Supporting Materials

Novel Hyaluronic Acid Conjugates for Dual Nuclear Imaging and Therapy in CD44-Expressing Tumors in Mice *In Vivo*

Ravindra Dhar Dubey[a], Rebecca Klippstein[b†], Julie Tzu-Wen Wang[b†], Naomi Hodgins[b], Kuo-Ching Mei[b], Jane Sosabowski[c], Robert C. Hider[b], Vincenzo Abbate[b], Prem N. Gupta[a], Khuloud T. Al-Jamal[b]*

[a]Formulation & Drug Delivery Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu-180001, India

[b]Institute of Pharmaceutical Science, Faculty of Life Sciences & Medicine, King's College London, Franklin-Wilkins Building, London SE19NH, United Kingdom

[c]Centre for Molecular Oncology, Bart's Cancer Institute, Queen Mary University of London, London, EC1M 6BQ, UK

*Corresponding author
Professor Khuloud T Al-Jamal
150 Stamford Street,
Institute of Pharmaceutical Science
King's College London
Franklin-Wilkins Building
150 Stamford Street
London SE1 9NH, UK
E-mail: khuloud.al-jamal@kcl.ac.uk

Dr Rebecca Klippstein, Dr Julie Tzu-Wen Wang, Dr Naomi Hodgins, Mr Kuo-Ching Mei, Prof. Robert Hider, Dr Vincenzo Abbate, Prof. Khuloud T. Al-Jamal
150 Stamford Street,
Institute of Pharmaceutical Science
King's College London
Franklin-Wilkins Building
150 Stamford Street
London SE1 9NH, UK

Dr Jane Sosabowski
Centre for Molecular Oncology

Bart's Cancer Institute, Queen Mary University of London,
London, EC1M 6BQ, UK

Dr Ravindra Dhar Dubey and Dr Prem N. Gupta
Formulation & Drug Delivery Division
CSIR-Indian Institute of Integrative Medicine
Canal Road, Jammu-180001, India

† Contributed equally to the work

Supplementary Information

Synthesis of HA-THP

To prepare HA-THP conjugate, the desalted HA (100 mg) was dissolved in 2 mL of anhydrous DMSO under nitrogen. Subsequently, EDC (96 mg) and NHS (58 mg) dissolved in anhydrous DMSO (1 mL), were slowly added to the HA solution. The mixture was stirred for 1 h at room temperature to activate the HA carboxylic group. After 1 h, THP-amine (5 mg) and TEA (70 μL) were added to the activated HA solution. Subsequently, the solution was allowed to react for 24 h at room temperature. The product was subsequently purified by successive dialysis for 4 days, changing media two times per day (MWCO 10 Da) against excess of 0.1 M NaCl, 25 % (v/v) ethanol solution and then deionized water at RT. The solution was then lyophilized and stored at -20 °C until further use. The amount of THP-amine in the conjugate was determined using a calibration curve of THP-amine measured at 288 nm **(Figure 3C)**. The calibration curve was prepared by dissolving known concentrations (2, 4, 8, 12, 20 μg/mL) of THP-amine in PBS (pH 7.4) **(Figure 3D)**.

Synthesis of HA-4'-AMF

To prepare HA-4'AMF conjugate, the desalted HA (100 mg) was dissolved in 2 mL of anhydrous DMSO under nitrogen. Subsequently, EDC (96 mg) and NHS (58 mg) dissolved in anhydrous DMSO (1 mL), were slowly added to the HA solution. The mixture was stirred for 1 h at room temperature to activate the HA carboxylic group. After 1 h, 4'-AMF (5 mg) and TEA (70 μL) were added to the activated HA solution. Subsequently, the solution was allowed to react for 24 h at room temperature. the produc was purified by successive dialysis for 4 days, changing media two times in a day (MWCO 10 kDa) against excess of 0.1 M NaCl, 25 % (v/v) ethanol

solution and then deionized water at room RT. The solution was then lyophilized and stored at -20 °C in the dark until further use. The amount of HA-4'-AMF in the conjugate was determined using a calibration curve of 4'-AMF prepared at emission spectrum of 515 nm when keeping excitation wavelength at 480 nm **(Figure 3E)**. The calibration curve was prepared by dissolving different concentration (0.5, 1, 2, 3, 4 μg/mL) of 4'-AMF in PBS (pH 10) **(Figure 3F)**.

Supplementary Figures

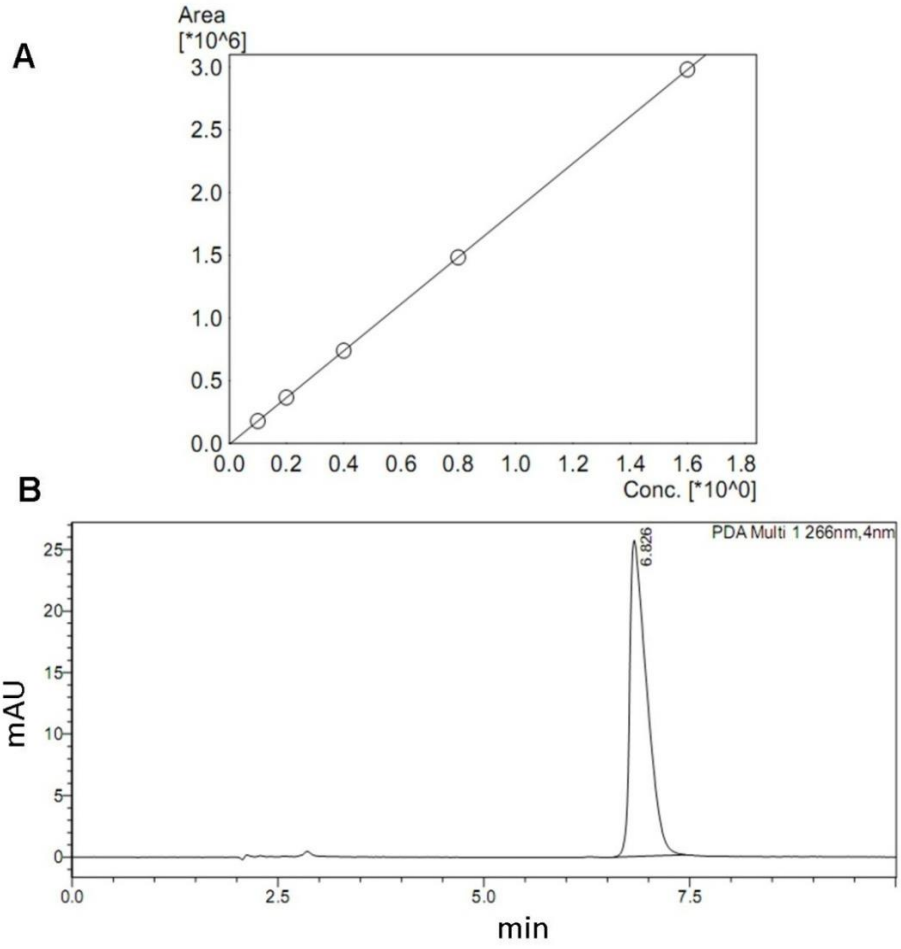

Figure S1: RP-HPLC quantification of Gem. (A) Calibration curve and **(B)** retention time of Gem in HPLC quantification. The experiment was done using RP-HPLC (SHIMADZU prominence, Japan), C-18 column (C-18G 250 x 4.5 mm) operated at 30˚C with PDA detector (SPD-M20A) at a wavelength of 266 nm. The mobile phase consisted of acetonitrile/water (70:30, v/v) with a flow rate of 1 mL/min. The retention time at 6.8 min was considered as Gem and the concentrations were calculated from Gem calibration curve prepared under the identical condition.

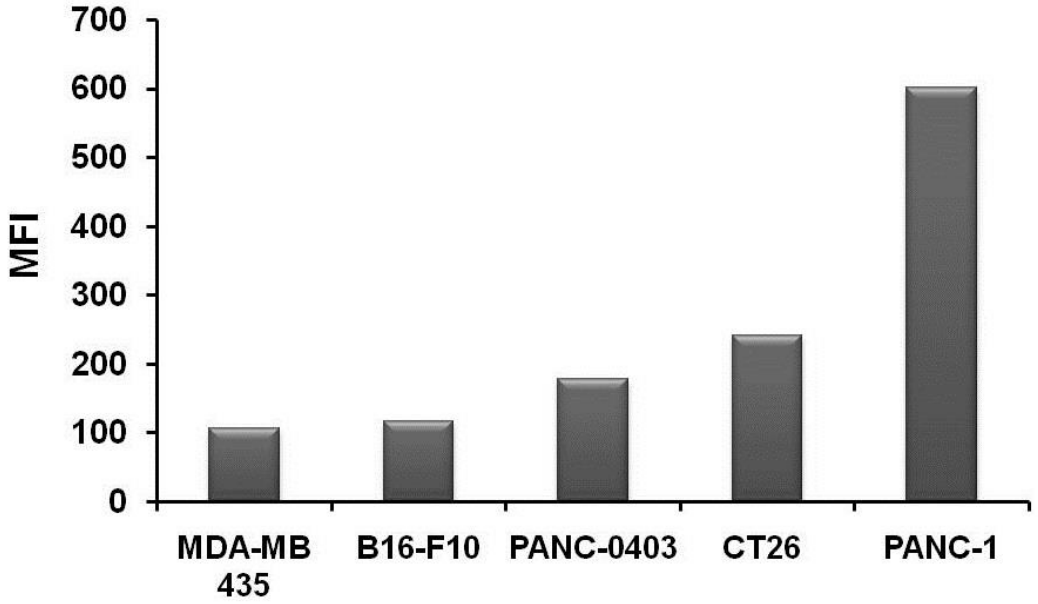

Figure S2: CD44 level of expression in a range of murine and human cancer cell lines. 5 x 10^5 cells of each cell line (PANC-1, PANC-0403, CT26, B16-F10 and MDA-MB 435) were incubated with rat anti-CD44 monoclonal antibodies (#ab119863, Abcam, UK) for 30 min at RT, stained with goat anti-rat antibodies conjugated with PE (#ab7010, Abcam, UK) for 30 min and then fixed with 4% PFA. The fluorescence was measured using BD FACS Calibur flow cytometer (BD Bioscience, USA). Mean fluorescence intensities (MFI) indicated the level of CD44 expression.

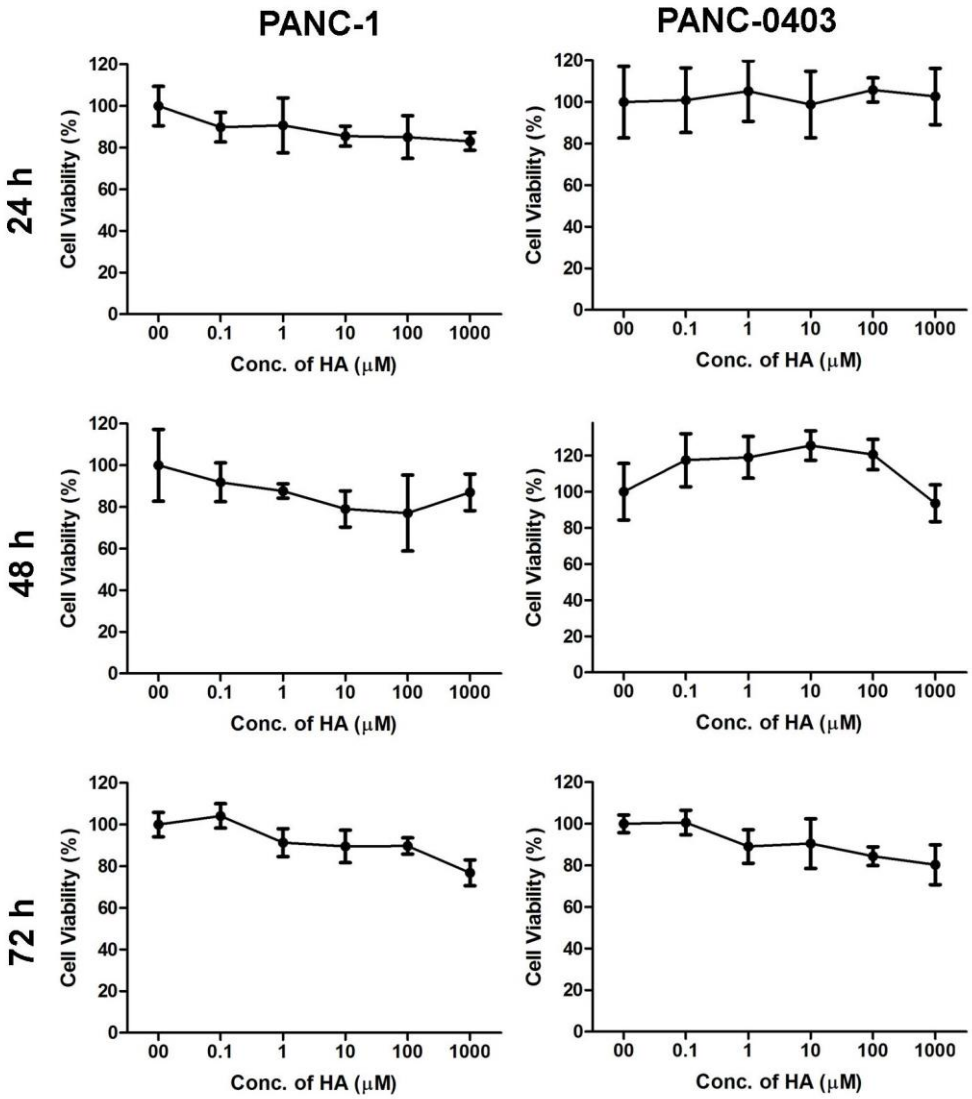

Figure S3: ***In vitro*** **cytotoxicity of HA.** Concentration-dependent cytotoxicity of hyaluronic acid (HA) on PANC-1 and PANC-0403 cells assessed with MTT assay after 24 h, 48 h and 72 h post-incubation. Percentage viability was calculated as a percentage of untreated cells and expressed as mean ± SD (n = 5).

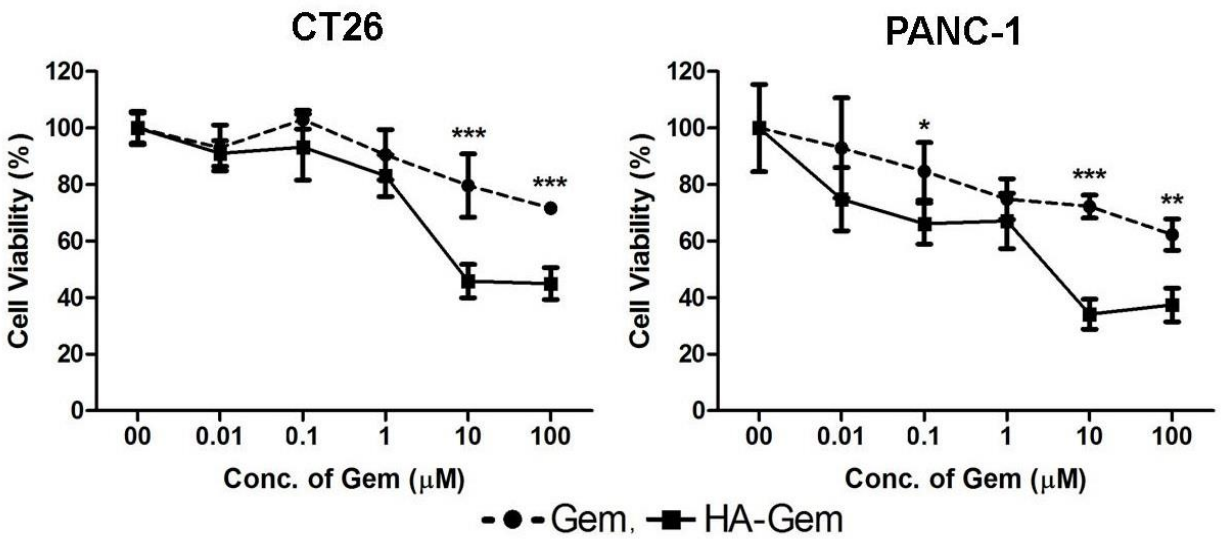

Figure S4: Nucleoside transporter inhibition assay. The effect of nucleoside transporter inhibition, using dipyridamol, on Gem or HA-Gem cytotoxicity, was studied with MTT assay, in CT26 and PANC-1 cells. Cells were pre-incubated with dipyridamol (10 μM) for 30 min, prior co-incubation with different concentration (0.01 to 100 μM) of Gem or HA-Gem for 24 h. Percentage viability was assessed with MTT assay and calculated as a percentage of untreated cells and expressed as mean ± SD (n = 5). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ Gem versus HA-Gem.

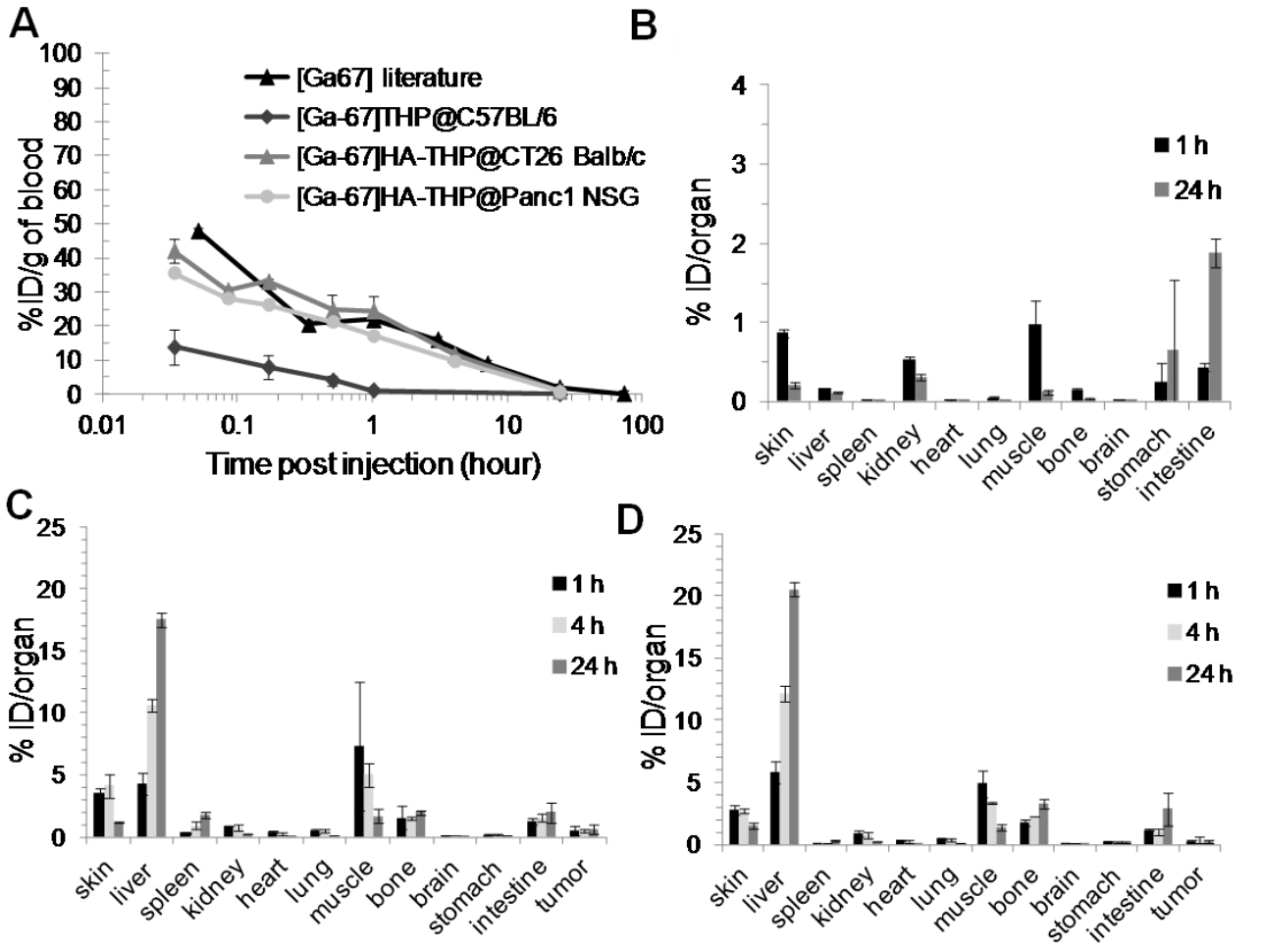

Figure S5: **Blood and organ biodistribution profiles of[^{67}Ga]THP-amine and [^{67}Ga]HA-THP.(A)** Blood profiles **(B-D)** Organ biodistribution of [^{67}Ga]THP-amine in normal C57BL/6 mice, [^{67}Ga]HA-THP in CT26 tumor-bearing BALB/C mice and [^{67}Ga]HA-THP in PANC-1 tumor-bearing NSG mice. Mice were i.v. injected with 1 MBq of [^{67}Ga]THP-amine (200 µg) or [^{67}Ga] HA-THP (200 µg) and major organs were excised at 1 , 4 and 24 h post injection. The radioactivity of the samples was measured by γ-scintigraphy with results expressed as %ID/organ. Data is presented as mean ± S.D. (n=3). The blood data of [^{67}Ga] in (A) is re-plotted from published paper Sephton et al., 1978.

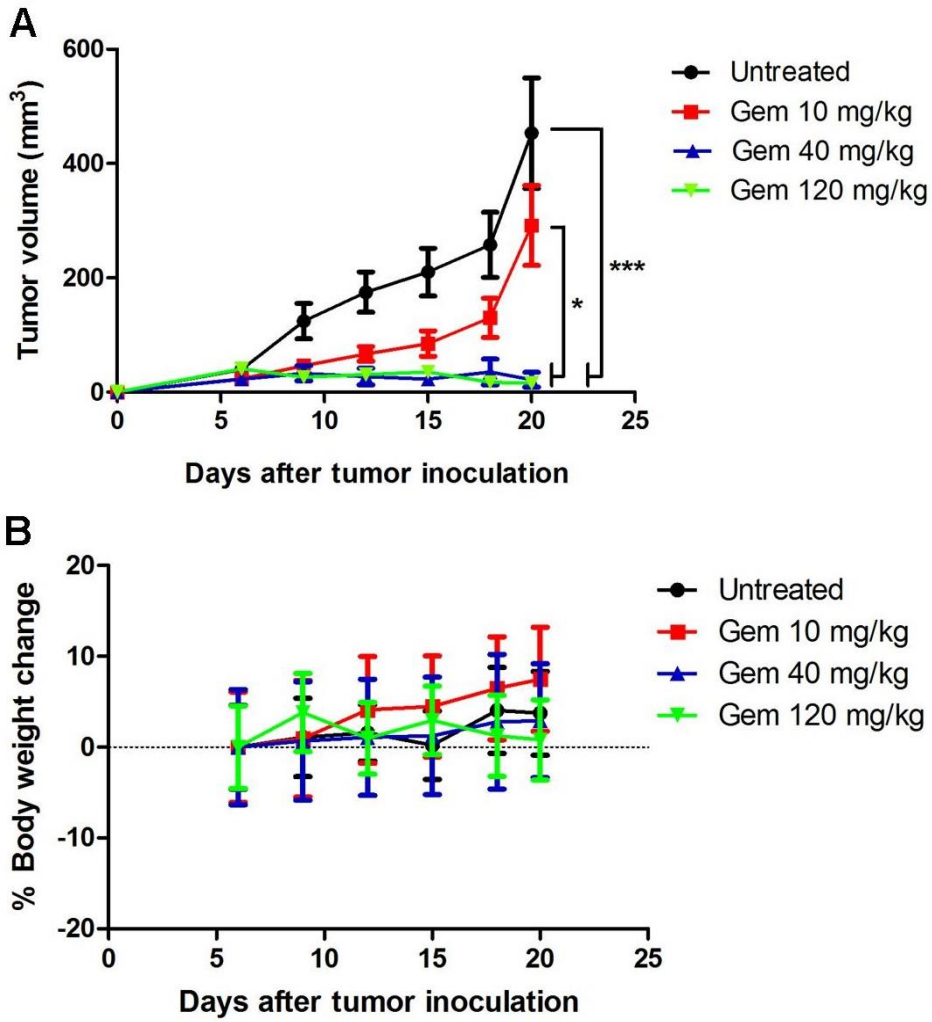

Figure S6: Evaluation of *in vivo* anti-tumor effect of Gem in CT26 tumor bearing BALB/c mice. (A) *In vivo* anti-tumor effect of gemcitabine (Gem) in the CT26 tumor bearing BALB/c mice. **(B)** Percentage change in body weight of mice. The mice were transplanted subcutaneously with 1×10^6 CT26 cells. The animals were then divided randomly into four groups: Untreated (control), 10 mg/kg Gem (free drug), 40 mg/kg Gem and 120 mg/kg Gem. Mice were administered a total of 4 injections intravenously on day 7, 10, 14 and 18 post-tumor inoculation at a drug dose of 10, 40 and 120 mg/kg of Gem. The size of the tumor was measured three times in a week. Data are presented as mean value ± SD (n = 9). The statics were applied using one-way ANOVA following Bonferroni post comparison test. *p< 0.05, *** P < 0.001.

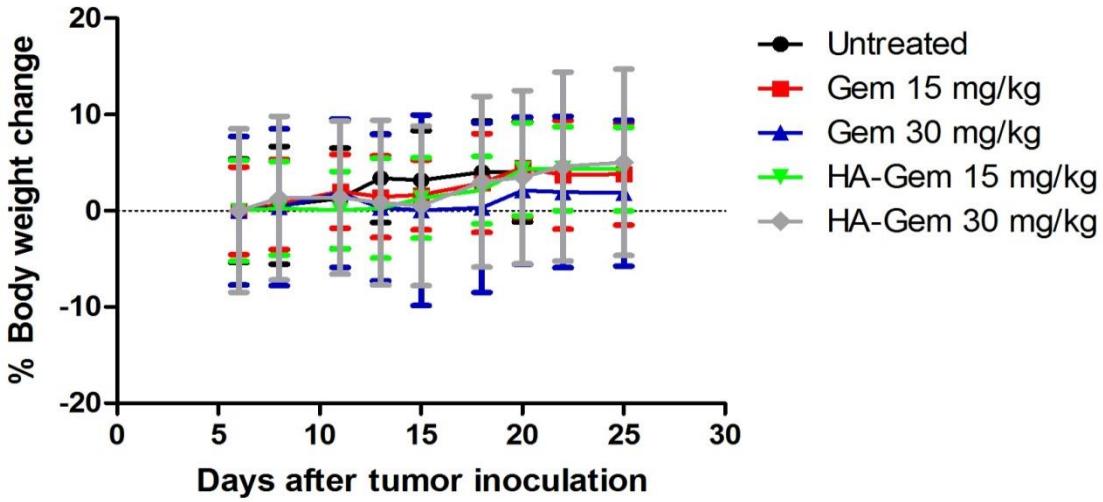

Figure S7: Percentage change body weight in CT26 tumor bearing BALB/c mice after treatment with Gem or HA-Gem. Percentage change in body weight after *in vivo* anti-tumor effect of gemcitabine (Gem) and hyaluronic acid conjugated gemcitabine (HA-Gem) in the CT26 tumor bearing BALB/c mice. The mice were transplanted subcutaneously with 1×10^6 CT26 cells. The animals were then divided randomly into five groups: Untreated (control), 15 mg/kg Gem (free drug), 30 mg/kg Gem, 15 mg/kg HA-Gem and 30 mg/kg HA-Gem. Mice were intravenously administered a total of 4 injections on day 7, 10, 14 and 18 post-tumor inoculation with the particular treatment. The body weight of the animal was measured three times in a week. Data are given as mean value ± SD (n = 8).

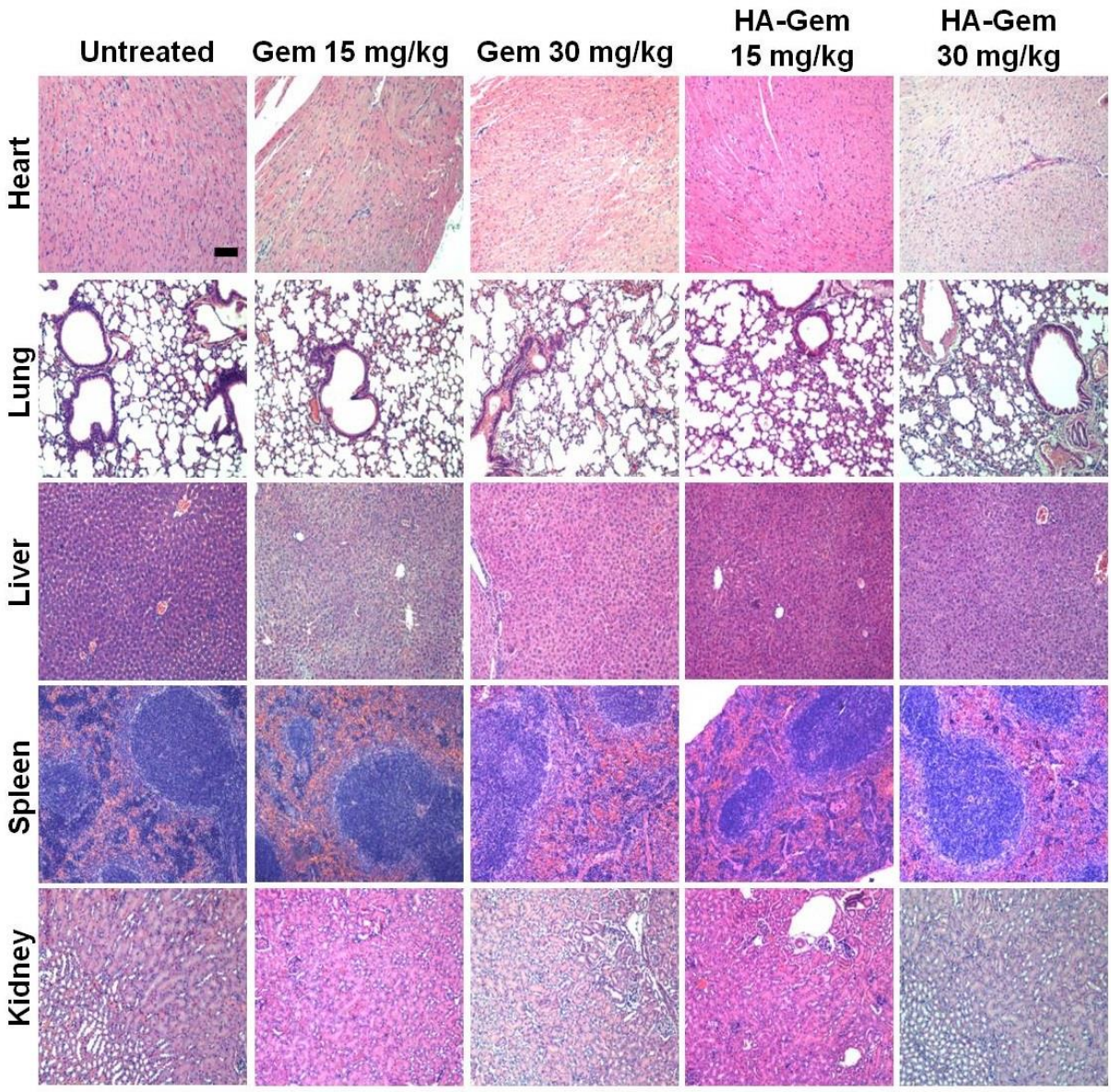

Figure S8: Histology examination of major organs in CT26 tumor-bearing BALB/c mice after treatment of Gem or HA-Gem. Mice transplanted subcutaneously with CT26 cells were divided randomly into five groups: Untreated (control), 15 mg/kg Gem (free drug), 30 mg/kg Gem, 15 mg/kg HA-Gem and 30 mg/kg HA-Gem. Mice were intravenously administered a total of 4 injections on day 7, 10, 14 and 18 post-tumor inoculation with the particular treatment. Major organs including heart, lung, liver, spleen and kidney are excised at the experimental end point of each group, and the tissue sections were preceded for H&E staining. No obvious histological changes were observed in these organs after treatments compared to the untreated group. Scale bar: 100 μm.

Supplementary Tables

Table S1: Major organ biodistribution at 1, 4 and 24 h post injection of HA in CT26 and PANC-1 bearing mice (μg (HA)/g tissue).

Organs	CT26 tumor-bearing mice (μg (HA)/g tissue)			PANC-1 tumor-bearing mice (μg (HA)/g tissue)		
	1 h	4 h	24 h	1 h	4 h	24 h
Skin	2.66±0.15	2.70±0.30	1.16±0.03	1.47±0.14	1.40±0.07	0.88±0.06
Liver	10.80±1.05	24.37±0.45	33.50±1.65	8.60±0.51	17.72±1.59	39.42±0.55
Spleen	5.64±1.18	15.54±2.59	38.91±2.98	4.03±0.32	10.45±1.53	33.52±0.97
Kidneys	7.31±0.23	6.10±0.72	1.85±0.07	4.45±0.73	3.92±0.41	1.43±0.05
Heart	8.51±0.88	6.17±1.23	0.82±0.04	4.93±0.47	3.37±0.69	0.53±0.03
Lungs	7.86±0.94	7.79±0.86	1.06±0.06	5.98±0.35	4.96±0.72	0.86±0.06
Muscle	1.73±0.59	1.16±0.12	0.45±0.07	0.78±0.08	0.56±0.00	0.28±0.02
Bone	3.09±0.88	2.90±0.10	4.47±0.18	2.43±0.14	3.12±0.06	5.24±0.22
Brain	0.79±0.08	0.52±0.03	0.06±0.01	0.60±0.00	0.32±0.12	0.06±0.00
Stomach	1.32±0.20	3.53±1.93	1.05±0.37	0.56±0.04	0.52±0.09	1.29±0.37
Intestine	2.16±0.18	2.01±0.24	4.70±1.08	1.83±0.42	1.08±0.10	4.30±1.14
Tumor	1.96±0.27	2.85±0.24	3.48±0.23	1.42±0.11	1.87±0.25	1.58±0.10