Electronic Supplementary Material (ESI) for Biomaterials Science.

Dimeric α-Helical Cell Penetrating Peptide Mounted with HER2-Selective Affibody

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Table of Contents

I. General Materials and Methods

Cell lines and cell culture

SKBR-3, *HCC-1937, BT474 and MCF-7* cells were purchased from Korean Cell Line Bank. *SKBR-3* and *HCC-1937* cells were maintained in Roswell Park Memorial Institute Media (RPMI-1670, Welgene) with 10% fetal bovine serum (FBS) at 37˚C under 5% CO_2 condition. *BT474* and *MCF-7* cells were maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM, welgene) with 10% FBS at 37˚C under 5% CO_2 condition.

Construction of the expression vector

The recombinant proteins with a histidine tag were prepared in pET28b vector system.

Construction of pET28b-LK-2-Domain Z (Z_{wt}) and pET28b-Domain Z(Z_{wt}):

For the construction of pET28b-LK-2-Domain Z (Z_{wt}), the synthetic gene encoding the *Domain Z* (Z_{wt}, Macrogen, Korea) was amplified by PCR using primers having *SacI* and *XhoI* restriction enzyme sites. This fragment was inserted into the vector *(pET28b-LK-2-eGFP)* from the previous study[1] after the eGFP part was removed with *SacI* and *XhoI* restriction enzymes (NEB, New England Biolabs). The ligation was carried out using Quick Ligation Kit (NEB, New England Biolabs) following the manufacturer's protocol. In order to create pET28b-Domain Z(Z_{wt}) from pET28b-LK-2-Domain Z vector, the unnecessary *LK-2* (LKKLLKLLKKLLKLGGLKKLLKLLKKLLKLAG) part was removed using blunt-end-ligation technique. The desired parts of genes were amplified by PCR using Q5 Hot Start Master Mix (NEB) and the blunt ends were ligated using KLD Enzyme Mix (NEB).

Construction of pET28b-LK-2-$Z_{HER2:342}$, pET28b-$Z_{HER2:342}$ and pET28b-LK-2-$Z_{HER2:342}$-cys:

The plasmid having *LK-2-Z_{HER2}* was prepared through exactly the same protocol how LK-2-Z_{wt} was prepared but the synthetic gene encoding $Z_{HER2:342}$ (Macrogen, Korea) was used instead of Z_{wt}. To prepare *pET28b-$Z_{HER2:342}$*, LK-2 part was removed through blunt-end-ligation technique. The genes were amplified using Q5 Hot Start Master Mix and ligated by KLD Enzyme Mix (NEB). For *pET8b-LK-2-Z_{wt}-cys*, a cysteine was inserted using site directed mutagenesis (NEB).

Purification of the recombinant proteins

The proteins were expressed in *E.coli Rosetta* (DE3) *pLysS* (Novagen). The *E.coli* was transformed with the plasmids containing the material information. The *E.coli* was resuspended in 10 mL of liquid luria-bertani (LB) broth culture with kanamycin (50 µg/mL) and chloramphenicol (35 µg/mL) for 16 h at 37°C. This 10 mL culture was transferred to 1L of fresh LB broth medium with the same concentrations of two antibiotics and the mixture was incubated at 37 °C with shaking for about 3 h until the culture density of OD_{600} reached 0.4-0.8. The protein expression was induced by the treatment of 1 mM isopropyl-β-thiogalactopyranoside (IPTG) and the culture was maintained at 16°C with a gentle shaking at 200 rpm for overnight. The *E.coli* cells were harvested by the centrifugation at 6000 rpm for 20 min at 4°C and then were resuspended in a lysis buffer (20 mM Tris, 500 mM NaCl, 35 mM imidazole, 0.05% Triton X-100, pH 7.0). To avoid the protein degradation during the lysis step, α-toluenesulfonyl fluoride (PMSF) was added to the suspension. The suspension was sonicated by the ultrasonic processor (Sonic & Materials, Inc.) and then centrifuged at 15000 rpm and 4°C for 40 min. The supernatant was collected and filtered using a 0.22 µm syringe filter and loaded on a nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography column (GE Healthcare). The proteins were eluted by an elution buffer. The salts in the final product were exchanged with a storage solution of 20 mM Tris, 200 mM KCl, 1 mM dithiothreitol (DTT) and 10% glycerol by the Amicon centrifuge filtration (Milipore). The concentrations of the purified proteins were calculated using the Quick Bradford assay (Bio-Rad) and the proteins were stored at -20°C until use.

FITC-labeling of proteins

The proteins were labeled with fluorescein-5-isothiocyanate (FITC, Invitrogen F1906) following the manufacturer's protocol. Each of LK-2-Z_{wt} (3.3 mg/mL, 300 µL), LK-2-Z_{HER2} (3.3 mg/mL, 300 µL) and Z_{HER2} (3.0 mg/mL, 300 µL) was mixed with 700 µL of a 0.1 M $NaHCO_3$ buffer (pH 8.5) and the mixture was added with 100 µL of a FITC solution (1 mg/mL in DMSO). After incubation at 37 °C for 3 h, the excess FITC was removed and the proteins were concentrated using a 10 KDa Amicon centrifuge filter (Millipore). The final products were stored in the solution containing 20 mM Tris, 200 mM KCl, 1 mM dithiothreitol (DTT) and 10% glycerol. The labeling degree was calculated via the following equation: Moles dye per mole protein = [A_{max} of the labeling protein / ε × protein concentration (M)] × dilution factor, where A_{max} was the absorbance at 494 nm and ε was the molar extinction coefficient of FITC (68,000 $M^{-1}cm^{-1}$). We adjusted the labeling degree to 15% for each sample.

Flow cytometry analysis (FACS)

SKBR-3, HCC-1937, BT474 or *MCF-7* (1.0×10^5 cells per well) was seeded on a 24-well plate and incubated for 24 h. In order to study the selective uptake of proteins (Figure 2A, Figure S3 and Figure S4), various concentrations of the FITC labeled recombinant proteins were treated on cells for 1 h. For the kinetic study (Figure 2C), the cellular uptake of the FITC labeled recombinant proteins at 200 nM were measured at various time points. The media were removed and the cells were thoroughly washed twice with DPBS. Cells were detached using trypsin-ethylenediaminetetraacetic acid (0.25% Trypsin/EDTA) for 10 min at 37°C and this step could digest the proteins attached on the cell surface. Then, the detached cells were suspended in DPBS and were centrifuged at 4000 rpm for 10 min. The supernatant was removed after the centrifugation and the pellets were resuspended in 200 µL DPBS containing 2% FBS. The FACS analysis was performed using the BD Accuri C6 (BD Bioscience, USA). A total of 1×10^4 cells were analyzed for each sample and dead cells were excluded by the gating strategy.

siRNA knockdown assay

SKBR-3 cells were seeded on a 24-well plate (1.0×10^5 cells/well) and were incubated under 37°C and 5% CO_2 condition for 24 h.

For HER2 knockdown study, HER2 siRNA (Santa Cruz, sc-156048) was used to knockdown the expression of HER2 receptors. As a control, siRNA-A (Santa Cruz, sc-37007) was used. The siRNA-lipofectamine 2000 complexes were prepared in opti-MEM media following the manufacturer's protocol. Each of the complexes including 40 pmol of siRNA in 500 µL of the culture media was treated to the cells in a well. After 48 h-treatment of siRNA, the cells were washed with PBS thoroughly. Then, 100nM, 200 nM and 500 nM of FITC-labeled LK-2-Z_{HER2} proteins were treated on the cells for 1 h. The cellular uptake was measured by the FACS analysis.

For xylosyltransferase-1 (XylT-1) knockdown study, XylT-1 siRNA (Santa Cruz, sc-61817) was used to inhibit the elongation of heparan sulfate, whereas siRNA-A (Santa Cruz, sc-37007) was used as a negative control. The cells were treated with the complexes of 40 pmol of siRNA and 1 µL of lipofectamine 2000 in 500 µM culture media for 48 h. Then, 200 nM of FITC-labeled LK-2-Z_{HER2} proteins was treated on the knock-down cells for 1 h.

The FACS analysis was performed using the BD Accuri C6 (BD Bioscience, USA). A total of 1×10^4 cells were analyzed for each sample and dead cells were excluded by the gating strategy.

Competitive blocking assay with anti-HER2 antibody

The competition assay was carried out with anti-HER2 antibody (abcam, ab201332 targeting human HER2 extracellular domain). *SKBR-3* cells were seeded on a 24-well plate (1.0×10^5 cells/well) and were incubated at 37°C and 5% CO_2 for 24 h. The anti-HER2 antibody was pretreated on the cells at the concentration of 2 μg/mL for 3 h. Cells were thoroughly washed twice with DPBS. Then, 100nM, 200nM and 500nM of FITC-labeled-LK-2-Z_{HER2} were treated for 1 h. The cellular uptake was measured by FACS (BD Accuri C6, BD Bioscience, USA). A total of 1×10^4 cells were analyzed for each sample and dead cells were excluded by the gating strategy.

Cellular uptake measurement on *SKBR-3* with endocytosis inhibitors

SKBR-3 cells (1.0×10^5 cells per well) were seeded on a 24-well plate and incubated in RPMI containing 10% FBS for 24 h. Cells were pre-treated with various endocytosis inhibitors for 3 h and the cells were washed thoroughly twice with DPBS. Then, 200 nM FITC-labeled LK-2-Z_{HER2} were treated on cells under various conditions: 3 mM methyl-β-cyclodextrin, 20 mM sodium azide (NaN_3), 0.45 M sucrose hypertonic media, hypo-K^+ buffer condition (140 mM NaCl, 50 mM HEPES, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$), or 50 nM wortmannin. For the inhibition study at low temperature, cells were pre-incubated at 4 °C for 3 h. The cellular uptake was analyzed via FACS analysis. A total of 1×10^4 cells were analyzed for each sample and dead cells were excluded by the gating strategy.

Confocal laser scanning microscopy (CLSM) observation of cells

Cells were seeded on a confocal dish (SPL) with the density of 1.5×10^5 cells/mL and were incubated at 37°C and 5% CO_2 for 24 h. The cells were then treated with the protein samples for 1 or 24 h. The cells were thoroughly washed with DPBS for twice and the nuclei were stained with Hoechst 33342 dye (Thermo Fisher Scientific) for 15 min. The images were acquired in RPMI + 10%FBS using the confocal microscope Leica TCS SP8 X (Leica, Germany). The FITC fluorescence was detected at the excitation and emission wavelengths of 495 nm and 519 nm, respectively. The DOX fluorescence was detected at the excitation and emission wavelengths of 470 nm and 595 nm, respectively.

To obtain the HER2-immunostaining images of *SKBR-3, HCC-1937, BT474 and MCF-7* cells, the cells were fixed with 4% paraformaldehyde. The cells were thoroughly washed twice with DPBS and blocked with 3% BSA in a PBS solution for 30 min. The fixed cells were labeled with an anti-HER2 antibody (abcam, ab241325), followed by Donkey anti-Rabbit IgG H&L (Invitrogen, A21207) following the manufacturer's protocol. Neu siRNA (HER2 siRNA, Santa Cruz, sc-156048) was used to knockdown the HER2 expression and siRNA-A (Santa Cruz, sc-37007) was used as a negative control at the concentrations of 80 pmol in 1 mL.

Preparation of protein-DOX and protein-Cy5.5 conjugates

LK-2-Z_{HER2}-cys and Z_{HER2}-cys proteins, which possess a cysteine at each *C*-terminus of LK-2-Z_{HER2} and Z_{HER2}, respectively, were prepared in the *E. coli* system. Aldoxorubicin (INNO-206, AdooQ, Cat no. A12326) and sulfo-cyanine 5.5 maleimide (Lumiprobe, Cat no.27380) were used to create protein-DOX and protein-Cy5.5 conjugates, respectively. Solutions of LK-2-Z_{HER2}-cys and Z_{HER2}-cys were prepared at 100 µM in a degassed 20 mM Tris buffer (pH 7-7.5) on ice. To reduce disulfide bond, 100× molar excess of TCEP (tris-carboxyethylphosphine) was added to the protein solutions and the mixture was placed in ice bath for 20 min. Either aldoxorubicin or sulfo-cyanine 5.5 maleimide was dissolved in DMSO and 20× molar excess of the reagent was added to the mixture. The mixture was thoroughly mixed for overnight at 16°C. The excess aldoxorubicin or sulfo-cyanine 5.5 maleimide was removed using a 10 KDa Amicon centrifuge filter (Milipore). The final products were stored in a solution containing 20 mM Tris, 200 mM KCl, 1 mM dithiothreitol (DTT) and 10% glycerol. The labeling degree was calculated via the following equation:

$$Labeling\ degree = \frac{A_{max}\ of\ the\ labeling\ protein}{\epsilon' \times protein\ concentration\ (M)} \times dilution\ factor$$

where A_{max} and ἐ were the absorbance at 481 nm and 10,410 $M^{-1}cm^{-1}$ for the doxorubicin labeling and those were the absorbance at 650 nm and 235,000 $M^{-1}cm^{-1}$ for the Cy5.5 labeling. The labeling degrees of doxorubicin and Cy5.5 were calculated as ~65% and 10-30%, respectively.

Cell viability assay

The cytotoxicity of each sample was measured using the cell counting kit-8 (CCK-8) (Dojindo, Japan). Both of *SKBR-3* and *HCC-1937* cells (8×10^3 per well) in RPMI+10%FBS were seeded on a 96-well plate and incubated for 24 h. The cells were treated with each sample in DMEM + 10%FBS with various concentrations for 24 h. Following the incubation, the cells were washed with PBS twice and were treated with 100 µL of DMEM + 10%FBS + 10% CCK-8. The cells were incubated in the cell culture incubator for 1 h and the absorbance at 450 nm was measured using a microplate reader (Molecular Device Co., Menlo Park, CA).

BT474 spheroid formation and the CLSM imaging

BT474 cells were seeded in a round-bottom 96 well plate at density of 2×10^4 cells per a well and cultured for 3 days. After aspiration of culture medium, Cy5.5-labeled Z_{HER2} or LK-2-Z_{HER2} was treated at 200 μM and the cells were further incubated for 1-24 h. After the incubation, the *BT474* spheroids were washed several times with DPBS, and then fixed with 4% paraformaldehyde for 30 min. The fixed spheroid was observed by CLSM (LSM 510, Zeiss, Germany) with a Cy5.5 filter set.

Tumor xenograft model

Female Nu/Nu nude mice were purchased from Koatech Technology Corporation (Pyeongtaek, Republic of Korea). To obtain the *BT474* and *MCF-7* xenograft models, 5×10^6 cells of each cell line were mixed with Matrigel (100 µL) and subcutaneously injected into the left and right thighs of Nu/Nu nude mice, respectively. An estradiol supplement (estradiaol cypionate 1 mg/mL, 100 µL) was injected subcutaneously into the neck area to increase tumor engraftment rate.

All procedures of animal research were provided in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent experiment provided by the IACUC (Institutional Animal Care and Use Committee) in school of medicine, The Catholic University of Korea (Approval number: CUMS-2020-0359-01).

In vivo and *ex vivo* imaging

100 µL of Cy5.5-labeled LK-2-Z_{HER2} (3.75 µM) was administered intravenously into the *BT474* and *MCF-7* tumor-bearing mice. Whole body fluorescence images were taken by IVIS Lumina XRMS (PerkinElmer, Inc, Waltham, MA, USA) with a Cy5.5 filter under respiratory anesthesia. Blood samples were collected at different time points and the fluorescence of the sample was analyzed by IVIS Lumina XRMS. Euthanasia was performed five hours post-injection and major organs and tumors were excised for *ex vivo* imaging and they were also analyzed by IVIS Lumina XRMS.

Immunofluorescence imaging of tissues

The tumor tissues excised from *BT474* tumor-bearing xenograft were fixed for 16 h with 4% paraformaldehyde, and immersed in a sucrose solution overnight. They were embedded in optimal cutting temperature (OCT) compound and stored at -80 ^{o}C. The frozen tissue was sectioned with a thickness of 10 μm, which were washed with PBS and then incubated with 20 μg/mL of CD31 antibody labeled with FITC (eBioscience, San Diego, CA, USA) for 2 h in a humidified chamber. They were counterstained with 1 μg/mL of Hoechst 33342. The sectioned tissues were observed by the CLSM (LSM 510, Zeiss, Germany).

Acute toxicity analysis

Blood samples (500 µL) were obtained from abdominal vena cava 24 h post intravenous injection of LK-2-Z_{HER2} (10 µM in 100 µL per a mouse) or saline. The plasma was isolated from the blood sample and the concentrations of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN) and creatinine (CREA) were measured using DRI-CHEM NX 500 (Fujifilm Corporation, Tokyo, Japan).

Table S1. Amino acid sequences of LK-2, Domain Z (Z_{wt}), and $Z_{HER2:342}$

	Sequence
LK-2	LKKLLKLLKKLLKLGGLKKLLKLLKKLLKLAG
Domain Z (Z_{wt})	VDNKFNKEQQNAFYEILHLPNLNEEQRNAFIQSLKDDPSQSANLLAEAKKLNDAQAPK
$Z_{HER2:342}$ (Z_{HER2})	VDNKFNKEMRNAYWEIALLPNLNNQQKRAFIRSLYDDPSQSANLLAEAKKLNDAQAPK

Table S2. Primer sequences for the recombinant DNA constructs

Gene constructs	Primers (5' to 3')	Method
Domain Z (Z_{wt}) amplifications	For: TTCGAGCTCCGTCGAC	Gene synthesis and amplification
	Rev : GTGCTCGAGTTATTTCGGGG	
Z_{HER2} gene amplification	For:CAAGAGCTCCGTCGACGTTGACAACAAATTCAACAA	Gene synthesis and amplification
	Rev. GTGCTCGAGTTATTTCGGGGCCTGCG	
pET28b - LK-2 – Z_{HER2}	for: AGCTCCGTCGACGTTG	Blunt-end ligation
	Rev: CTTGAACTCAGAGCCACCA	
pET28b - LK-2 – Z_{wt}	for: AGCTCCGTCGACGTTG	Blunt-end ligation
	Rev: CTTGAACTCAGAGCCACCA	
pET28b – Z_{HER2}	For: GTCGACGTTGACAACAAATTCAAC	Blunt-end ligation
	Rev : GAATTCCATATGGCTGCCG	

Table S3. Acute toxicity analysis of mouse plasma

Groups	ALT [a] [U/L]	AST [b] [U/L]	ALP [c] [U/L]	BUN [d] [mg/dL]	CREA [e] [mg/dL]
Saline	33±2.64	81±33.29	375.0±116.2	21.9±1.68	0.10±0.04
LK-2-Z_{HER2}	32±6.93	89±14.73	282.3±47.3	20.8±0.5	0.14±0.03

[a] alanine transaminase; [b] aspartate transaminase; [c] alkaline phosphatase; [d] blood urea nitrogen; [e] creatinine

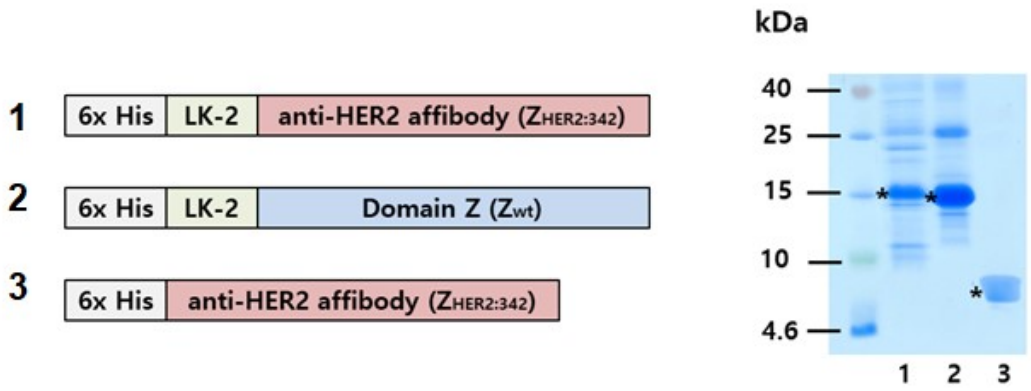

Figure S1. The constructions and purification of the recombinant proteins. The constructions of LK-2-Z_{HER2} (1) , LK-2-Z_{wt} (2) and Z_{HER2} (3). The SDS-PAGE analysis of the purified proteins: LK-2-Z_{HER2} (13.7kDa), LK-2-Z_{wt} (13.7 kDa) and Z_{HER2} (9.5 kDa).

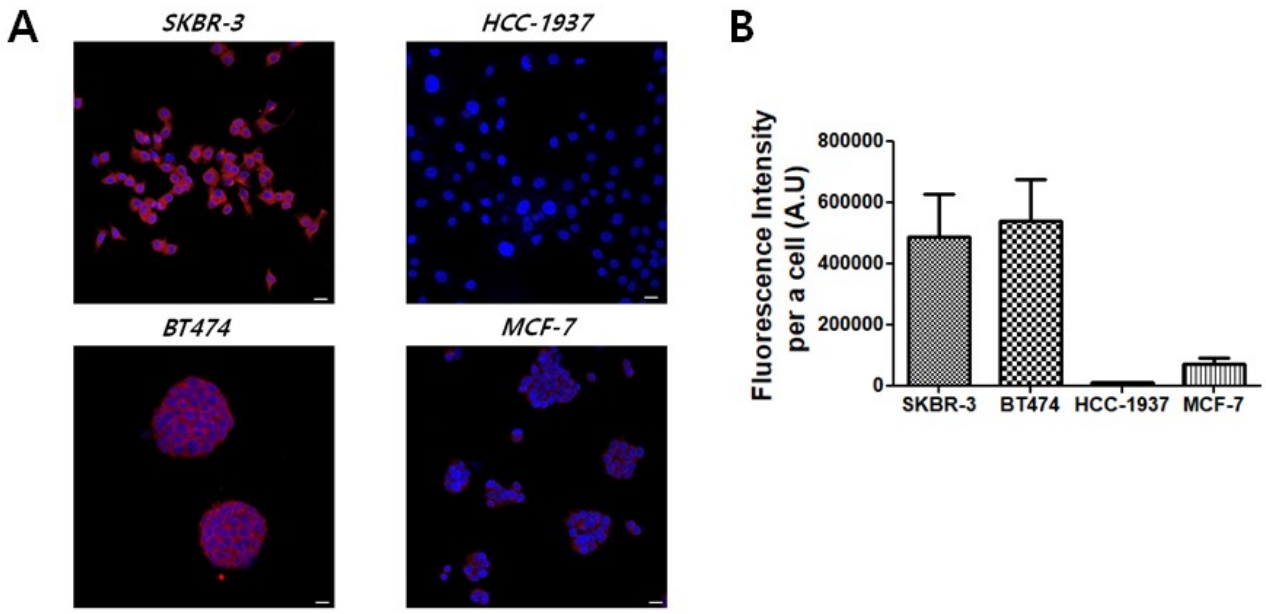

Figure S2. HER2 expression levels of *SKBR-3, HCC-1937, BT474* and *MCF-7* (A) Representative confocal laser microscopy (CLSM) images of *SKBR-3*, *HCC-1937*, *BT474*, and *MCF-7* cells. The cells were fixed 4% paraformaldehyde. The fixed cells were labeled with anti-HER2 antibody, followed by Donkey anti-rabbit IgG H&L (Alexa 594) antibody for the visualization. Scale bar = 20 μm. (B) Comparison of the fluorescence level on each cell type. The fluorescence intensity of each cell was quantified by Image-J program and the average value of more than 35 cells was compared. The data points are shown as the average values ± standard deviation.

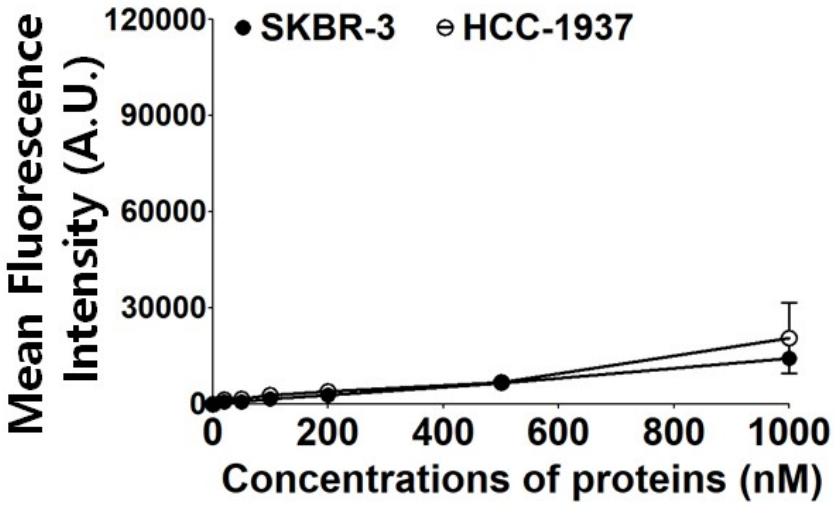

Figure S3. Cellular uptake of FITC-labeled LK-2-Z_{wt} at various concentrations on *HCC-1937* (HER2-) and *SKBR-3* (HER2+) cells after 1h-incubation. Mean fluorescence intensity (MFI) of 10,000 cells are shown. The data points are shown as the average values ± standard deviation (*n*=3).

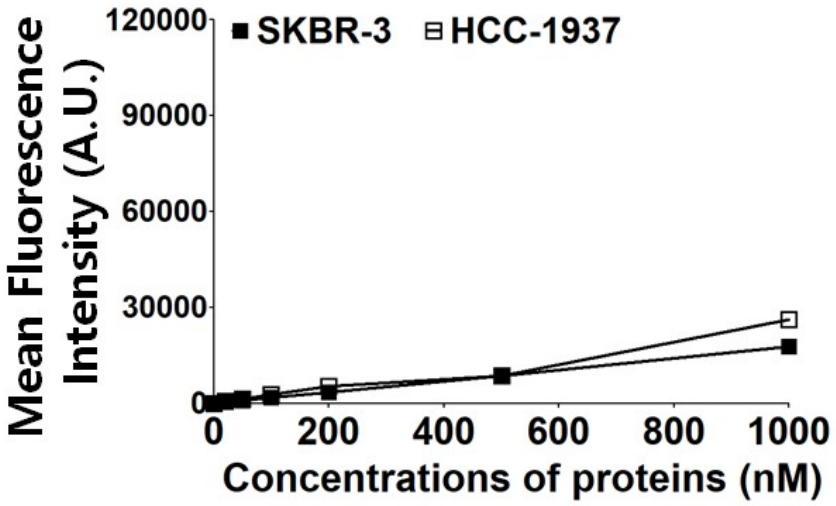

Figure S4. Cellular uptake of FITC-labeled $Z_{HER2:342}$ at various concentrations on *HCC-1937* (HER2-) and *SKBR-3* (HER2+) cells after 1h-incubation. Mean fluorescence intensity (MFI) of 10,000 cells are shown. The data points are shown as the average values ± standard deviation (*n* = 3).

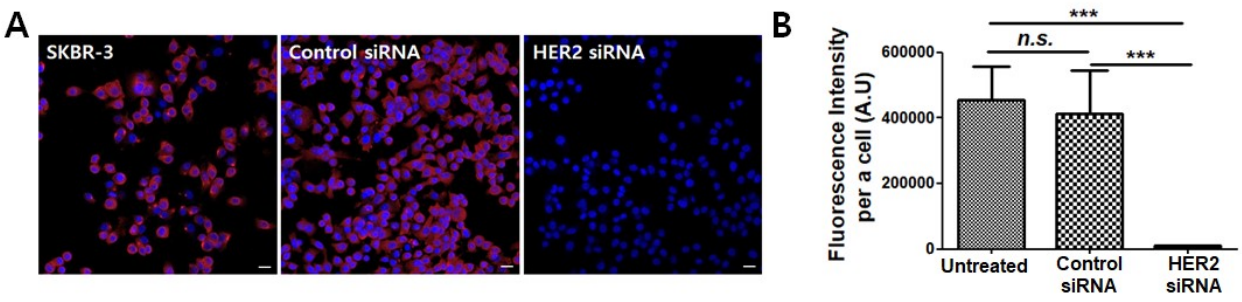

Figure S5. HER2 expression levels of HER2-knockdown *SKBR-3.* (A) The HER2 expression levels of *SKBR-3* cells treated either with control siRNA or HER2 siRNA were analysed. Cells were fixed 4% paraformaldehyde. The fixed cells were labeled with anti-HER2 antibody, followed by Donkey anti-rabbit IgG H&L (Alexa 594) antibody. Scale bar = 20 µm. (B) Quantification of HER2 receptors labeled with antibodies through Image-J program. The average values of fluorescent intensity per a cell were plotted. The data points are shown as the average values ± standard deviation. 35 cells were analyzed. The data were analyzed using two-tailed Student's *t*-tests. (***) indicates $p \leq 0.001$.

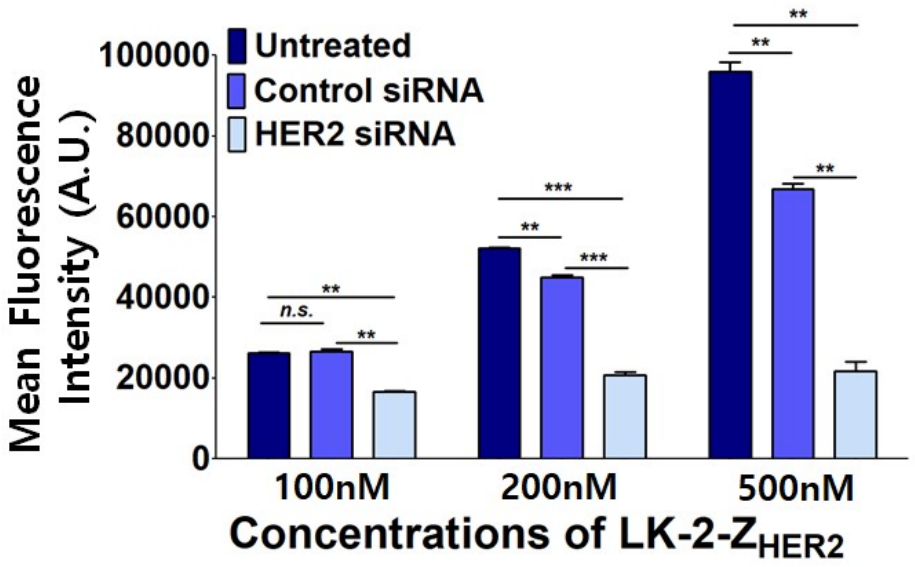

Figure S6. Cellular uptake of FITC-labeled LK-2-Z_{HER2} on *SKBR-3* and HER2-knockdown *SKBR-3*. The cellular uptake levels of FITC-labeled LK-2-Z_{HER2} were compared in untreated cells, control siRNA-treated cells, and HER2 siRNA-treated cells by the FACS analysis. Cells were transfected with siRNA for 48 h and then were treated with FITC-labeled LK-2-Z_{HER2} for 1 h. The data points are shown as the average values ± standard deviation (n = 3). The data were analyzed using two-tailed Student's t-tests. (**) and (***) indicate $p \leq 0.01$ and $p \leq 0.001$, respectively.

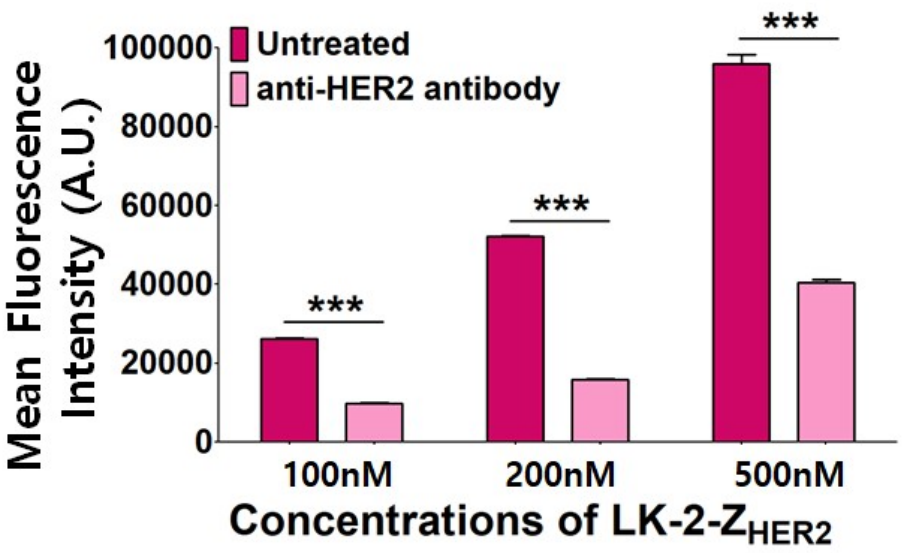

Figure S7. Cellular uptake of FITC-labeled LK-2-Z_{HER2} on *SKBR-3* and on HER2-blocked *SKBR-3*. HER2 receptors were blocked by anti-HER2 antibody targeting extracellular domain of HER2 for 3 h and the cells were treated with various concentrations of FITC-labeled LK-2-Z_{HER2} for 1 h. The cellular uptake of FITC-labeled LK-2-Z_{HER2} was measured by the FACS analysis. The data are shown as the average values ± standard deviation (n=3). The data were analyzed using two-tailed Student's t-tests. (***) indicates $p \leq 0.001$.

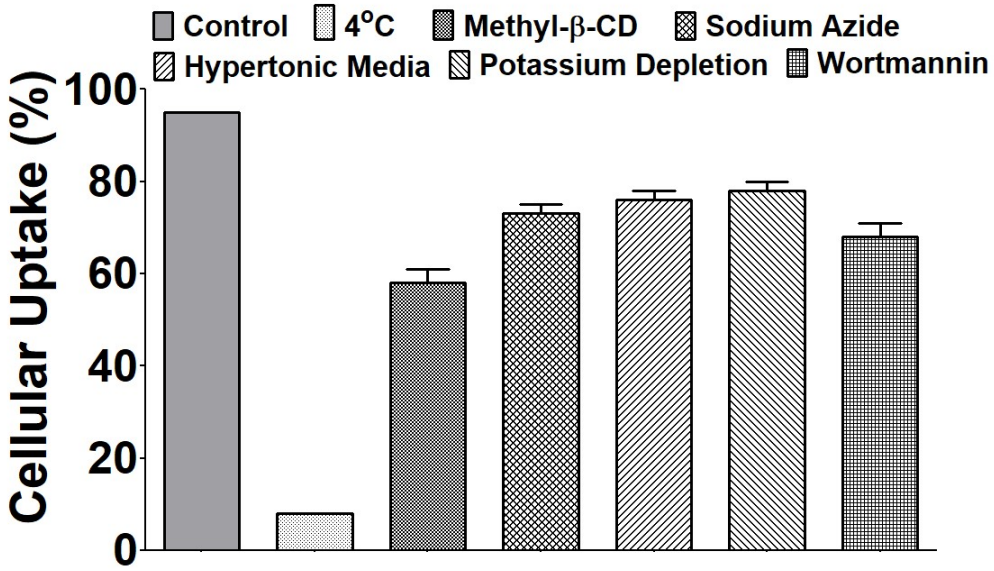

Figure S8. Cellular uptake of FITC-labeled LK-2-Z_{HER2} on *SKBR-3* treated with various endocytosis inhibitors. *SKBR-3* cells were pre-treated with various endocytosis inhibitors for 3 h and they were treated with 200 nM of FITC-labeled LK-2-Z_{HER2} for another 1 h. PBS treated cells were used as a control. The data points are shown as the average values ± standard deviation.

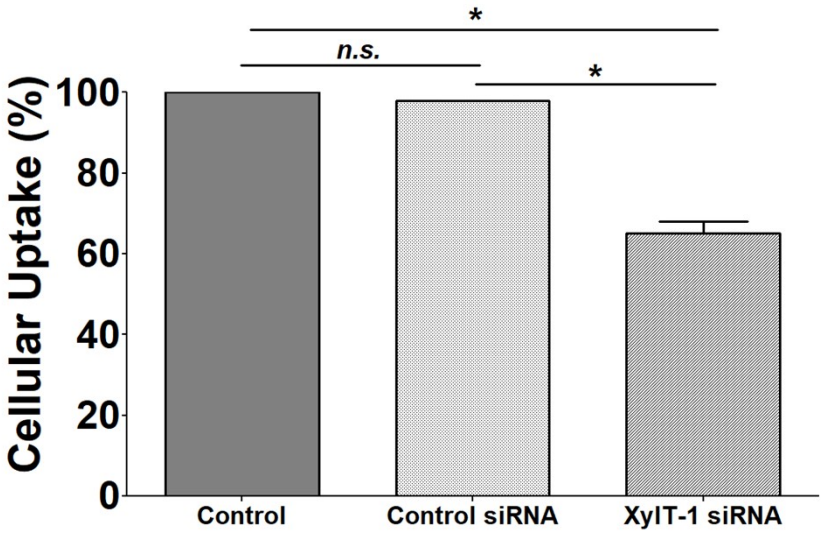

Figure S9. Cellular uptake of FITC-labeled LK-2-Z_{HER2} on *SKBR-3* treated with XylT-1 siRNA. *SKBR-3* cells were treated either with control siRNA and XylT-1 siRNA for 48 h and then, they were treated with 200 nM of FITC-labeled LK-2-Z_{HER2} for 1 h. The data points are shown as the average values ± standard deviation (*n* = 3). The data were analyzed using two-tailed Student's *t*-tests. (*) indicates $0.01 < p \leq 0.05$.

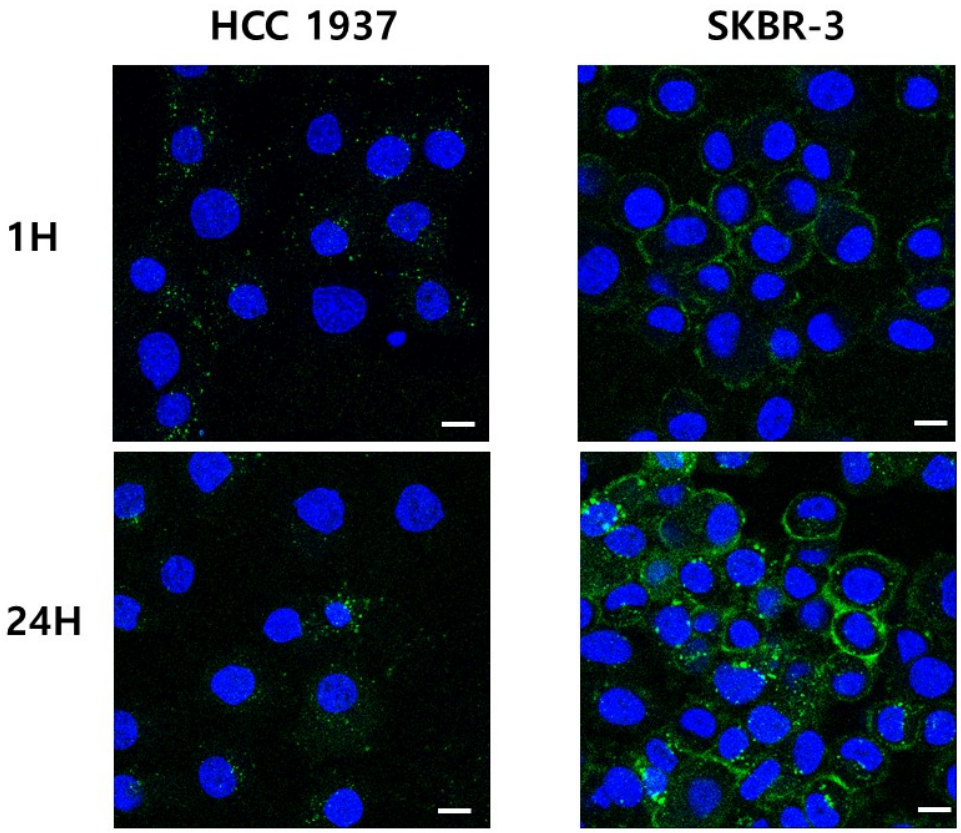

Figure S10. Representative CLSM images of cells treated with FITC-labeled LK-2-Z_{HER2}. *HCC-1937* and *SKBR-3* cells were treated with FITC-labeled LK-2-Z_{HER2} at 200 nM for 1 h and 24 h. Nuclei were stained with Hoechst 33342. Scale bar = 10 µm.

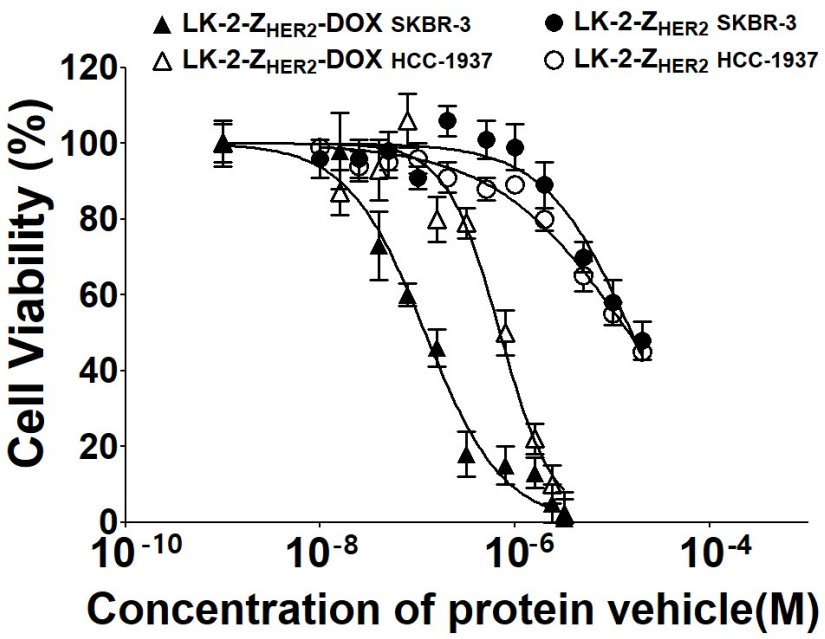

Figure S11. Comparison of toxicity of LK-2-Z$_{HER2}$ and LK-2-Z$_{HER2}$-DOX on *HCC-1937* and *SKBR-3* cells. The cells were treated with each protein for 24 h and cell viability was measured using the CCK-8 assay kit. The data points are shown as the average values ± standard deviation (*n* = 3).

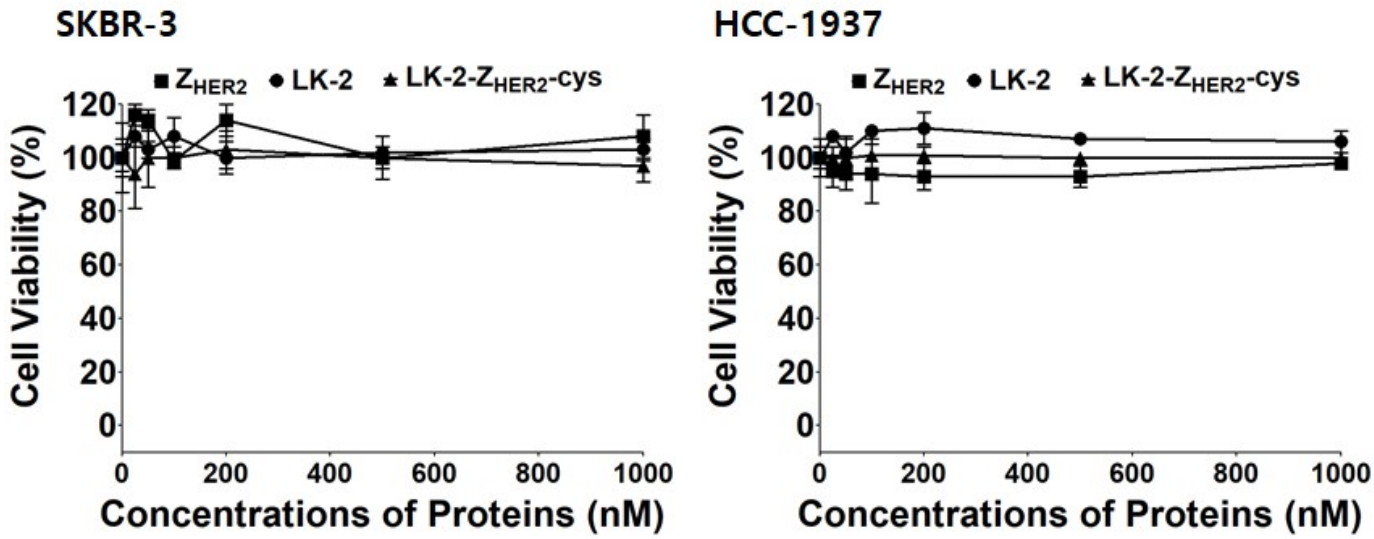

Figure S12. Cytotoxicity of LK-2, Z_{HER2} and LK-2-Z_{HER2}-cys on *SKBR-3* and *HCC-1937* cells after 24h-incubation. The cells were treated with proteins for 24 h and cell viability was measured using the CCK-8 assay kit. The data points are shown as the average values ± standard deviation (*n* = 3).

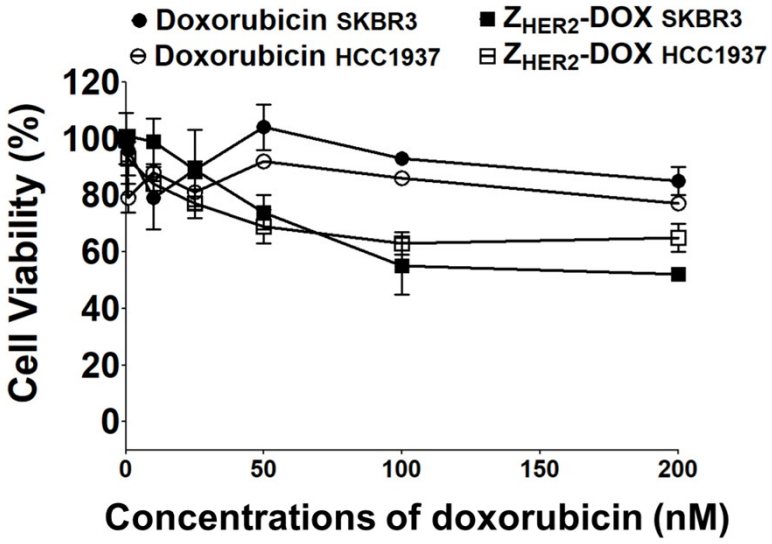

Figure S13. Cytotoxicity of free DOX and Z_{HER2}-DOX conjugates on *SKBR-3* and *HCC-1937* cells. The cells were treated with the samples for 24 h and the toxicity was measured using the CCK-8 assay kit. The data are shown as the average values ± standard deviation (*n* = 3).

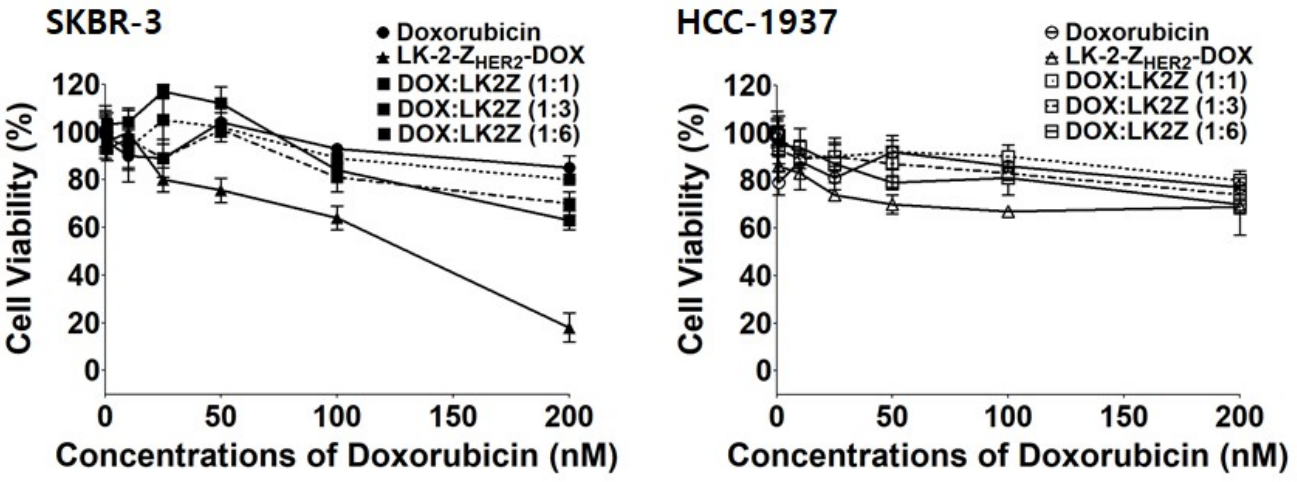

Figure S14. Cytotoxicity of non-covalent mixtures of LK-2-Z_{HER2} and free DOX. Comparison of anti-cancer effect of doxorubicin, LK-2-Z_{HER2}-DOX and the non-covalent simple mixtures of doxorubicin and LK-2-Z_{HER2} with various ratio (1:1, 1:3, 1:6) on *SKBR-3* (left) and *HCC-1937* (right). The data are shown as the average values ± standard deviation (*n* = 3).

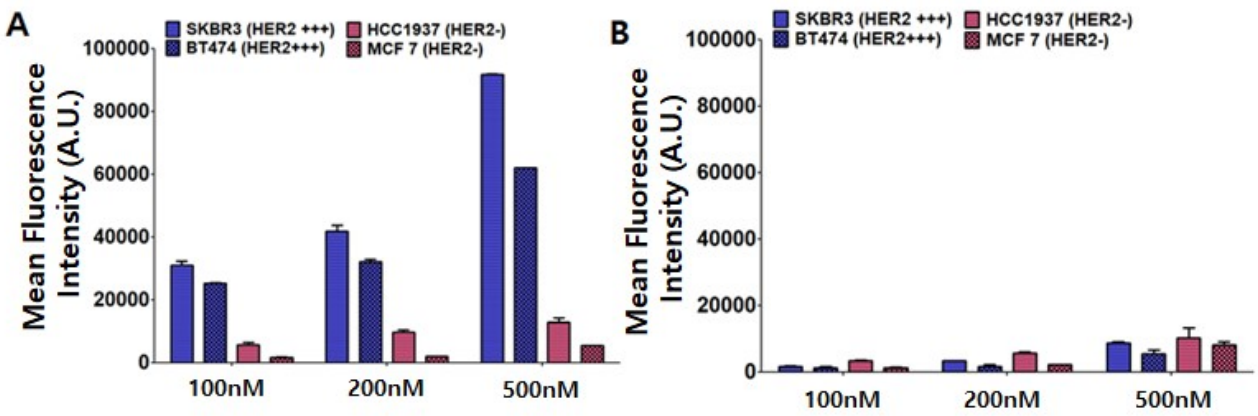

Figure S15. Cellular uptake of FITC-labeled LK-2-Z_{HER2} and LK-2-Z_{wt} on *SKBR-3, HCC-1937, BT474* and *MCF-7* cells. (A) FACS results for selective cellular uptake of FITC-labeled LK-2-Z_{HER2} on HER2-overexpressed cells (*SKBR-3* and *BT474*) and HER2-negative cells (*HCC-1937* and MCF-7). (B) FACS results for the cellular uptake of FITC-labeled LK-2-Z_{wt} on HER2-overexpressed cells (*SKBR-3* and *BT474*) and HER2-negative cells (*HCC-1937* and *MCF-7*). The cells were treated with proteins for 1 h. The data points are shown as the average values ± standard deviation (*n* = 3).

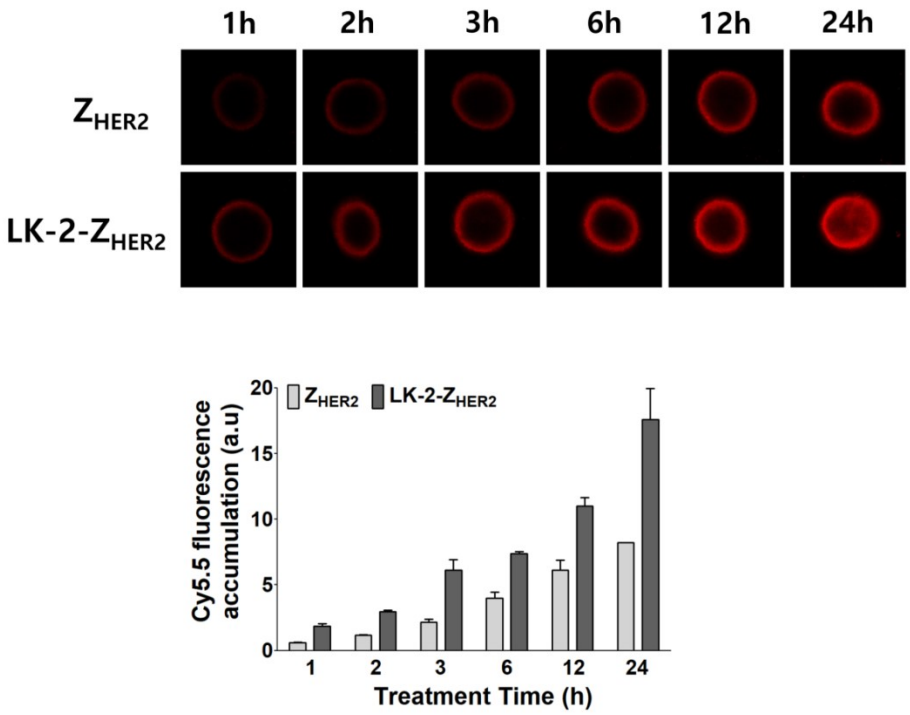

Figure S16. Penetration into *BT474* spheroid. (A) The CLSM images of *BT474* spheroids treated with Cy5.5-labeled Z_{HER2} and LK-2-Z_{HER2} at 200 nM with varying the incubation time. (B) Accumulation of Cy5.5 fluorescence inside the spheroids treated with Z_{HER2} and LK-2-Z_{HER2}. The data are shown as the average values ± standard deviation (*n*=3).

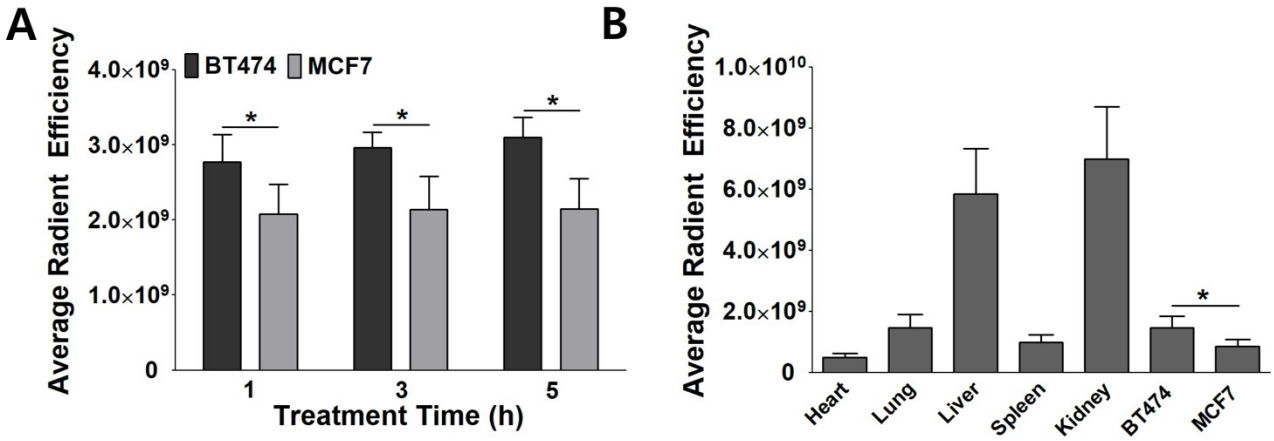

Figure S17. ***In vivo*** **biodistribution of LK-2-Z_{HER2} in** ***BT474*** **and** ***MCF-7*** **tumor-bearing nu/nu mice.** (A) Quantitative comparison of the fluorescence accumulation on *BT474* and *MCF-7* tumors. (B) Quantitative comparison of the fluorescence intensity in various organs and tumors. The data are shown as the average values ± standard deviation (n=3). The data were analyzed using two-tailed Student's t-tests. (*) indicates $p \leq 0.05$.

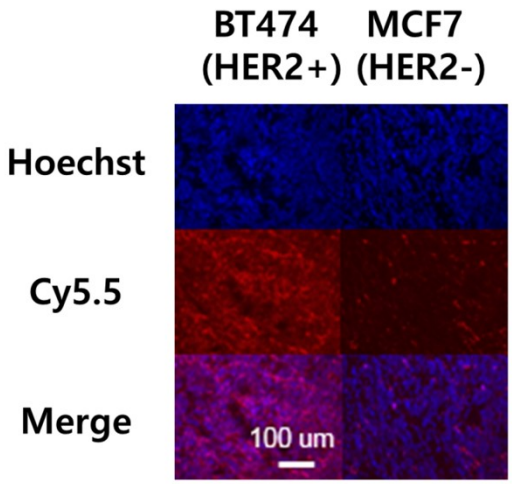

Figure S18. Representative CLSM images of the sliced *BT474* and *MCF-7* tumor tissues. The tissues were harvested 5 h after the intravenous injection of Cy5.5-labeled LK-2-Z_{HER2} (red). Nuclei were stained with Hoechst 33342 (blue).

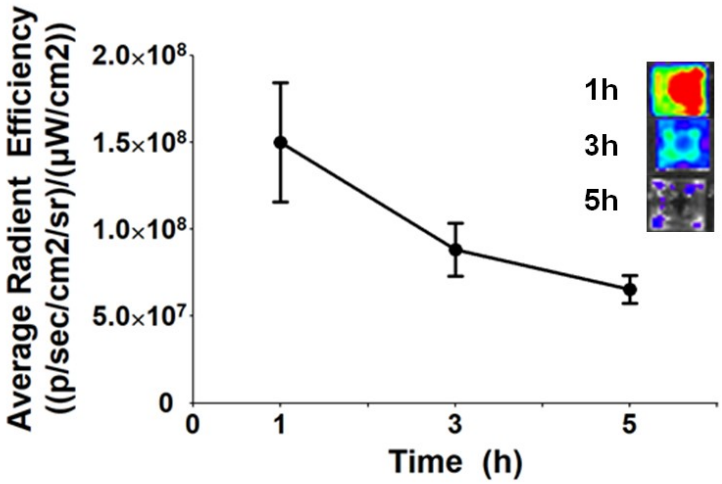

Figure S19. Remaining Cy5.5 fluorescence in blood samples after the intravenous injection. The data are shown as the average values ± standard deviation (*n*=3).

References

[1] J. H. Oh, S. Chong, S. Nam, S. Hyun, S. Choi, H. Gye, S. Jang, J. Jang, S. W. Hwang, J. Yu, Y. Lee, *Adv. Sci.* **2018**, 5, 100840.