes	no toxicity
SQCOOH/isoCA-4 NAs	no toxicity	no toxicity
SQCOOH NAs	no toxicity	no toxicity
Saline	no toxicity	no toxicity

Table S7. Plasma pharmacokinetic parameters.

	AUC ∞ (nmol mL^{-1} h^{-1})		
	Gem+isoCA-4 (1)	SQ-gem/isoCA-4 NAs (2)	Factor 2/1
Gemcitabine	72.5	226.3	3.12
isoCA-4	272.5	102.1	0.37
SQ-gem	-	263.8	-
Total Gem*	72.5	490.1	6.8

*The total quantity of gemcitabine including gemcitabine and SQ-gem.

Table S8. Tumor pharmacokinetic parameters.

	AUC ∞ (nmol g^{-1} h^{-1})		
	Gem+isoCA-4 (1)	SQ-gem/isoCA-4 NAs (2)	Factor 2/1
Gemcitabine	134.5	6.8	0.05
isoCA-4	634.0	30.8	0.05
SQ-gem	-	1053.1	-
Total Gem*	134.5	1059.9	7.9

*The total quantity of gemcitabine including gemcitabine and SQ-gem.