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Supplementary Information

for

A Brain Tumor-homing Tetra-peptide Delivers a Nano-Therapeutic for More Effective Treatment of a Mouse Model of Glioblastoma

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Table of Contents

Materials and Methods

General information

For the peptide synthesis, all chemicals, including Rink-amide resin, Fmoc-protected amino acids, 1-hydroxybenzotriazole (HOBt), and O-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU) were purchased from Novabiochem (Germany). Human serum (Product No. H4522), fluorescein isothiocyanate (FITC), *N*,*N*′-diisopropylethylamine (DIPEA), dimethylformamide (DMF), piperidine, trifluoroacetic acid (TFA), triisopropylsilane (TIS), and acetonitrile were from Sigma-Aldrich (USA). Endocytic inhibitors, such as chlorpromazine hydrochloride (CPZ), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), dynasore, methyl-β-cyclodextrin (MβCD), heparan sulfate, and chloroquine were from Sigma-Aldrich. Also, the conjugated streptavidin-FITC and biotin-NHS were from Sigma-Aldrich. The chemical reagents were purchased from TCI (Japan), Nanocs (USA), Merck (USA) and Samchun chemicals (Rep. of Korea). Commercially available reagents and anhydrous solvents were used without further purification. Chemical reaction and centrifugation was performed in an open-air environment at room temperature (25 °C). Maleimide-PEG-NHS (M.W. = 5 kDa) (Product No. PG2-MLNS-5k, USA) was purchased from Nanocs (USA). (3-Mercaptopropyl) triethoxysilane (MPTES) was purchased from TCI (Product No. M1505, Japan). Ethanol was purchased from Samchun Chemicals (Product No. E0223, Rep. of Korea). Dimethyl sulfoxide (DMSO, product No. 1.02952.1000) was purchased from Merck (USA). 7-Ethyl-10-hydroxycamptothecin (SN-38, product No. FE29579) was purchased from Carbosynth (USA). Dulbecco's Modified Eagle's Medium (DMEM, Product No. SH30243.01), Dulbecco's phosphate buffered saline (DPBS, Product No. SH30028.02), and fetal bovine serum (FBS, SH30084.03) were purchased from Hyclone (USA). Trypsin-EDTA (Product No. 25200-056) and Penicillin streptomycin (Product No. 15140-122) were purchased from Gibco (USA). Tribromoethanol and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (USA). 4′,6-diamidino-2-phenylindole (DAPI, product No. D1306), CellLight reagent ER-GFP, (product No. C10590), CellLight Golgi-GFP (product No. C10592), CellLight Actin-GFP (product No. C10582), and CellMask™ Deep Red Plasma membrane stain (product No. C10046) were purchased from ThermoFisher Scientific (USA). 4% Paraformaldehyde (PFA, Product No. CBPF-9004) was purchased from Chembio (USA).

Stability of SIWV in human serum

100 μM SIWV peptide incubated in 100% human serum at 37 °C for 60 min. At each time interval, the stability of SIWV in human serum was measured by dynamic light scattering (DLS) that compared to human serum.

Construction of GFP-conjugated SIWV

Enhanced GFP (EGFP) regions of the pEGFP vector (provided by Y. S. Yang, Korea Institute of Science and Technology (KIST), Seoul, Rep. of Korea) were generated using polymerase chain reaction. The EGFP protein was fused at the C-terminus of SIWV-CPP and inserted into the pET-23(+) vector to express the SIWV-GFP protein in Escherichia coli.

Expression and purification of the SIWV-GFP fusion protein

The recombinant plasmids were transformed into E. coli strain BL21 (DE3) pLysS for the expression of fusion proteins with or without SIWV-CPP. The bacteria were grown to an OD600 of approximately 0.5–0.8 OD (optical density) prior to induction. For the expression of SIWV-GFP and GFP, the cells were induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 h. The cells were then harvested, suspended in binding buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl), and sonicated. The fusion proteins were purified via Ni^{2+}-affinity chromatography using a chelating Sepharose resin column (ELPiS Biotech, Rep. of Korea). After the column was washed with 100 mM imidazole in binding buffer, the fusion protein was eluted with 1 M imidazole in binding buffer. The purified fusion protein was used immediately or aliquoted and stored frozen in 10% glycerol at –70 °C. For the transduction of SIWV-GFP fusion proteins, cells were cultured to 70–80% confluence in 6-well microtiter plates. Immediately before transduction, the culture medium was removed and replaced with 0.5 mL fresh medium, to which SIWV-GFP fusion proteins (10 μM) were then added. After incubation for the indicated intervals, the cells were washed with phosphate-buffered saline (PBS) at least four times to avoid possible protein aggregation. The cells were then examined directly via fluorescence microscopy and used for western blot analysis.

Cell culture

The HeLa and U87MG cell lines used in this study were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained at 37 °C in 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin. A549 and Huh-7 cell lines were grown in Roswell Park Memorial Institute-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin.

Cell viability assay

The cell viability was assessed using Cell Counting Kit-8 (CCK-8) assays (Dojindo, Japan). HeLa cells were seeded on 96-well plates, cultured to 80% confluence, and treated with truncated CPPs (0–100 μM) for 24 h in DMEM containing 10% serum. WST-1 reagent (10 μL) was added to each well, followed by incubation for 2 h. The absorbance of each well was measured using a microplate reader system (Molecular Devices, San Jose, CA, USA) at 450–650 nm. The cell viability is presented as the relative percentage of untreated HeLa cells. Data is presented as means ± SDs.

Localization of SIWV-CPPs by subcellular fractionation

Purification of cytoplasmic, organelle membrane (including endoplasmic reticulum (ER) and mitochondria), nuclear, and cytoskeletal matrix fractions was performed using a ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Sigma-Aldrich, USA), with slight modifications. We analyzed fractions using western blotting with specific antibodies to mark organelles (tubulin for the cytoplasm, poly [ADP-ribose] polymerase [PARP] for the nuclei, caveolin-1 for organelle membranes, and actin for the cytoskeleton).

Immunogenicity assay

Five-week-old BALB/c mice were injected lipopolysaccharide (LPS, 10 μg/mL) or SIWV (10 μM, 5 μg/mL) used for immunogenicity assays. After 24 h, blood was collected using heart puncture and centrifuged at 1,500 × g for 10 min. For the detection of IL-6, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), we used mouse ELISA kits (Thermo Fisher Scientific, Waltham, MA, USA). The results from triplicate experiments were analyzed using Prism software. Data is presented as means ± SDs.

Calcium sealing of pSiNP(SN-38)

To prepared a stock solution 2 M in calcium chloride ($CaCl_2$), we added 2.94 g of solid $CaCl_2$ (M.W: 147.01, dihydrate, Sigma-aldrich) to 10 mL of DI H_2O. The drug-loaded pSiNP(SN-38) (1 mg/100 μL DI H_2O) were mixed with the 2 M $CaCl_2$ stock solution (900 μL). The mixture was agitated for 90 min and purified by successive dispersion in/centrifugation from DI H_2O, 30% ethanol, 70% ethanol, and 100% ethanol.

Nanoparticle characterization

The hydrodynamic size and zeta-potential pSiNPs, pSiNP(SN-38), and SIWV-pSiNP(SN-38) were measured using Malvern Instruments Zetasizer Nano ZS90 (Worcester-shire, UK). The measurements were carried out with particles dispersed in DI H_2O. The morphologies of nanoparticles were characterized by transmission electron microscopy (Tecnai, G2 F30ST, FEI Company, OR, USA). Attenuated total reflection Fourier transform infrared (FT-IR) spectroscopy was collected on a Thermo Scientific Nicolet™ iS™ 5 FT-IR spectrometer instrument.

Verification of SN-38 encapsulation within the pore of pSiNPs

SIWV-pSiNP(SN-38) (1 mg) were centrifuged with DMSO at 14,000 rpm for 15 min to wash out the SN-38, which is attached on the suface of pSiNPs. Next, in order to verify the encapsulation of SN-38 within the pore of pSiNPs, the SIWV-pSiNP(SN-38) was suspended in DMSO (1 mL) and maintained at 37 °C with mild shaking in an incubator, and the emission spectra of SN-38 (λ_{ex} = 367 nm) were monitored at designated time point to confirm the released SN-38 from the interior pore of pSiNPs.

Drug release profile assay

Release kinetics of the loaded SN-38 from SIWV-pSiNP(SN-38) in PBS at 37 °C was measured by using a spectro-fluorophotometer. At each time interval, the samples (0.1 mg/mL) were removed from the aqueous phase using centrifugation at 14,000 rpm for 15 min, and fluorescence of free SN-38 left in the supernatant at 561 nm (λex = 367 nm) was measured to determine the amount of released SN-38.

Cellular uptake mechanism anaylsis of nano-formulation

In order to confirm the uptake of nano-formulation by U87MG and Huh-7 cells, the cells were seeded onto 48-well plates (3×10^4 cells/well). After 24 h, the cells were washed twice with serum-

free DMEM and incubated with TAMRA labelled-SIWV (1 μM) in serum-containing DEME at 37 °C. The cells were incubated in trypsin-EDTA solution (0.01% trypsin, 100 μL) for 5 min at 37 °C then suspended in 200 μL PBS. The cell suspension was spin down using a centrifuge (1000 rpm, 3 min), and the supernatant was removed. Finally, the cells were resuspended in 200 μL PBS for flow cytometry. For treatment with endocytosis inhibitors, the cells were pre-incubated with four inhibitors (10 μM CPZ, 3 mM MβCD, 50 μg/mL dynasore, or 30 μM EIPA) for 30 min in serum-containing medium, and TAMRA labeled conjugated pSiNPs (100 μg/mL) was then added. Samples for flow cytometry analysis were prepared as described above. Analysis of nanoparticle internalization was performed with a BD FACSLyrics (BD Bioscience, USA), following the manufacturer's instructions, using the TAMRA channel (552/578nm). The results are presented as the relative percentage of untreated cells. Data are presented as mean ± standard deviation (SD).

Confocal laser scanning microscopy for nano-formulation uptake study

Intracellular distributions of nanoparticles were evaluated using U87MG cells with confocal laser scanning microscopy (CLSM, Carl Zeiss, Oberkochen, Germany). The cells were seeded on the confocal dish containing 2 mL of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotics at a density of 1×10^5cells/mL. After 24 h incubation, the original medium was replaced with DMEM containing 100 μg/mL samples. After 6 h, the cells were washed three times with DBPS. The nuclei were stained with DAPI, and the cell membranes were stained with CellMask™ Deep Red Plasma membrane Stain (Thermo Fisher). After washing 3 times with PBS, then they were fixed with 4% paraformaldehyde for 20 min. Finally, SN-38 in nanoparticles distribution was observed with CLSM.

Cytotoxicity assay of nano-formulation

The cytotoxicity of pSiNPs, pSiNP(SN-38), and SIWV-pSiNP(SN-38) against U87MG cells was evaluated using Cell Counting Kit-8 (CCK-8) assays according to the manufacturer's instructions. The cells (5×10^4 per well) were seeded on 96-well plates and incubated for 24 h at 37 °C in a humidified 5% CO_2 incubator. The cells were then treated with 6.25, 12.5, 25, 50 and 100 μM SN-38 concentrations in the nanoparticles, and the cell toxicity was measured after a 6 h incubation. 10% WST-1 reagent was added to each well, followed by incubation for 2 h. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Multiskan FC, Thermo Fisher, Waltham, MA, USA). Cell viability is presented as the relative percentage of untreated U87MG cells.

***In vivo* distribution of SIWV in healthy mice**

The nude mice were injected intravenously with 1 mM SIWV-TAMRA in 0.2 mL PBS. The TAMRA signal was examined over time after intravenous injection. After 4 h, mice were sacrificed and perfused with PBS. The main organs (brain, liver, lungs, kidneys, heart, and spleen) were harvested and fixed with 4% formaldehyde solution (Merck, Darmstadt, Germany). The six-organ embed tissue in OCT compound (Sakura Fineteck, CA, USA) were stored at -20 °C freezer overnight then transfer to -80 °C. For the tissue sectioning, the organs were cut into 15-μm-thick sections on a cryostat. The sections were examined by confocal laser scanning microscopy (LSM Zeiss 700). The

results from triplicate experiments were analyzed using Prism software. Data is presented as means ± SDs.

***In vivo* distribution of SIWV-pSiNP(SN-38) in healthy mice**

ICR mice were randomly divided into 4 groups (n=3 per group) randomly and were injected intravenously each formation as follows: (1) Control group (PBS, *i.v.*), (2) pSiNPs group (injected with empty pSiNPs, *i.v.*), (3) pSiNP(SN-38) group (injected with pSiNP(SN-38), *i.v.*), and (4) SIWV-pSiNP(SN-38) group (injected with SIWV-pSiNP(SN-38), *i.v.*). Each pSiNP was diffused in PBS and injected intravenously (*i.v.*). At 2 h circulation point after injection, the mice were anesthetized with tribromoethanol, perfused transcardially with 0.05 M PBS and then fixed with cold 4% PFA. Brain, lung, heart, spleen, kidney and liver were removed and immersed in buffer solution pH 12 (product No. 2058-3700, Daejung, Rep. of Korea) for 1 h to analyze the fluorescence intensity using the xenogen IVIS 200 luminescence and flourescence animal imaging system (GFP excitation filter (445-490 nm) 0.2 s exposure time, and the emission filter was DsRed (575-650 nm).

Supporting Figures

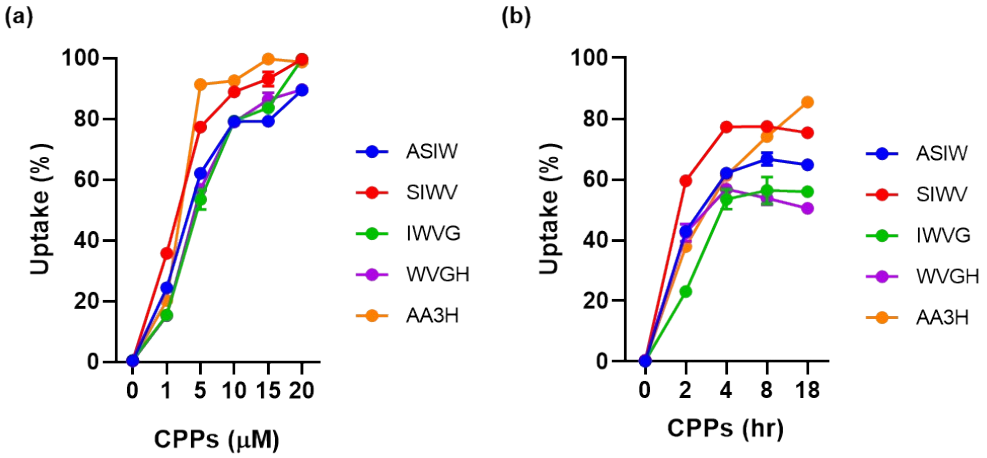

Figure S1. Quantification of uptake of truncated peptides in HeLa cells. (a) Comparison of uptake efficacy. Fluorescence intensity from FITC-labeled truncated peptides ASIW, SIWV, IWV, WVGH, and AA3H administered to HeLa cells at the indicated concentrations and then assayed 4 h later. (b) Time course of uptake. Fluorescence intensity from FITC-labeled truncated peptides ASIW, SIWV, IWV, WVGH, and AA3H administered to HeLa cells at a fixed concentration (5 μM) and then assayed at the indicated times. Results from triplicate experiments were analyzed by Prism software. Data are presented as means ± SDs.

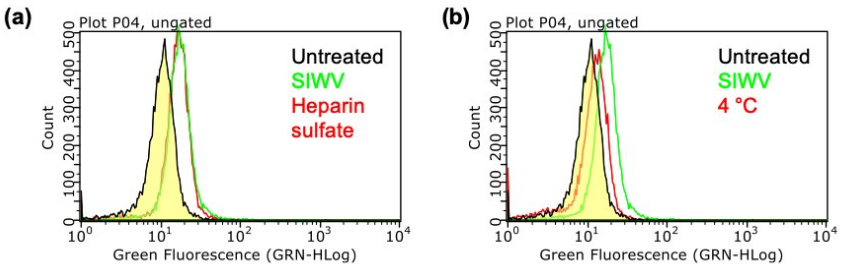

Figure S2. Cellular uptake pathway of FITC-labeled SIWV in HeLa cells, quantified by flow cytometry. (a) The uptake of SIWV after treating the HeLa cells with heparan sulfate for 4 h. (b) The uptake of SIWV peptide at 37 °C (green) and 4 °C (red).

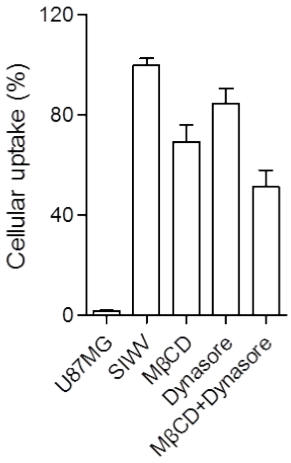

Figure S3. Effects of endocytosis inhibitors on SIWV penetration in U87MG cells. SIWV was labeled with FITC and uptake was quantified by flow cytometry. Cells were treated with a caveolin inhibitor (MβCD), a dynamin inhibitor (dynasore), and both before SIWV treatment, and then analyzed triplicate using flow cytometry. Data is presented as means ± SDs. U87MG indicates control cells administered PBS. SIWV indicates uptake of the peptide (1 μg/mL) under control conditions and is taken at the 100% value. Inhibitors were administered at concentrations of 30 μg/mL (dynasore) or 1.3 mg/mL (MβCD).

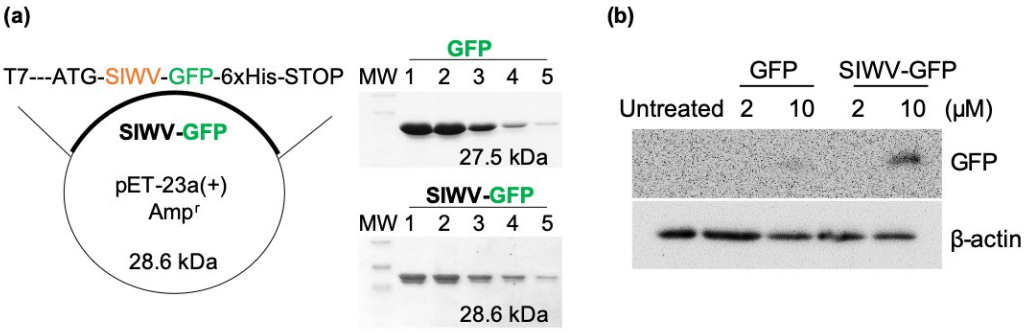

Figure S4. Cloning and purification of SIWV-GFP. (a) Construction of the SIWV-GFP cloning vector and (b) detection of purified GFP and SIWV-GFP by SDS-PAGE.

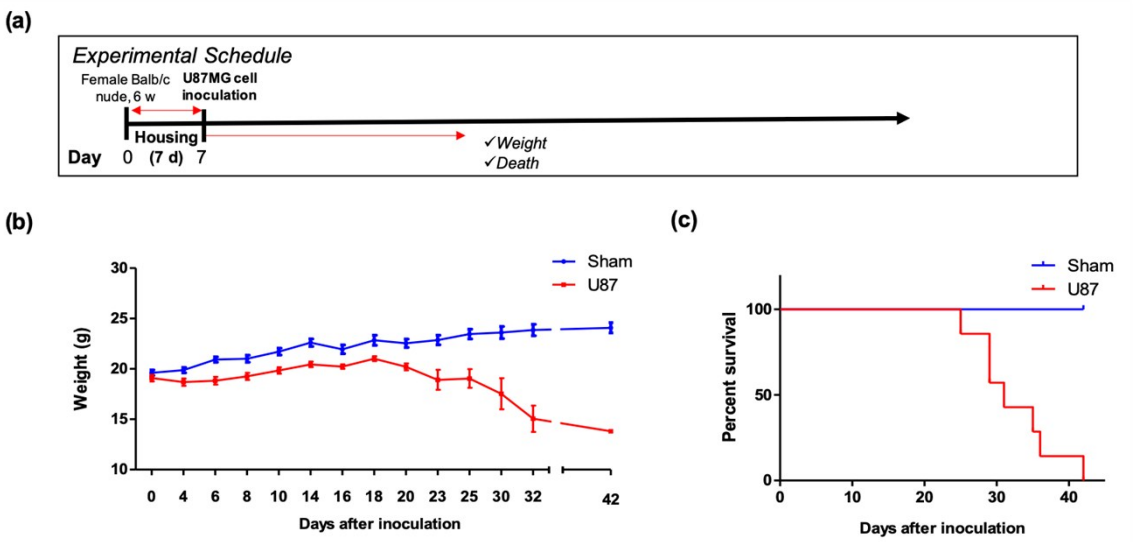

Figure S5. Establishment of intracranial U87MG xenograft (tumor implantation) model in mice. (a) Scheme of U87MG cell xenograft mouse experimental design and schedule. (b) Mice body weight after U87MG cell xenografts, compared to PBS control (n = 7). (c) Mouse survival percentage as a function of time after implantation of the U87MG cell xenograft, compared to PBS control (n = 7).

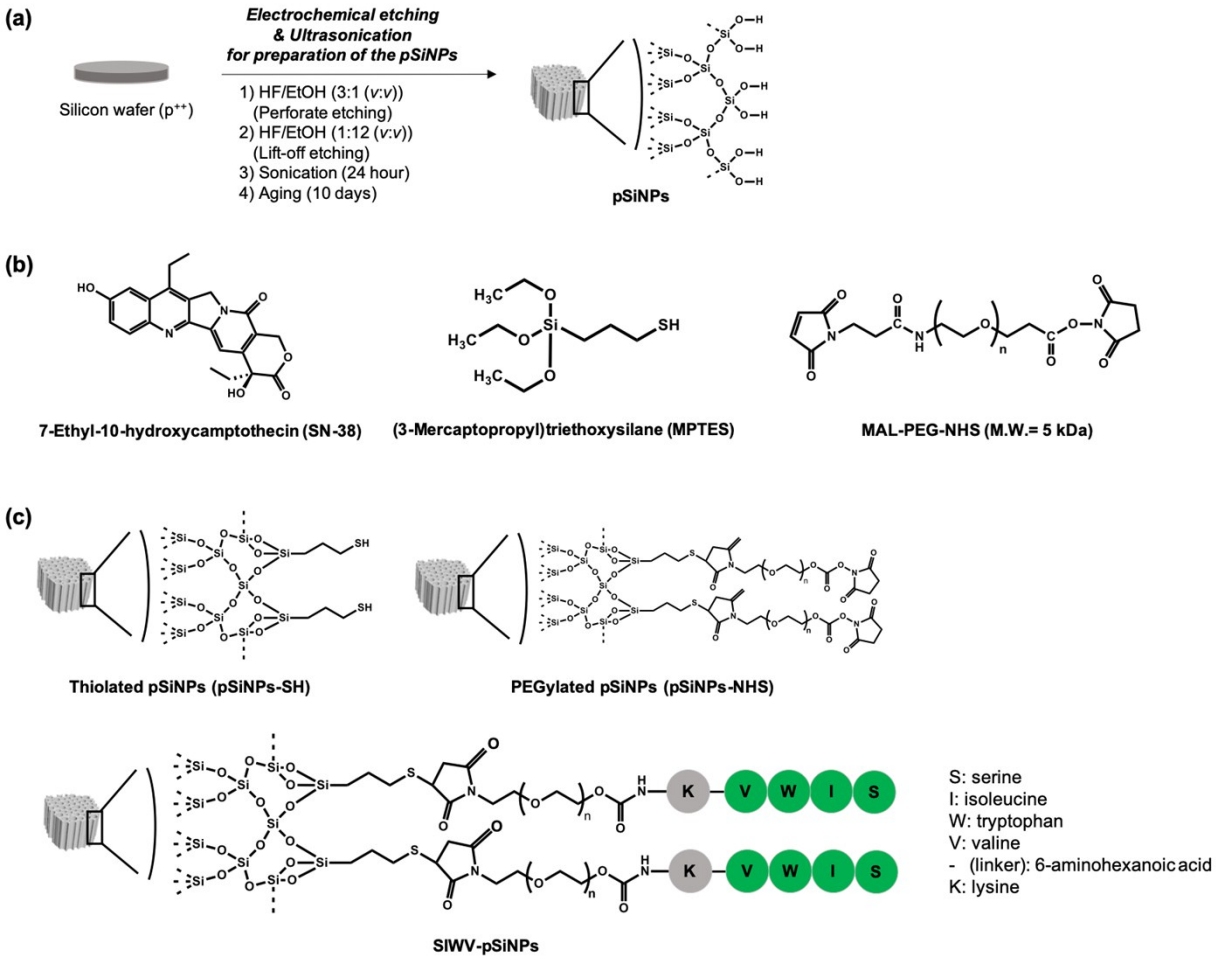

Figure S6. Outline of the preparation of the SIWV-pSiNP(SN-38) construct. (a) The porous silicon nanoparticles are prepared by electrochemical etching. (b) Chemical structure of the therapeutic 7-Ethyl-10-hydroxycamptothecin (SN-38), the surface grafting agent (3-mercaptopropyl)-triethoxysilane (MPTES), and the linking agent MAL-PEG-NHS (M.W. = 5 kDa) that were used in this study. (c) Chemical structure of the surfaces of the pSiNPs formulations at intermediate stages and of the final peptide-targeted nanoparticle surface. The scheme only shows the surface functionalization of the pSiNP surface; the loading step for SN-38 is not described.

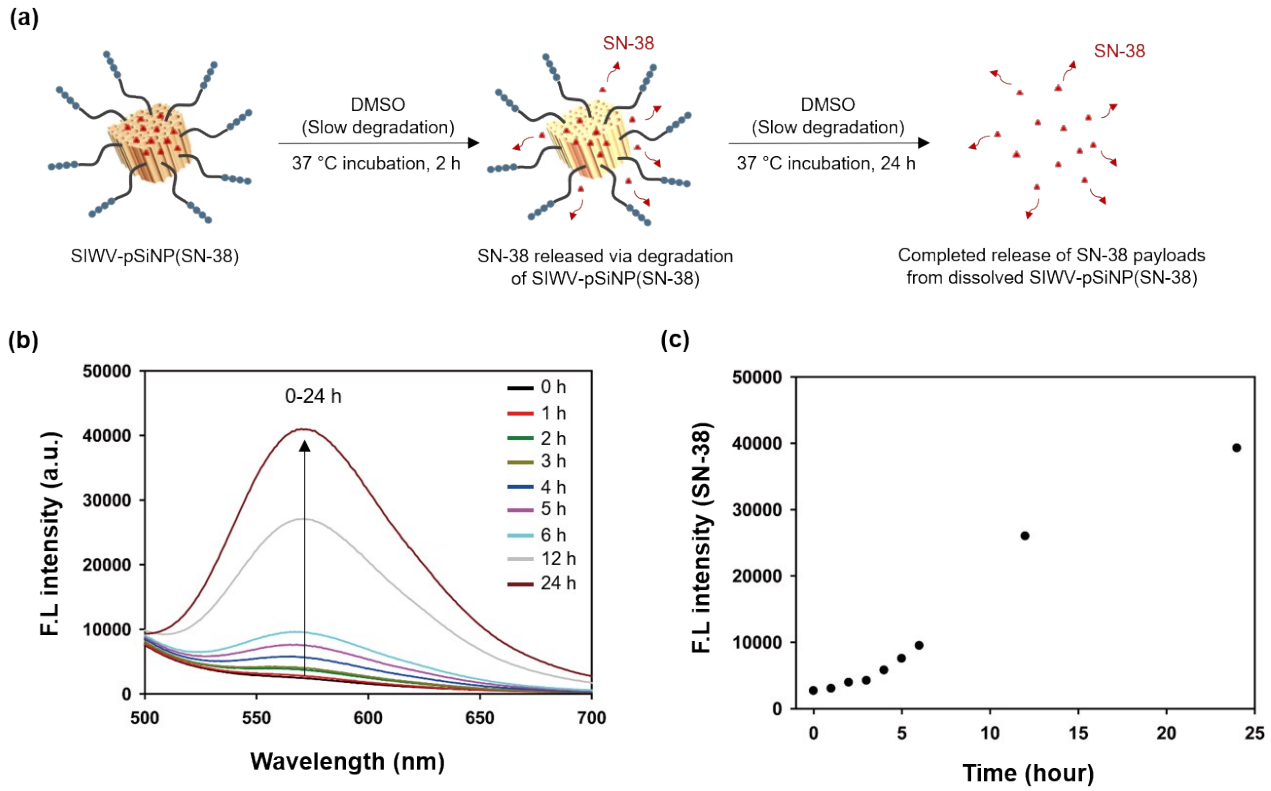

Figure S7. Verification of SN-38 encapsulation within the pore of pSiNPs. (a) Schematic illustration for the SN-38 release from the interior pores of SIWV-PSiNP(SN-38). The SIWV-pSiNP(SN-38) was incubated within DMSO and measured emission intensity changes of the solution after spin-down the particles. In this condition, the stacked SN-38 on the surface of pSiNPs is rapidly washed away, but the encapsulated SN-38 is slowly released out by slow degradation of pSiNPs in the DMSO. (b) Emission spectra of DMSO solution that contain SIWV-pSiNP(SN-38) (1 mg/mL) at 37 °C during incubation (0–24 h). (c) A plot of fluorescence intensity (peak height at 567 nm for SN-38) from panel (b).

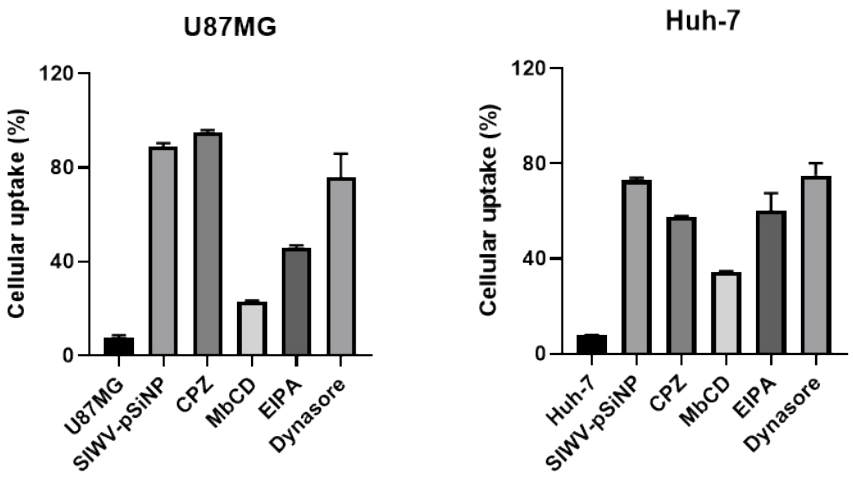

Figure S8. Cellular uptake analysis of SIWV-pSiNP penetration in U87MG and Huh-7 cells. Cells were co-incubated with the inhibitors CPZ, MβCD, EIPA, and Dynasore before nano-formulation (100 μg/mL) treatment, and then analyzed triplicate using flow cytometry. Uptake was quantified by TAMRA fluorescence intensity from flow cytometry data. Data are presented as means ± SDs.

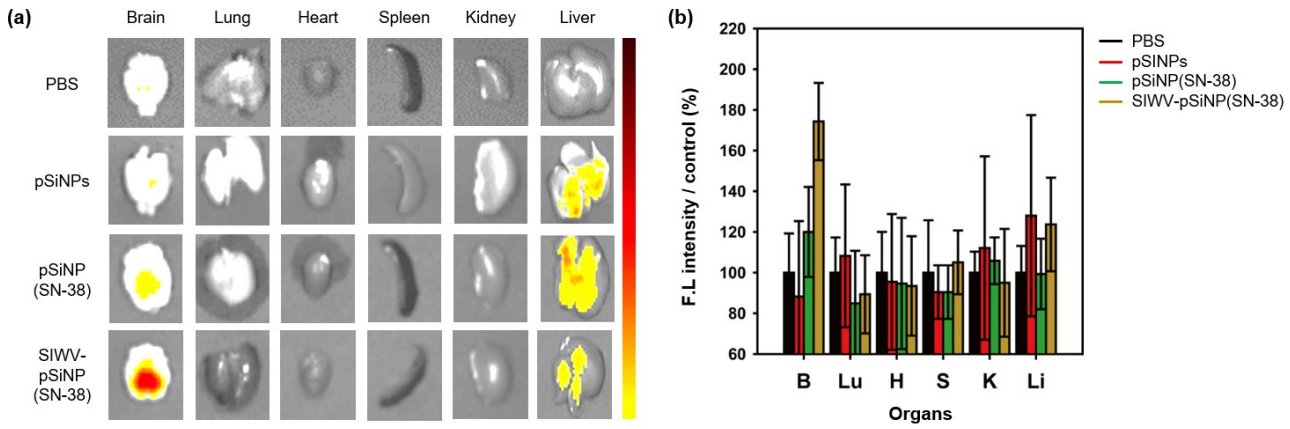

Figure S9. *In vivo* distribution of nano-formulation of SIWV in healthy mouse. (a) Ex vivo fluorescence images of organs were taken using the IVIS imaging system. (GFP excitation filter (445-490 nm) 0.2 s exposure time, and the emission filter was DsRed (575-650 nm)). (b) Plot of relative fluorescence intensity of organs 2 h after the intravenous injection of pSiNPs, SN-38, pSiNP(SN-38), and SIWV-pSiNP(SN-38) into a tail vein. PBS served as the control.

Supporting Tables

Table S1. Hydrodynamic size/PDI and zeta-potential of pSiNPs, pSiNP(SN-38), and SIWV-pSiNP(SN-38). Hydrodynamic size/PDI and zeta-potential values are measured in DI H_2O. Each mean and standard deviation was determined in triplicate. The standard deviations are calculated from 3 replicate measurements.

	pSiNPs	pSiNP(SN-38)	SIWV-pSiNP(SN-38)
DLS (PDI)	139.7 ± 18.8 nm (0.206)	196.3 ± 32.2 nm (0.103)	306.2 ± 32.7 nm (0.217)
Zeta potential (mV)	-44.5 ± 8.39	-24.3 ± 6.73	-32.8 ± 3.68