Supporting Information for:

Improving Cancer Chemoradiotherapy Treatment by Dual Controlled Release of Wortmannin and Docetaxel in Polymeric Nanoparticles

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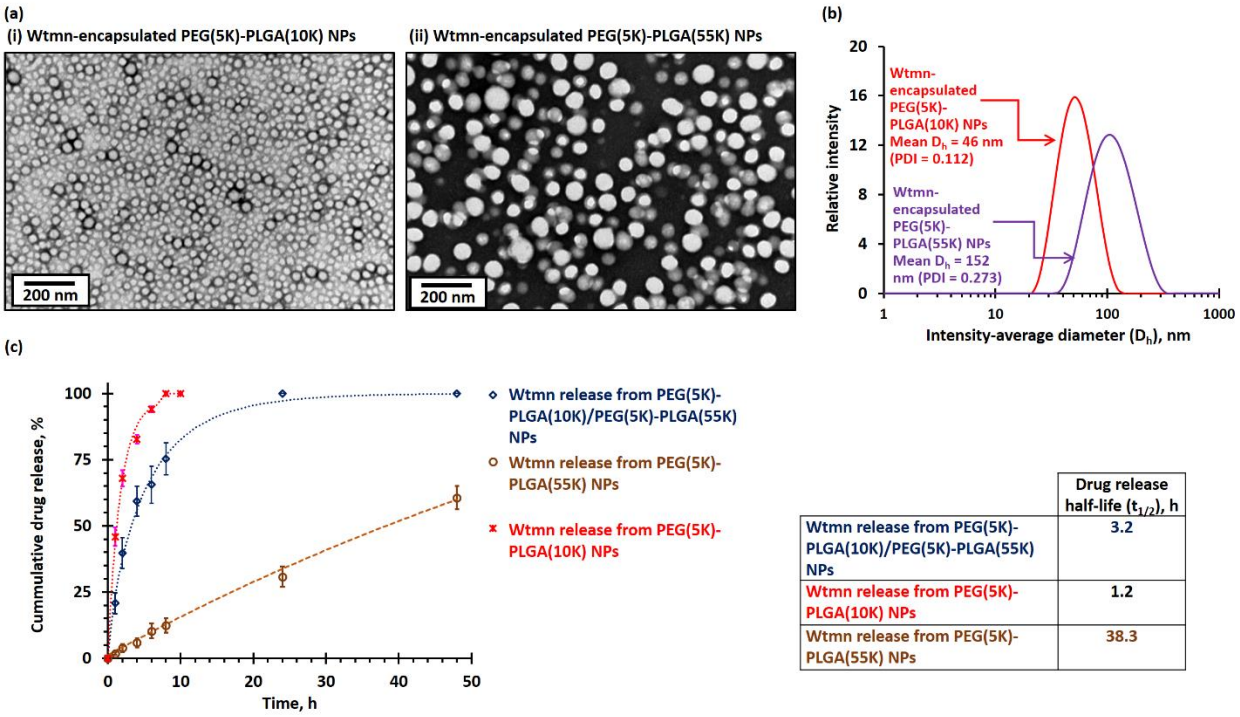
SUPPORTING FIGURES



	Drug release half-life ($t_{1/2}$), h
Wtmn release from PEG(5K)-PLGA(10K)/PEG(5K)-PLGA(55K) NPs	3.2
Wtmn release from PEG(5K)-PLGA(10K) NPs	1.2
Wtmn release from PEG(5K)-PLGA(55K) NPs	38.3

Figure S1. (a) TEM images recorded for Wtmn-encapsulated PEG-PLGA nanoparticles prepared from (i) pure PEG(5K)-PLGA(10K) and (ii) pure PEG(5K)-PLGA(55K) di-block copolymers. The average number-average diameter were found to be 32 ± 4 nm and 59 ± 13 nm, respectively. (b) Intensity-average diameter distribution curves recorded for Wtmn-encapsulated PEG-PLGA nanoparticles prepared from pure PEG(5K)-PLGA(10K) and pure PEG(5K)-PLGA(55K) di-block copolymers. The mean intensity-average diameter (D_h) of the Wtmn-encapsulated PEG(5K)-PLGA(10K) and pure PEG(5K)-PLGA(55K) nanoparticles were found to be 46 nm (PDI = 0.112) and 152 nm (PDI = 0. 273), respectively, as determined by dynamic light scattering (DLS) method. The Wtmn-encapsulated PEG(5K)-PLGA(55K) nanoparticles are larger and less monodisperse than the Wtmn-encapsulated PEG(5K)-PLGA(10K) nanoparticles. (c) Drug release kinetics of PEG-PLGA nanoparticles prepared from 4:1 mixture of PEG(5K)-PLGA(10K)/PEG(5K)-PLGA(55K), PEG(5K)-PLGA(10K) and PEG(5K)-PLGA(55K).

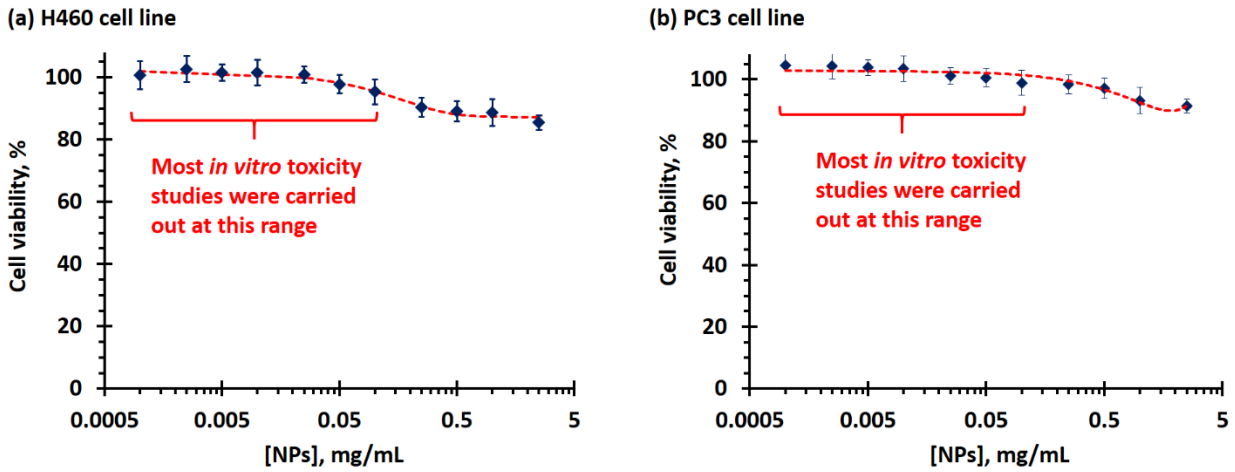


Figure S2. Toxicity of drug-free ("empty") PEG-PLGA nanoparticles in H460 and PC3 cell lines.

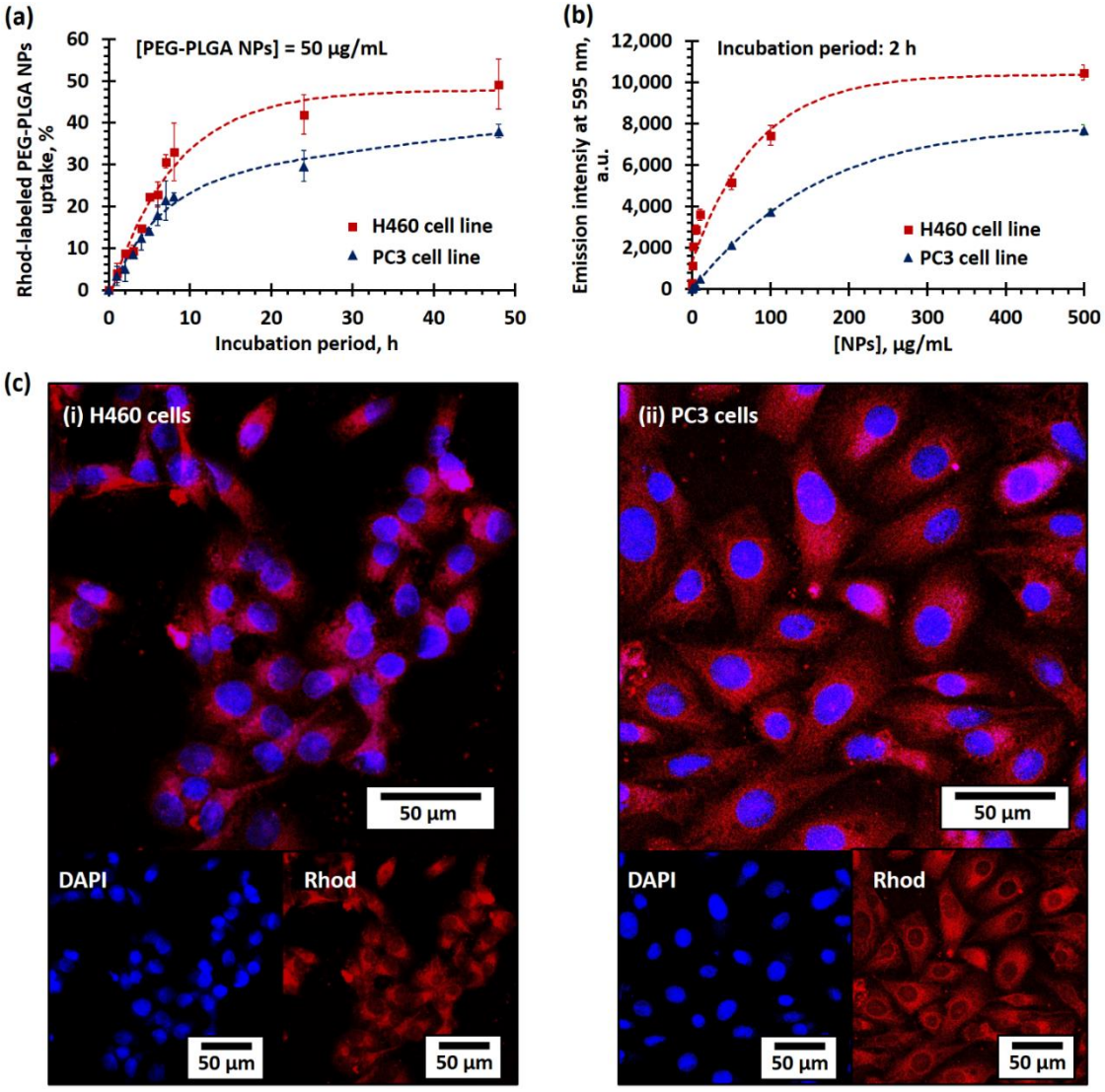


Figure S3. *In vitro* cellular uptake of PEG-PLGA nanoparticles. (a) Time-dependent cellular uptake of 50 µg/mL (4.68×10^{10} particles per mL) of Rhod-labeled PEG-PLGA NPs at physiological conditions. (b) Concentration-dependent fluorescence intensity at 595 nm of H460 and PC3 cells after incubation with different concentrations of Rhod-labeled PEG-PLGA NPs. The fluorescence intensity at 595 nm is directly proportional to the amount of Rhod-labeled PEG-PLGA NPs taken up by the cancer cells. (c) and (d) Representative confocal fluorescent images recorded for (c) H460 and (d) PC3 cells after incubation with 50 µg/mL of Rhod-labeled PEG-PLGA NPs (red) for 30 min. The nuclei were counter stained with 4',6-diamidino-2-

phenylindole (DAPI, blue). The confocal images indicate that the sub-50 nm diameter PEG-PLGA nanoparticles rapidly localized at the cytoplasm of the cancer cells.

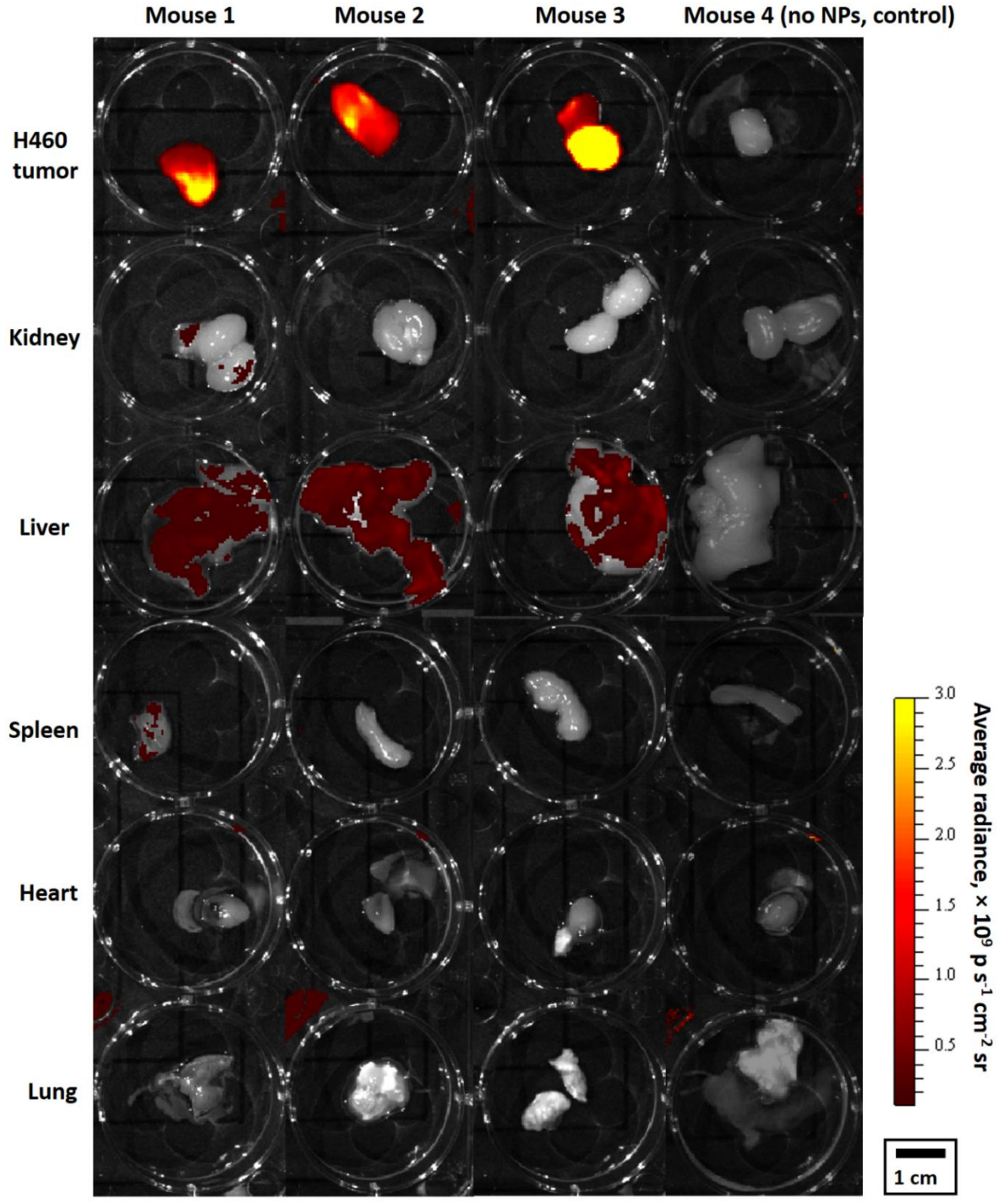


Figure S4. Biodistribution of Rhod-labeled PEG-PLGA nanoparticles in H460 tumor-bearing Numice. *Ex vivo* fluorescent images of xenograft tumor, kidney, liver, spleen, heart, and lung

collected 48 h after tail-vein i.v. injection of Rhod-labeled PEG-PLGA nanoparticles or PBS (control).

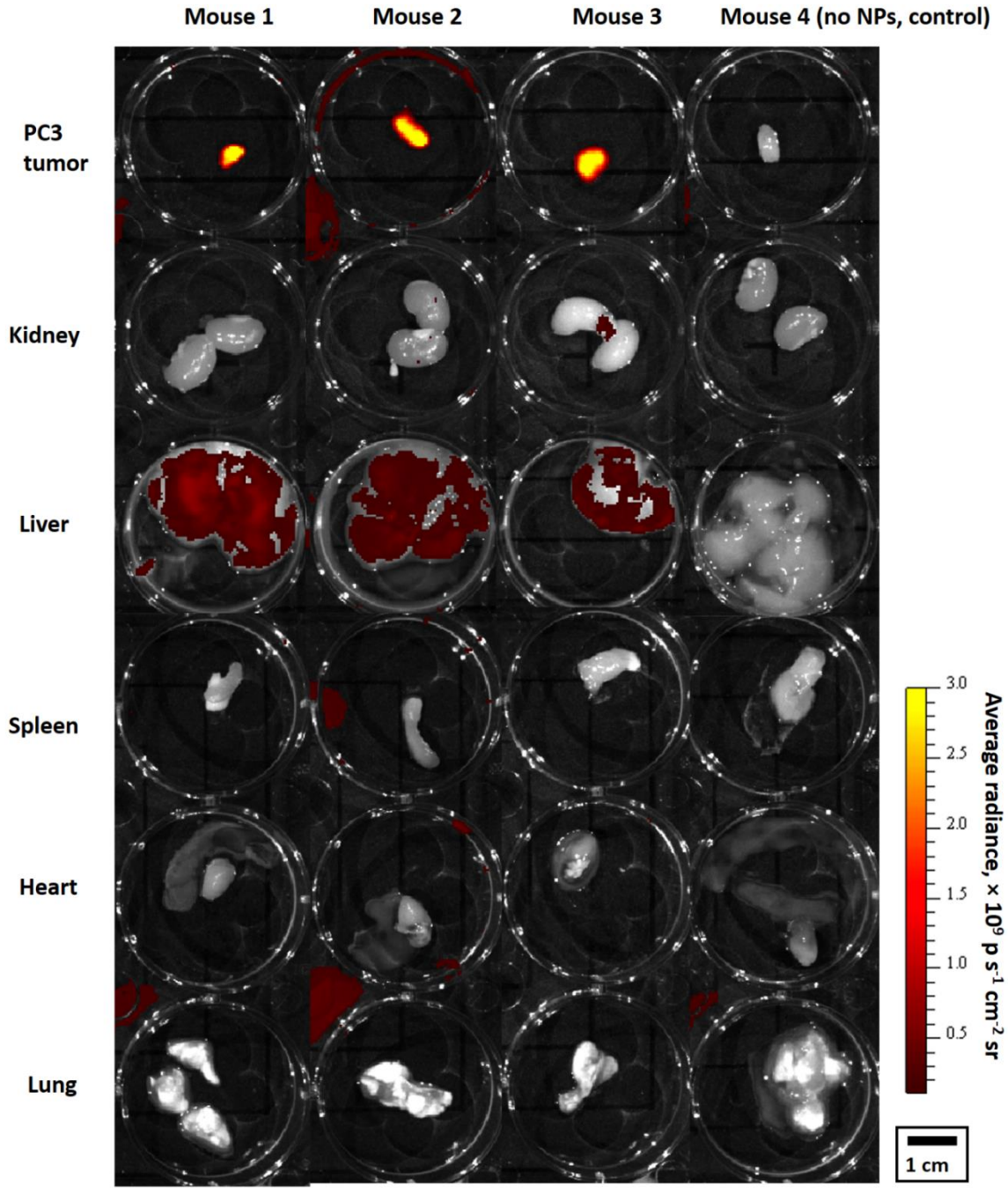


Figure S5. Biodistribution of Rhod-labeled PEG-PLGA nanoparticles in PC3 tumor-bearing Nu mice. *Ex vivo* fluorescent images of xenograft tumor, kidney, liver, spleen, heart, and lung

collected 48 h after tail-vein i.v. injection of Rhod-labeled PEG-PLGA nanoparticles or PBS (control).

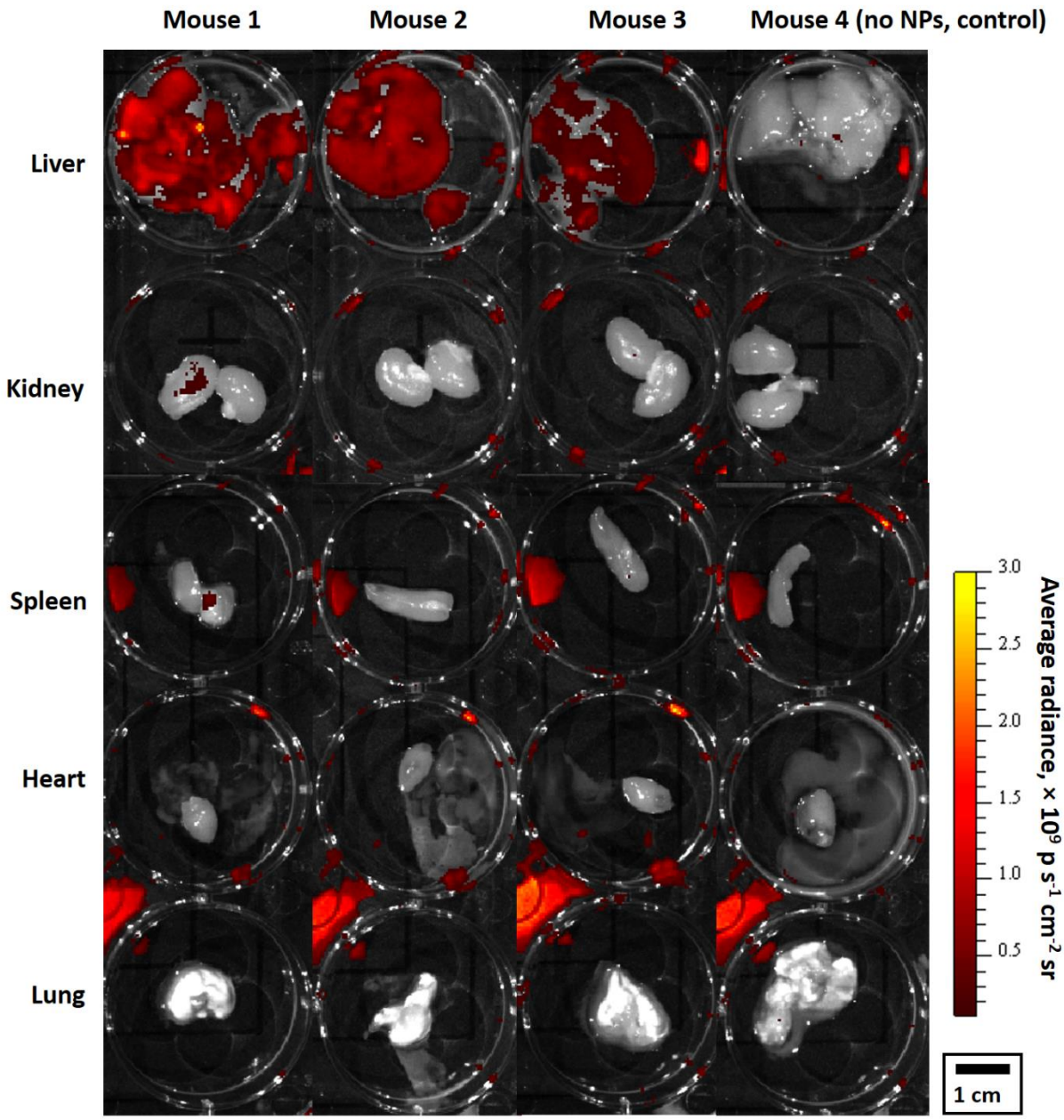


Figure S6. Biodistribution of Rhod-labeled PEG-PLGA nanoparticles in healthy tumor-free Nu mice. *Ex vivo* fluorescent images of kidney, liver, spleen, heart, and lung collected 48 h after tail-vein i.v. injection of Rhod-labeled PEG-PLGA nanoparticles or PBS (control).

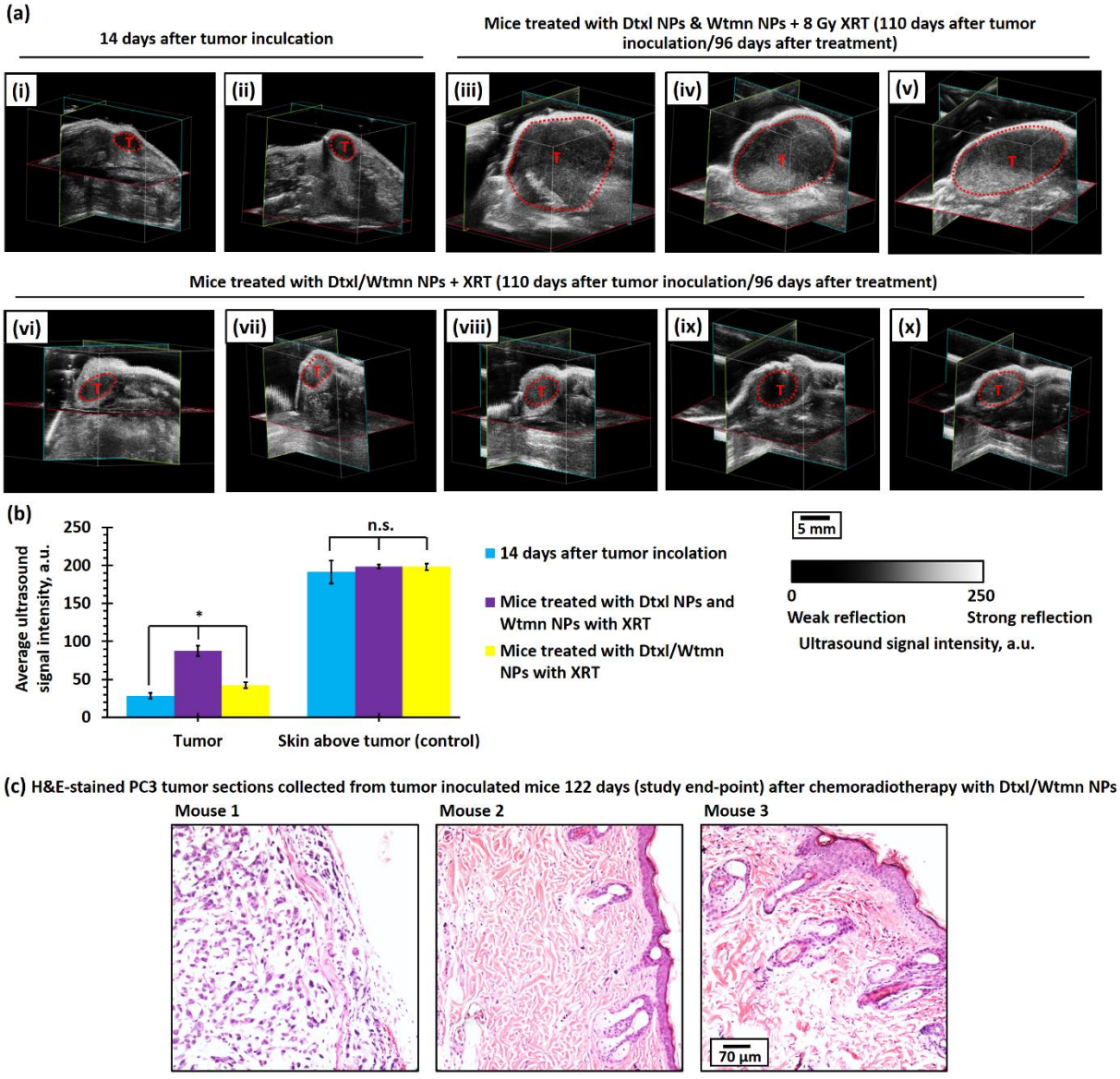


Figure S7. Morphology of PC3 xenograft tumors after different combination chemoradiotherapy treatments. (a) 3D ultrasound images of xenograft tumors recorded (i and ii) before and (iii - x) 96 days after different combination chemoradiotherapy treatments (either with (iii-v) Dtxl NPs and Wtmn NPs or (vi - x) Dtxl/Wtmn NPs). (b) The plot of average ultrasound signal intensity of different xenograft tumors after different treatments. The average ultrasound intensities were based on the average of five B-scans recorded from each mice (n = 3 - 5 per group). (d)

Representative H&E stained PC3 tumor sections of tumor-bearing mice treated Dtxl/Wtmn NPs and 8 Gy fractionated radiotherapy collected at the end-point of the *in vivo* study (122 days after chemoradiotherapy).

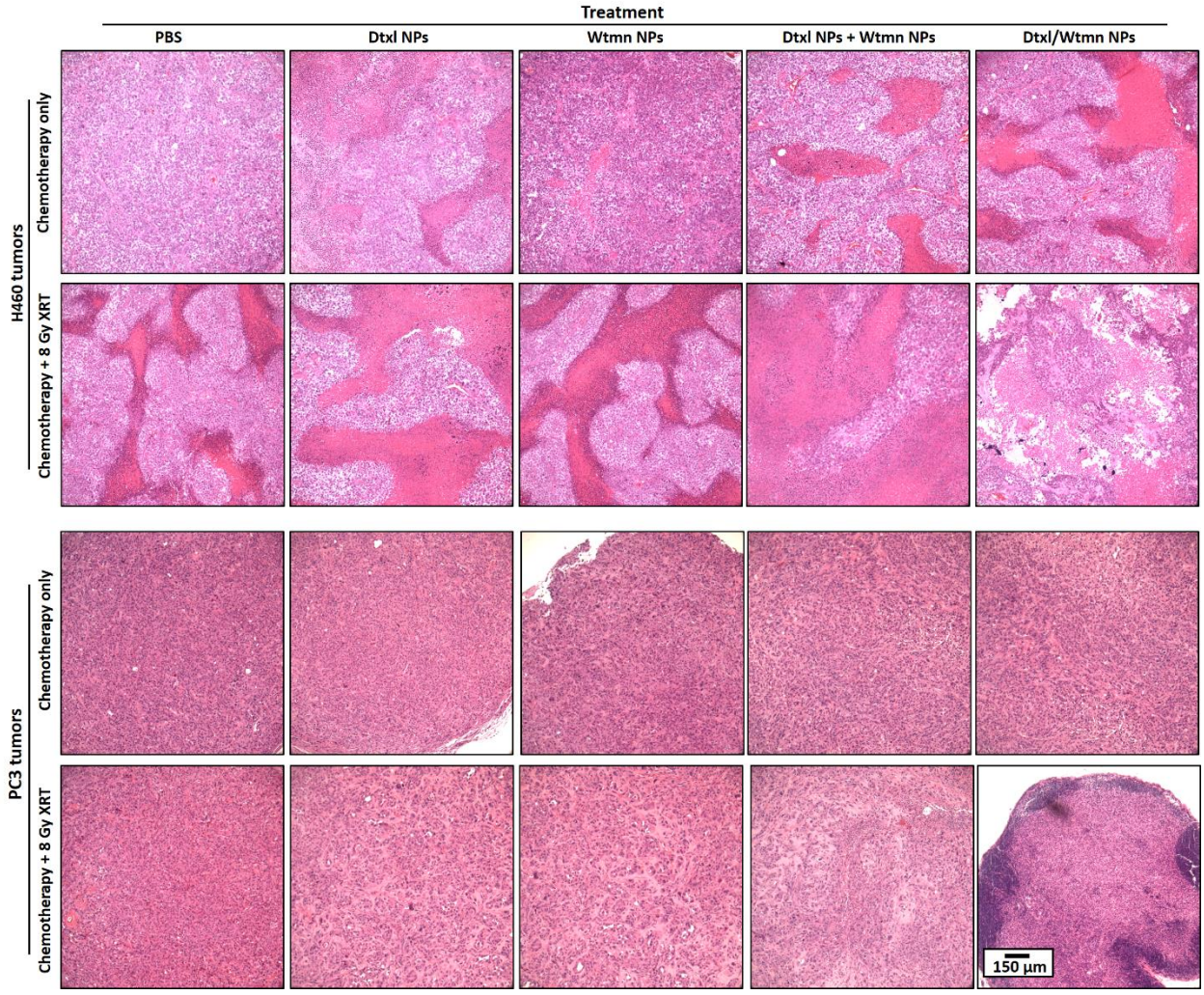


Figure S8. Representative H&E-stained H460 and PC3 xenograft tumor sections collected from xenograft tumor-bearing mice 4 days after i.v. administration of Dtxl NPs, Wtmn NPs, a combination of Dtxl NPs and Wtmn NPs, or Dtxl/Wtmn co-encapsulated NPs for chemotherapy/chemoradiotherapy.

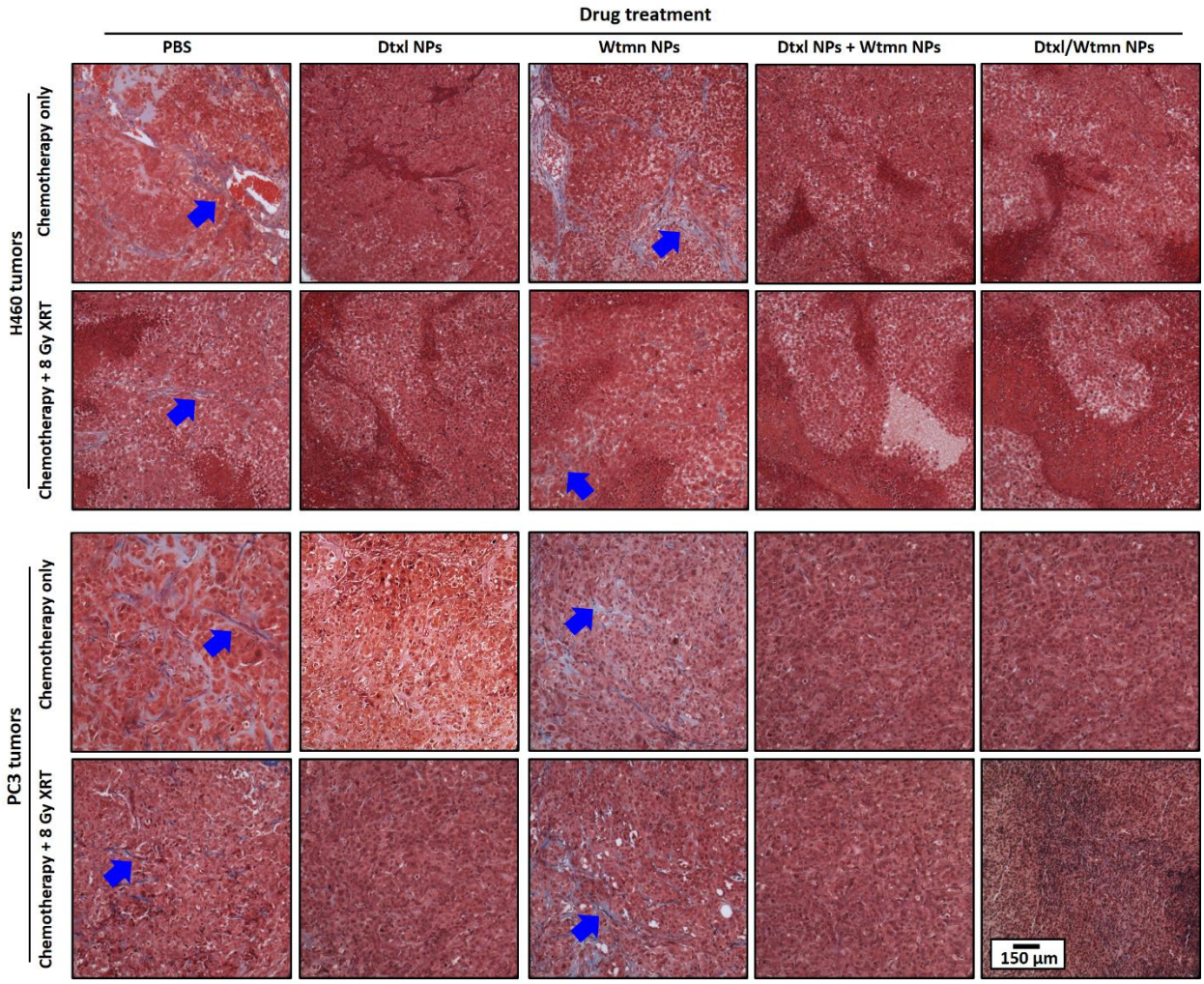


Figure S9. The change of tissue microenvironments in H460 and PC3 xenograft tumors after different chemotherapy or chemoradiotherapy treatments. Representative optical micrographs of Masson's trichrome-stained tumor sections collected from xenograft tumor-bearing mice 4 days after i.v. administration of Dtxl NPs, Wtmn NPs, a combination of Dtxl NPs and Wtmn NPs, or Dtxl/Wtmn co-encapsulated NPs for chemotherapy/chemoradiotherapy. The Masson's trichrome stain labeled the porous collagen fibers in blue (labeled by ➔). Normalization of tissue microenvironments can be observed in tumor sections that received treatment with Dtxl NPs, Dtxl NPs and Wtmn NPs or Dtxl/Wtmn NPs due to the antiangiogenic effect of Dtxl.

SUPPORTING TABLES

	Mean D_n, nm[a]	Mean D_n, nm[b]	Average number of particles formed from 1 mg of diblock copolymers[b]	Mean D_h, nm (and PDI)[c]	Mean zeta potential, mV[d]	Encapsulation efficiency, %[e]
Empty NPs	33 ± 5	55	1.19×10^{12}	63 (0.063)	- 0.8	/
Wtmn NPs	34 ± 6	56	1.07×10^{12}	67 (0.055)	- 1.2	24.5
Dtxl NPs	36 ± 6	55	1.04×10^{12}	63 (0.059)	- 0.7	9.1
4:1 Dtxl/Wtmn NPs[f]	37 ± 7	57	1.06×10^{12}	64 (0.069)	- 1.4	Dtxl: 24.2[f] Wtmn: 8.9[f]
8:1 Dtxl/Wtmn NPs[g]	36 ± 4	54	1.08×10^{12}	68 (0.067)	- 0.9	Dtxl: 23.6[g] Wtmn: 8.6[g]
Rhod-labeled empty NPs	39 ± 9	59	9.36×10^{11}	69 (0.124)	- 2.1	/

[a] determined by TEM on dried PEG-PLGA NPs (counted from at least 150 particles); [b] determined by NTA on 5 μg/mL of PEG-PLGA NP dispersions; [c] determined by DLS on PEG-PLGA NP dispersions (in 0.1 M PBS); [d] determined by aqueous electrophoresis method; [e] determined by quantitative HPLC method; [f] *target* Dtxl/Wtmn molar ratio = 3:2, *actual* Dtxl/Wtmn molar ratio ≈ 4:1; [g] *target* Dtxl/Wtmn molar ratio = 3:1, *actual* Dtxl/Wtmn molar ratio ≈ 8:1.

Table S1. The mean D_n, average particle concentration, mean D_h (and polydispersity index, PDI), and mean zeta potential of different drug(s)-loaded PEG-PLGA nanoparticles, as determined by TEM, NTA, DLS, and aqueous electrophoresis methods.

	Drug release kinetics (Weibull model)					
	$Q_{0,48h}$, %	b	t_{lag}, h	t_{scale}, h	R^2	$t_{1/2}$, h
Dtxl NPs	93.5	1.8	1.5	83.9	0.998	15.5
Wtmn NPs	100.0	0.8	0.0	3.8	0.994	3.2
4:1 Dtxl/Wtmn NPs						
encapsulated Dtxl	93.2	1.8	1.5	84.4	0.995	14.7
encapsulated Wtmn	100.0	0.8	0.1	4.0	0.996	3.7
8:1 Dtxl/Wtmn NPs						
encapsulated Dtxl	94.3	1.8	1.5	89.5	0.994	15.7
encapsulated Wtmn	100.0	0.9	0.1	3.8	0.996	4.0

Table S2. Key drug release parameters of different single and dual-drug loaded PEG-PLGA nanoparticles obtained after being fitted to Weibull model and drug release half-life ($t_{1/2}$).

Free/encapsulated drug(s)	IC_{50}, μM	
	H460 cell line	PC3 cell line
Free Dtxl	0.28	7.4
Dtxl NPs	1.1	12.1
Free Wtmn	14.0	23.1
Wtmn NPs	20.0	34.7
4:1 free Dtxl + free Wtmn		
Dtxl pre-treatment	0.51	11.2
Wtmn pre-treatment	0.04	0.9
Dtxl and Wtmn co-treatment	0.31	10.1
4:1 Dtxl NPs + Wtmn NPs	0.17	1.1
4:1 Dtxl/Wtmn NPs	0.15	1.2
8:1 free Dtxl + free Wtmn		
Dtxl pre-treatment	0.49	7.6
Wtmn pre-treatment	0.18	1.9
Dtxl and Wtmn co-treatment	0.37	5.5
8:1 Dtxl NPs + Wtmn NPs	0.38	3.5
8:1 Dtxl/Wtmn NPs	0.48	4.8

Table S3. *In vitro* toxicities of small molecules and encapsulated drugs. IC_{50} of different single and dual small molecule ("free") and encapsulated drug treatments obtained from the MTS assay of H460 and PC3 cell lines.

	H460 cell line			PC3 cell line		
	Quasi threshold dose (D_q), Gy	Dose required to achieve survival fraction of 0.1, Gy	Dose enhancement factor (E.F.) to achieve survival faction of 0.1	Quasi threshold dose (D_q), Gy	Dose required to achieve survival fraction of 0.1, Gy	Dose enhancement factor (E.F.) to achieve survival faction of 0.1
PBS (control)	3.38	5.16	/	3.70	5.36	/
Free Dtxl	2.43	4.24	1.22	2.54	4.22	1.27
Dtxl NPs	2.77	4.53	1.14	2.18	4.22	1.27
Free Wtmn	1.78	3.44	1.50	2.36	3.54	1.52
Wtmn NPs	1.64	3.25	1.59	2.92	3.81	1.41
Free Dtxl/free Wtmn (co-treatment)	1.62	2.78	1.86	1.79	2.89	1.86
Free Dtxl ➔ free Wtmn	1.81	3.04	1.69	2.22	2.96	1.81
Free Wtmn ➔ free Dtxl	1.20	2.61	1.97	1.45	2.74	1.96
Dtxl NPs + Wtmn NPs	1.18	2.34	2.21	1.70	2.73	1.96
Dtxl/Wtmn NPs	0.94	2.27	2.27	1.42	2.51	2.14

Table S4. Table summarizes the Quasi threshold dose (D_q), radiation dose required to achieve a survival fraction of 0.1 and the corresponding dose enhancement factor (E.F.) obtained after being fitted the radiation dose-dependent survival fraction to linear-quadratic model (see Experimental for details).

	Tumor growth delay			
	Time in day required for tumor to grow to 40 times of its initial volume	Absolute growth delay (A.G.D.), day(s) [a]	Normalized growth delay (N.G.D.), days [b]	Enhancement factors (E.F.) (change in tumor sensitivity to XRT, %)[c]
PBS only (control)	19.2 ± 0.8			
Empty NPs	19.8 ± 0.7	0.6 ± 0.7		
Dtxl NPs	20.0 ± 0.9	0.8 ± 0.9		
Wtmn NPs	19.6 ± 1.2	0.4 ± 1.2		
Dtxl NPs + Wtmn NPs	21.6 ± 0.7	2.4 ± 0.7		
Dtxl/Wtmn NPs	24.0 ± 0.6	4.8 ± 0.6		
PBS + XRT	31.6 ± 1.2	12.4 ± 1.2		
Empty NPs + XRT	32.1 ± 1.5	12.7 ± 1.5	12.1 ± 1.5	0.99 (- 1 %)
Dtxl NPs + XRT	33.9 ± 0.7	14.7 ± 0.7	13.9 ± 0.7	1.12 (+ 12 %)
Wtmn NPs + XRT	36.2 ± 0.9	17.0 ± 0.9	16.6 ± 0.9	1.34 (+ 34 %)
Dtxl NPs + Wtmn NPs + XRT	40.4 ± 1.2	21.2 ± 1.2	18.8 ± 1.2	1.52 (+ 52 %)
Dtxl/Wtmn NPs + XRT	52.4 ± 1.1	33.2 ± 1.1	28.4 ± 1.1	2.29 (+ 129 %)

[a] Absolute growth delay (A.G.D.) caused by Wtmn and/or Dtxl (co)treatment with/without concurrent XRT is defined as the time in day(s) tumors required to reach 40 times of its initial volumes minus the time in days untreated tumors required to grow to 40 times of its initial volume.
[b] Normalized growth delay (N.G.D.) is defined as time in days for tumors to reach 40 times of its initial volume in mice treated by drug-free ("empty") PEG-PLGA NPs, Wtmn NPs and/or Dtxl NPs, or Dtxl/Wtmn NPs and radiation minus the time in days for tumors to reach 40 times of it is volume in mice only received chemotherapy.
[c] Enhancement factors (E.F.) : obtained by dividing normalized tumor growth delay in mice treated with different chemotherapy plus radiation by the absolute growth delay in mice treat with radiation only.

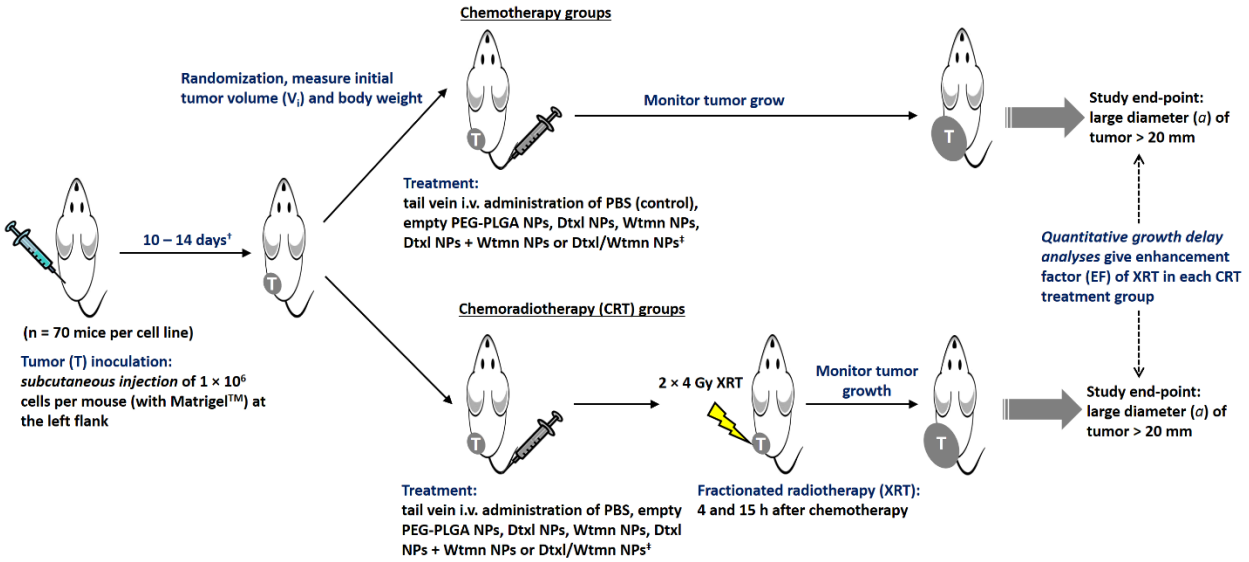
Table S5. Effect of (co-)encapsulated Dtxl and Wtmn on radioresponse of H460 xenograft tumor measured by tumor growth delay.

	Tumor growth delay			
	Time in day required for tumor to grow to 40 times of its initial volume [a]	Absolute growth delay (A.G.D.), day(s) [b]	Normalized growth delay (N.G.D.), days [c]	Enhancement factors (E.F.) (change in tumor sensitivity to XRT, %)[d]
PBS only (control)	43.5 ± 0.8			
Empty NPs	69.8 ± 1.1	0.3 ± 1.1		
Dtxl NPs	50.4 ± 1.1	6.9 ± 1.1		
Wtmn NPs	48.6 ± 1.3	5.1 ± 1.3		
Dtxl NPs + Wtmn NPs	59.4 ± 1.2	15.9 ± 1.2		
Dtxl/Wtmn NPs	65.4 ± 1.7	21.9 ± 1.7		
PBS + XRT	67.5 ± 0.7	26.3 ± 0.7		
Empty NPs + XRT	69.0 ± 1.3	25.5 ± 1.3	25.2 ± 1.3	0.96 (- 4 %)
Dtxl NPs + XRT	80.2 ± 1.7	36.7 ± 1.7	29.8 ± 1.7	1.14 (+ 14 %)
Wtmn NPs + XRT	85.4 ± 0.6	36.8 ± 0.6	36.8 ± 0.6	1.40 (+ 40 %)
Dtxl NPs + Wtmn NPs + XRT	99.6 ± 1.1	56.1 ± 1.1	40.2 ± 1.1	1.53 (+ 53 %)
Dtxl/Wtmn NPs + XRT	> 126	> 82.5	> 60.6	> 2.30 (+ > 130 %)

[a] Absolute growth delay (A.G.D.) caused by Wtmn and/or Dtxl (co)treatment with/without concurrent XRT is defined as the time in day(s) tumors required to reach 40 times of its initial volumes minus the time in days untreated tumors required to grow to 40 times of its initial volume.
[b] Normalized growth delay (N.G.D.) is defined as time in days for tumors to reach 40 times of its initial volume in mice treated by drug free ("empty") PEG-PLGA NPs, Wtmn NPs and/or Dtxl NPs, or Dtxl/Wtmn NPs and radiation minus the time in days for tumors to reach 40 times of it is volume in mice only received chemotherapy.
[c] Enhancement factors (E.F.): obtained by dividing normalized tumor growth delay in mice treated with different chemotherapy plus radiation by the absolute growth delay in mice treat with radiation only.

Table S6. Effect of (co-)encapsulated Dtxl and Wtmn on radioresponse of PC3 xenograft tumor measured by tumor growth delay.

SUPPORTING SCHEME



Scheme S1. Flow diagram summarize the *in vivo* treatment plan for the investigation of tumor inhabitation efficiencies of chemotherapy and chemoradiotherapy after i.v. administration of different nanoformaulations. (N.B. †H460-bearing mice started in vivo treatment 10 days after tumor inoculation, PC3-bearing mice started in vivo treatment 14 days after tumor inoculation. ‡*In vivo* treatment dose: PBS (control group), 33 mg/Kg of drug-free empty PEG-PLGA NPs, 0.38 mg/kg of encapsulated Dtxl, 0.05 mg/Kg of encapsulated Wtmn, or 0.05 mg/Kg of encapsulated Wtmn and 0.38 mg/kg of encapsulated Dtxl (either duel drug-loaded NPs or combination of two single-drug loaded NPs))