Electronic Supplementary Information (ESI)

A nanocarrier based on a genetically engineered protein cage to deliver doxorubicin to human hepatocellular carcinoma cells

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1. Experimental details

1-1 Genetic engineering of HCC-targeted HspG41C-SP94

As reported previously, a pET21a(+) vector encoding HspG41C, in which Gly41 was substituted with a Cys residue, was prepared by PCR-mediated site-directed mutagenesis. This HspG41C vector was used as a template to prepare vectors encoding HspG41C-SP94 recombinant proteins, which have additional peptides at the C-terminus, by PCR-mediated mutagenesis using appropriate forward and reverse primers. Successful mutagenesis was confirmed by DNA sequencing.

1-2 Expression and purification of Hsp cages

All recombinant proteins were expressed from E. coli and purified by anion-exchange chromatography and size-exclusion chromatography (SEC). BL21-CodonPlus(DE3) and
BL21-Gold(DE3) competent cells (Novagene) were used to express HspG41C-SP94 and HspG41C, respectively. *E. coli* cells containing pET21a(+) plasmid vector were grown in 2× yeast extract tryptone medium containing 100 µg/mL ampicillin at 37°C. When the culture reached an OD at 600 nm of 0.5 to 0.6, expression of recombinant protein was induced with 1 mM IPTG for 4 h at 37°C. Cells were collected by centrifugation, resuspended in 25 mM KH$_2$PO$_4$ solution (containing 1 mM EDTA and 2 mM DTT, pH 7.0), and stored at −80°C until purification. The cell suspension was sonicated to disrupt cell membranes and the lysate was centrifuged at 15,000 rpm for 30 min at 4°C. Recombinant proteins were purified from the supernatant using a HiLoad 26/10 Q Sepharose HP anion-exchange column (GE Healthcare, Tokyo, Japan) and a TSKgel G3000SW SEC column (Tosoh, Tokyo, Japan). Purity was confirmed by 15% SDS-PAGE analysis.

1-3 Measurement of size of Hsp cages

The size distribution of Hsp cages was determined using a Zetasizer (Malvern Instruments, UK) with an He/Ne laser (λ = 633 nm) at a detection angle of 173° and a temperature of 25°C. The concentration of Hsp cages was adjusted to 0.1 mg/mL in PBS (pH 7.4).

1-4 Labeling of Hsp cages with Alexa488–maleimide

Hsp cages and Alexa Fluor 488 C$_5$ maleimide (Alexa488; 1.2 eq. to protein monomer) (Invitrogen, Grand Island, NY, USA) were dissolved in phosphate buffer (0.1 M, pH 7.2) at 4°C. The mixture was stirred for 2 h at room temperature and incubated for another 20 h at 4°C. Unreacted Alexa488 was removed by ultrafiltration using Amicon Ultra centrifugal filters with a molecular weight cut-off (MWCO) of 100,000 (Millipore, MA, USA).
1-5 Cell culture

Human hepatocellular carcinoma (HCC) cells (Huh-7, HepG2, and Hep3B), human cervical adenocarcinoma (HeLa) cells, and rat normal liver (RLN-8) cells were cultured in appropriate media containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (all from Invitrogen) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Dulbecco’s modified Eagle’s medium was used for cultivation of Huh-7 and HepG2 cells, Eagle’s minimal essential medium containing non-essential amino acids was used for Hep3B and HeLa cells, and minimal essential medium α was used for RLN-8 cells (all from Wako Pure Chemical Industries, Osaka, Japan).

1-6 Cell study

Cells were seeded on 96-well culture plates at an initial density of 10,000 cells/well and cultured for 1 day in 100 µL of appropriate medium containing 10% FBS. Final concentrations of Hsp cages were adjusted to 0.1 or 1 µM in Opti-MEM (Gibco) and 100 µL of diluted Hsp cages was added to each well. After incubation for 4 or 24 h, medium was changed to fresh Opti-MEM containing 5 µg/mL Hoechst 33342 (Dojindo, Kumamoto, Japan) and plates were incubated for 1 h. Fluorescence images were obtained using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). In the competition assay, Alexa488-labeled Hsp cages were added to wells with a 30-fold higher concentration of unlabeled HspG41C-SP94(L11).
1-7 Synthesis of (6-maleimidocaproyl) hydrazone derivatives of doxorubicin (DOX-EMCH)

\[
\text{DOX-EMCH} \quad \text{MeOH} \quad \text{trifluoroacetic acid (cat.)} \quad \text{overnight, room temperature}
\]

DOX-EMCH was synthesized according to a previous report. Briefly, doxorubicin (DOX; 25 mg, 43 µmol) (Wako) and EMCH (N-(ε-maleimidocaproic acid) hydrazide, trifluoroacetic acid salt; 44 mg, 129 µmol) (Thermo Fisher Scientific, Waltham, MA, USA) were dissolved in 12 mL of methanol, and then two drops of trifluoroacetic acid were added by a Pasteur pipette. The reaction solution was stirred overnight at room temperature in the dark. The reaction solution was then concentrated to 1 mL by evaporation and thrice precipitated in ethyl acetate. The precipitate was collected by centrifugation and dried under vacuum.

\(^1\)H NMR (DMSO-\(d_6\), 298K, 500 MHz), \(\delta\) (ppm from TMS): 10.29 (1H, s, –N-NH-CO–), 7.93 (1H, br, Ar), 7.80 (1H, br, Ar), 7.67 (1H, br, Ar), 6.97 (2H, s, double bond of maleimide group), 4.97 (1H, m, –CH\(_2\)-CH(–O–)\(_2\) of sugar ring), 4.17 (2H, m, –CH– of sugar ring), 4.04 (3H, s, CH\(_3\)-O-Ar), 3.36 (2H, t, –CH\(_2\)-CH\(_2\)-N=), 2.63–3.23 (3H, m, –CH\(_2\)= of aliphatic ring, –CH– of sugar ring), 2.10–2.36 (6H, m, –CH\(_2\)= of sugar ring and aliphatic ring), 1.29–1.68 (6H, m, –CH\(_2\)-(CH\(_3\))\(_3\)-CH\(_2\)=), 1.17 (3H, m, CH\(_3\)-CH of sugar ring).
1-8 Synthesis of DOX-modified Hsp cages

Aqueous solutions of DOX-EMCH (4 equivalents to Cys residues of Hsp cages) were added to the Hsp cages dissolved in pre-chilled 0.1 M phosphate buffer saline (pH 7.2). Mixture was allowed to stand for 3 h at room temperature, and then further incubated for 1 day at 4°C. Unreacted DOX-EMCH was removed by ultrafiltration using Amicon Ultra centrifugal filters with MWCO 100,000 (Millipore, MA, USA).

2. Amino acid sequences of engineered Hsp cages

**HspG41C** (147 a.a., MW=16498)

MFRGDPFDSL FERMFKKEFFA TPMTGTTMIQ SSTGIQISGK CFMPISIIEG DQHIKVIAWL PGVNKEDIIL NAVGDTLEIR AKRSPLMITE SERIIYSEIP EEEEIYRTIK LPATVKEENA SAKFENGVLS VILPKAESSI KKGINIE

**HspG41C-SP94(L5)** (164 a.a., MW = 18204)

MFRGDPFDSL FERMFKKEFFA TPMTGTTMIQ SSTGIQISGK CFMPISIIEG DQHIKVIAWL PGVNKEDIIL NAVGDTLEIR AKRSPLMITE SERIIYSEIP EEEEIYRTIK LPATVKEENA SAKFENGVLS VILPKAESSI KKGINIEGSP SGFSIIHTP ILPL

**HspG41C-SP94(L11)** (170 a.a., MW=18650)

MFRGDPFDSL FERMFKKEFFA TPMTGTTMIQ SSTGIQISGK CFMPISIIEG DQHIKVIAWL PGVNKEDIIL NAVGDTLEIR AKRSPLMITE SERIIYSEIP EEEEIYRTIK LPATVKEENA SAKFENGVLS VILPKAESSI KKGINIETSG GSGGSPGFSIIHTP ILPL
3. SEC analyses of Hsp cages

SEC analysis of Hsp cages was performed on a Shimadzu SEC system with a TSKgel G3000SW SEC column (Tosoh, Tokyo, Japan) at room temperature. Phosphate buffer (0.025 M, pH 7.0, containing 0.1 M NaCl) was used as an eluent (elution rate, 3.8 mL/min).

![Size exclusion chromatography profile of the Hsp cages](image)

**Figure S1.** Size exclusion chromatography profile of the Hsp cages. Absorbance of the samples at 272 nm was monitored for 30 min.
4. SDS-PAGE analyses of Alexa488-modified Hsp cages

![SDS-PAGE analysis](image)

**Figure S2.** SDS-PAGE analyses of Hsp cages. Lanes 1–3 and lanes 4–6 are unlabeled Hsp cages and Alexa488-labeled Hsp cages, respectively. Lane 1, HspG41C; lane 2, HspG41C-SP94(L5); lane 3, HspG41C-SP94(L11); lane 4, HspG41C-Alexa488; lane 5, HspG41C-SP94(L5)-Alexa488; lane 6, HspG41C-SP94(L11)-Alexa488.
5. Cellular uptake of HspG41C-SP94 cages

Figure S3. Evaluation of cellular uptake of Alexa488-labeled HspG41C-SP94. Alexa488-labeled HspG41C-SP94 cages (cage concentration 40 nM) were added to cells and uptake of cages was observed under a fluorescence microscope 24 h later. Both HspG41C-SP94(L5) and HspG41C-SP94(L11) were taken up by Hep3B cells (HCC cells) but not by HeLa cells (human cervical carcinoma cells), consistent with the results shown in Fig. 2A. However, uptake of HspG41C-SP94(L11) cages by Hep3B cells was greater than uptake of HspG41C-SP94(L5) cages. These results, together with those shown in Fig. 2A, indicate that the linker length between the Hsp cage and the SP94 peptide affects uptake of HspG41C-SP94 cages by Hep3B cells but has little effect on uptake by Huh-7 cells and HepG2 cells.
6. Subcellular localization of Hsp cages

Figure S4. Subcellular localization of Hsp cages in Huh-7 cells. After transfection of (A) Alexa 488-labeled HspG41C (HspG41C-Alx) (10 µM) and (B) HspG41C-SP94(L11) (HspG41C-SP94(L11)-Alx) (10 µM) into Huh-7 cells, acidic organelles (red) and nuclei (blue) were stained with LysoTracker-Red and Hoechst33342, respectively. The merged fluorescence (yellow) of Hsp cages and LysoTracker shows that both HspG41C and HspG41C-SP94(L11) cages are mainly localized in acidic organelles (endosomes and/or lysosomes) at 24 and 48 h post transfection.
7. Cytotoxicity assay of genetically engineered protein cages (HspG41C-SP94(L11))

**Figure S5.** Cytotoxicity of HspG41C-SP94(L11) towards (A) RLN-8 cells (normal rat hepatocytes) and (B) Huh-7 cells (human hepatocellular carcinoma). Data are means ± SD of three independent experiments.
8. SEC analyses and size distributions of HspG41C-SP94 after DOX-EMCH modification.

**Figure S6.** Comparisons of (C) SEC profiles and (D) size distributions of HspG41C-SP94(L11) cages before and after modification with DOX-EMCH. Measurements were taken at least two times and showed little variation.
9. Fluorescence microscopic observations of DOX-modified Hsp cages in Huh-7 and RLN-8 cells.

(A) RLN-8 (24 h treatment)

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(B) Huh-7 (24 h treatment)

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(C) Huh-7 (48 h treatment)

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Figure S7. Fluorescence microscopy observations of intracellular localization of DOX. (A) Intracellular distribution of DOX in RLN-8 cells 24 h after treatment with Hsp cages containing DOX (10 µM). Intracellular distribution of DOX in Huh-7 cells (B) 24 h and (C) 48 h after treatment with Hsp cages containing DOX (10 µM). DOX and nucleus (stained by Hoechst 33342) are shown in red and blue, respectively. Colocalization of DOX and nucleus is shown in purple.

HspG41C-DOX cages were taken up by RLN-8 cells but HspG41C-SP94-DOX cages were not. Conversely, in the case of Huh-7 cells, both HspG41C-DOX and HspG41C-SP94-DOX were taken up. These results show that HspG41C-SP94 cages were specifically taken up by HCC cells even after DOX modification.