이학석사 학위논문

Visualization of NIR-fluorescence labeled Affibody and Trastuzumab in HER2 overexpressed Gastric Cancer

HER2 과발현 위암에서 근적외선 형광 표지 Affibody 및 Trastuzumab 의 영상 연구

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정 경 윤

정 경 윤의 석사 학위논문을 인준함

2019 년 07 월

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위 원 _____ (인)

Visualization of NIR-fluorescence labeled Affibody and Trastuzumab in HER2 overexpressed Gastric Cancer

by

Kyoungyun Jeong

A thesis submitted to the Interdisciplinary Graduate Program in partial fulfillment of the Requirements for the Degree of Master of Philosophy in Cancer Biology at Seoul National University College of Medicine

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Approved by Thesis Committee:

Professor _____ Chairman

Professor _____ Vice chairman

Professor _____

ABSTRACT

Visualization of NIR-fluorescence labeled Affibody and Trastuzumab in HER2 overexpressed Gastric Cancer

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Introduction: Human Epidermal Receptor-2(HER2) is highly overexpressed in many kinds of cancers with a poor prognosis. Recently, near-infrared (NIR) fluorescence-based imaging is a growing field for both pre-clinical and clinical application. We developed molecular probe for *in vivo* imaging of HER2 using prospective NIR fluorescence, IRDye800CW. This study aimed to image HER2 overexpression in gastric cancer.

Methods: The human gastric carcinoma NCI-N87 and SNU601 and two patients-derived xenograft (PDX) cases were chosen by the level of HER2 expression. We obtained HER2-target probe under Click reaction.

Trastuzumab (Roche, USA) and HER2-target affibody (Affibody AB, Sweden) were conjugated with IRDye800CW. The resulting conjugates were characterized by Reichart and Biacore affinity measurements.

Female BALB/c nude (6 to 8 weeks old) mice were subcutaneously implanted with gastric cancer cell lines and PDX tissues. Fluorescence intensity was quantified by a Lumina II (Perkin Elmer) and laparoscopic NIR camera (InTheSmart, Seoul, Korea).

Results: Trastuzumab-IRDye800CW showed higher affinity to HER2 (K(D) = 2.093(3)pM) than unlabeled trastuzumab(K(D) = 25.75pM). This probe showed significantly high signal in HER2 positive gastric cancer cell mouse tumor model, while no or low signal retention was observed in HER2 negative group. On the other hand, HER2-target affibody-IRDye800CW showed insufficient affinity (K(D) = 4.71 nM) compared to both unlabeled affibody(K(D) = 1.42 nM) and trastuzumab molecules. The renal clearance of HER2-target affibody conjugated with IRDye800CW was so fast that we could not detect the signal.

Conclusion: Our results suggest that trastuzumab conjugated with IRDye800CW can be a feasible tool to monitor HER2 status in pre-clinical cancer imaging. Moreover, this probe can provide complementary means for assessment of HER2 expression in gastric cancer patients and/or be used to further detection of HER2-positive lesions during image-guided surgery.

Keywords: Gastric cancer, Near-infrared, cancer specific imaging, trastuzumab, affibody, HER2

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INTRODUCTION

Visualizing the lesion of gastrointestinal tract with minimally invasive procedure can be effective guidance to physicians. Image-guided biopsy and image-guided surgery can be option for precision medicine, which enables surgeons to examining the appropriate region during the operation and gastroscopy.(1, 2)

There are several kinds of image-guided surgery using various types of molecules such as radioisotope, near-infrared. Radioisotope can generate genetic mutation, damages tissues(3, 4). Compare to this, near-infrared fluorescence guided surgery is most effective and non-invasive, real-time method.(5, 6) Because of ability of the fluorescent to bind high proportion of plasma proteins and its effectiveness for navigating sentinel lymph node, ICG is commonly used in these days in gastric cancer surgery.(7-9) However, ICG is limited by its aqueous instability and rapid degradation, fast dispersion,(10, 11) while IRDye800CW have excellent water solubility, low auto-fluorescent background. Considering of auto-fluorescence, absorbance of the light and the scattering areas, it is more attractive that using near-infrared (NIR) fluorescence, IRDye800CW, to navigate deeper-located tumor with sensitivity and accuracy.(12-14) In the spectrum of 700-900nm, auto- fluorescence signals are minimized, and there is the lowest

tissue absorption of light, resulting in optimal tissue penetration.(15-17) Therefore, IRDye800CW is a fluorescent that is expected to be widely use in clinical practice.

Cancer specific imaging or targeted imaging of near-infrared dye is specific delivery of cancer tracer to tumor cells for guiding surgeons to reach right lesion of tumor during surgery, which has huge advantage of sighting of deep penetration of signals and high sensitivity compared to virtual dyes.(18) Azais H et al. developed a small ligand like folate linked to a fluorophore, which targets the folate-α receptor in ovarian cancer patients specifically.(19) To distinguish tumor cells, near-infrared based cancer specific molecules have been developed to target molecular markers such as membrane proteins. Tumor specific markers should be more expressed in tumor cells than normal cells and/or monopolistic for tumor cells.

The transmembrane glycoprotein HER2 is overexpressed in many types of cancer, especially breast and gastric cancers. The overexpression of this peptide has been correlated with poor prognosis.(20, 21)According to ToGa trial, doctors characterize HER2 positive and negative by identifying IHC and FISH level of patients and prescribe adjuvant chemotherapy with trastuzumab to people who have amplified HER2(22).

Numerous antibody mimetics or fragments are used to be modified as a tracer. One of the smallest peptides of antibody mimetics is affibody (Affibody AB, Sweden). The size of the HER2-target-affibody is 6 ~ 7kDa and it does not bind same structure as trastuzumab. Since it is very small compared to monoclonal antibody, trastuzumab (Roche, USA) and it does not share the same region of epitope as trastuzumab, many researchers tried to modify this small molecule as a tumor targeting probe. Many previous studies were conducted with the conjugation of small molecules(23, 24). Antibodies are the most widely used types of targeting agent today, and trastuzumab is a monoclonal antibody of HER2 and this antibody can be modified as a tracer of HER2. The large size of antibody molecules can, however, lead to poor solid tumor penetration and slow elimination from the circulation. To avoid these problems, smaller antibody constructs, such as Fab' s, minibodies and scFv, affibodies have successfully been made. Affibody molecules are stable three-helix molecules comprising 58 amino acids derived from a domain of staphylococcal protein A with 13 positions randomized. As affibody molecules contain no disulphite bonds, they can easily be produced in the cytoplasm and can be chemically synthesized. In this study, we focus on the visualizing of HER2 of gastric cancer by antibody and/or affibody and examining the *in vivo* targeting potential of the construct and demonstrate its utility as a tumor-imaging agent.

There are variety of studies which specifically image HER2 in different kinds of cancer cells. The use of radiolabeled HER2 specific antibodies(25-27) and fragments(28-32) for imaging HER2 expression has been reported. Since the HER2 target peptide conjugated with near-infrared fluorescence can non-invasively attach tumor cell and can penetrate tissue with low backgrounds, people have synthesized various types of probe using antibodies(33, 34) and fragments such as affibodies(35).

However, so far, no study has yet been done in gastric cancer with near-infrared fluorescence. We compared which near-infrared fluorescent conjugated peptide can well detect HER2 expression in gastric cancer.

Purpose of this study

We hypothesized that fluorescence labeled HER2-target affibody can visualize HER2 overexpressed gastric cancer more effectively than labeled with HER2 monoclonal antibody, trastuzumab. Therefore, the aim of this study is to visualize HER2 overexpression in gastric cancer and to compare those HER2 specific fluorescent tracer. Through this investigation, we would like to use this modified molecule as a diagnostic and monitoring tool for HER2 overexpressed and/or amplified gastric cancer patients.

Materials and Methods

Cell culture

Four human gastric cancer cell lines, SNU-216, SNU-601, NCI-N87 and SNU-638 cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and maintained in Rosewell Park Memorial Institute media (RPMI 1640) (Welgene, Daegu, Korea) with 10% Fetal bovine serum (FBS, Gibco, Invitrogen, UK) and 2% penicillin-streptomycin (Gibco, Invitrogen, UK). All cell lines were cultured and maintained in the conditions of 37℃ in a 5% CO2 humidified environment.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cell lines using Trizol reagent (Invitrogen, Carlsbad, USA). cDNA was synthesized to analyze mRNA expressions using a TOP script cDNA synthesis kit (Enzynomics, Daejeon, Korea) according to the manufacturer' s protocols. The expression level of HER2 was measured using SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA, USA) by normalizing to GAPDH housekeeping gene. Primers are indicated in

Table 1. All the reaction were performed and analyzed by comparative △△Ct methods using Step One Plus Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA).

Table 1. Primers for HER2 expressing, gene expression analysis by real-time PCR

Gene		Sequence
HER2	Forward	TGCCTGTGCCCACTATAAG
	Reverse	AGGAGAGGTCAGGTTTCACAC
GAPDH	Forward	TGACTTCAACAGCGACACCCA
	Reverse	CACCCTGTTGCTGTAGCCAAA

Western blotting

Cells were grown to 80% confluence and washed with cold PBS and lysed with RIPA buffer (ThermoFisher Scientific, MA, USA) plus protease inhibitor cocktails (Roche, Swiss). Proteins were isolated from cell lysates, and the lysate was collected by centrifugation at 15,000 RPM for 15min at 4℃ and the protein concentration was measured by bicinconinic acid (BCA) assay (Thermo Scientific, MA, USA). Lysate containing 25ug of protein was seperated by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), Bolt 4-12% Bis-Tris Plus gel, and transferred to a polyvinylidene difluoride (PVDF) membrane(Millapore, Watford,

UK). The membrane was probed with GAPDH(1:3000 dilution, Abcam, UK, cat. ab8245) to ensure equal protein loading, followed by blocking with 5% skim milk and incubation with primary antibody; HER2/neu(1:1,000 ; Cell signaling, USA, cat. 2165). After wasing and incubating with HRP-conjugated secondary antibodies; mouse IgG(1:3,000; Cell signaling technology, USA, cat. 7076P2) and rabbit IgG(1:3,000; Cell signaling technology, USA, cat. 7074). The membrane was washed with tris-buffered saline and tween 20(TBS-T) and visualized with Enhanced chemiluminescent (ECL) HRP substrate (ThermoFisher Sci. cat. 34577), imaged by ChemiDoc(Bio-Rad, USA).

Synthesis of HER2-specific probe

The IRDye800CW azide infrared dye(Li-COR Bioscience, USA) contain a azide that allows for site-specific labeling. Dibenzocyclooctyne group (DBCO)-PEG4-maleimide were purchased from Futurechem. Co., Ltd (Korea). PD-10 column were purchased from GE Healthcare(USA). Trastuzumab(Roche, Swiss) and HER2-target affibody(Affibody AB, Sweden) were conjugated

with IRDye800CW under click chemistry. All of the fluorescently labeled protein samples were aliquoted and sotred in the dark fridger at −20° C.

Synthesis of Trastuzumab−DBCO (TRZ−DBCO)

4 ㎕ of 0.3 M ethylenediaminetetraacetic acid(Sigma−Aldrich, MO, USA) in DW, 4 ㎕ of 1 M sodium bicarbonate(Sigma−Aldrich, MO, USA) in distilled water(DW) and 5 ㎕ 1.5 M β−mercaptoethanol(Thermo Scientific, MA, USA) in DW were added to trastuzumab solution for injection(2 mg/96 ㎕, 13.8 nmol). The mixture was incubated at 37℃ for 1 hr and purified using PD−10 column with PBS. To this 2 ml of TRZ−SH solution in PBS, 13.4 ㎕ of DBCO−PEG4−Maleimide in dimethyl sulfoxide (DMSO, 248.4 nmol) was added and incubated at 37℃ for 1.5 hr. The reaction mixture was purified using PD−10 column with PBS to give 13.5 nmol of Trastuzumab−DBCO (TRZ−DBCO).

Synthesis of Trastuzumab−IRDye800CW (TRZ−IRDye)

IRDye 800CW Azide in water (0.14 mg/13.7 ㎕, 108 nmol) was added to 2 ml of TRZ−DBCO (13.5 nmol) solution in PBS. The mixture was vortexed at 38° C for 3 hrs and purified using PD−10

column with PBS to give 13.2 nmol of Trastuzumab-IRDye800CW (TRZ-IRDye).

Synthesis of Affibody-DBCO (ABY-DBCO)

Affibody (0.5 mg, 35.7 nmol) was dissolved in 0.5 ml PBS and this solution was added to 298 ㎕ 0.1 M Dithiothreitol (DTT, Thermofisher sci. USA). The mixture was incubated at 37℃ for 1 hr and purified using PD-10 column with PBS. To this 2 ml of ABY-SH solution in PBS, 19.6 ㎕ of DBCO-PEG4-Maleimide in DMSO (363.3 nmol) was added and incubated at 37℃ for 1.5 hr. The reaction mixture was purified using PD-10 column with PBS to give 35.0 nmol of Affibody-DBCO (ABY-DBCO).

Synthesis of Affibody-IRDye800CW (ABY-IRDye)

IRDye 800CW Azide in water (0.37 mg/36.4 ㎕, 287 nmol) was added to 2 ml of ABY-DBCO (35.0 nmol) solution in PBS. The mixture was vortexed at 38° C for 3 hrs and purified using PD-10 column with PBS to give 34.3 nmol of Affibody-IRDye800CW (ABY-IRDye).

Synthesis of ^{111}In-NOTA-PEG$_3$-N$_3$

2 mCi (0.5 ml) of $^{111}InCl_3$ solution was dissolved in 0.5 ml sodium acetate buffer (pH=5.6). The mixture was added to 7.5 ㎕ of NOTA-PEG_3-N_3 (11.25 nmol) solution in DW. The mixture was incubated at 70° C for 10 min to give 2 mCi of ^{111}In-NOTA-PEG_3-N_3 (11.25 nmol). The radiolabeling efficiency (LE = 100 %) was determined using ITLC-SG after the radiolabeling with 0.1 M citric acid as the mobile phase. The Rf of ^{111}In-NOTA-PEG_3-N_3 = 0.6; Rf of free radioisotope = 0.9-1.0.

Synthesis of ^{111}In-Affibody (^{111}In-ABY)

2 mCi (1 ml) of ^{111}In-NOTA-PEG_3-N_3 (11.25 nmol) solution in sodium acetate buffer was added to 0.36 ml of Afb-DBCO (11.25 nmol) solution in PBS. The mixture was vortexed at 38° C for 3 hrs to give 2 mCi of 111In-Afb. The radiolabeling efficiency (LE = 100 %) was determined using ITLC-SG with 0.1 M citric acid as the mobile phase. The Rf of 111In-Afb = 0.2.

Stability of the near-infrared probes

The fluorescence stability of Affibody-IRDye800 (100 μg/mL) and Trastuzumab-IRDye800 (100 μg/mL) was determined using a fluorescence spectrophotometer, Varioskan LUX microplate reader (Thermofisher, USA). The aqueous solutions were prepared and placed in the 96 well black plate(SPL Life Science, Korea). The fluorescence emission spectra were measured at an excitation wavelength of 720 nm. The maximum fluorescence (λem = 840 nm) intensity of each sample at a given time was normalized by the corresponding fluorescence emission. The average normalized fluorescence intensities for each triplicate experiment were plotted.

Cell viability assay

To evaluate the cytotoxicity of trastzuaumab molecules and/or affibody molecules, cell viability assays were performed in SNU-601, NCI-87 cell line using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) at absorbance 450nm for 48hr. Cells were plated in 96-well flat bottomed plates at a density of 5×10^3 per 100uL growth medium in quintuplicate. Affibody and trastuzumab molecules were dissolved in PBS. Cells were incubated for 48 h after treatments at increasing concentrations 10uM. Luminoskan Ascent(ThermoFisher Sci. USA) was used to measure luminescence.

Binding affinity assay

Surface Plasmon resonance experiments were done on a Reichert SR7500C (Reichaert Technologies, USA) and Biacore T200 instrument (GE Healthcare, Sweden). For HER2/FC (R&D Systems, NE, USA), 1,100 relative unit (RU) protein was captured on the protein modified PEG sensor ship via an immobilization capture wizard. To verify the kinetic data, we evaluated using

software Scrubber2 and Control software version 2.0.1 & BIA evaluation software version 3.0 for each Reichert and Biacore result.

Immunofluorescence

Prepare coating coverslips in 6-well dish with poly-L-lysine (Sigma aldrich, cat. 25988) and plate 1 x 10^4cells. After the confluency of cultured dish reach ~80%, apply 4% paraformaldehyde solution (Thermo Scientific, MA, USA) for 10min, wash cells in PBS containing Triton X-100(Sigma-Aldrich, MO, USA) and 0.05% Tween20. Permealize cell with 0.5% Triton and PBS for 20min in room temperature. After remove the solution, apply blocking buffer (PBS, TritonX-100, Tween, 1% of BSA) for 30minutes in room temperature. Add near-infrared dye conjugated with HER2-target peptides overnight to cells. Wash with PBS several times, drop 20ul of DAPI ProLongGold (Invitrogen, CA, USA) Cultured cells were scanned and verified using established *in vitro* fluorescence methods with ImageXpress (Molecular Devices, San Jose, CA, USA).

In vivo near-infrared imaging experiments

Each cell line was harvested using Trypsin-EDTA(Gibco) and re-suspended at $1x10^6$ cells in 0.1 mL of 50% of growth factor reduced Matrigel (BD Bdioscience, USA, cat. 80712368) kept at 4° C and injected subcutaneously into the right flank of 6-8 weeks-old female BALB/c nu/nu mice (Orient Bio., Sungnam, Korea). Patient-dervied xenograft(PDX) tissues were chopped and implanted to 6-8 weeks BALB/C nu/nu mice by using trocar to establish tumor xenograft models.

Tumor size was measured using calipers twice every week, and the tumor volume was calculated by 1/2 × lenghth (L) × width (W) × height (H) (mm^3). All injections and tumor measurement were performed under isoflurane anesthesia to minimize stress.

To analyze the specific target accumulation of the imaging probes, mice were anesthetized by inhalation of isoflurane. Trastuzumab molecules (100ug) and affibody molecules (50ug) were injected into the tail vein and imaged by time point after injection (n = 5 / each group, n = 2 in control). At the end-point of experiments, all mice was sacrificed, and an autopsy was conducted to collect the primary tumors and fixed in 10% neutral buffered formalin solution (Sigma-Aldrich, MO, USA) for 12-24h and

paraffin-embedded and pathologically checked by hematoxylin and eosin (H&E) staining.

Fluorescence intensity was captured using Lumina II IVIS system (Perkin Elmer, USA) and NIR-camera camera (InTheSmart, Seoul, Korea). All images proceeded with IVIS were quantified using Living image 2.50.1(Perkin Elmer, USA) and laparoscopic near-infrared camera images were analyzed by Image J(http://rsb.info.nih.gov/ij/; public comain free software developed by National Institutes of Health) This animal experiment was approved by the Institutional Animal Care and Use Committee(IACUC) of Clinical Reseach Institute at Seoul National University Hospital (SNU-180308-1-2)

Small animal SPECT/CT

Mice were injected with 50 μg of ^{111}In-NOTA-Affibody. 5 min and 1 and 4 hours after injection, mice were scanned on an animal SPECT/CT device, using a 1.0-mm-diameter pinhole rat collimator cylinder. SPECT scans were acquired for 45 min (1 h after injection) or 60 min (4 h after injection), followed by CT scans for anatomic reference. Scans were reconstructed with VivoQuant reconstruction software (Invicro, MA, USA).

Staining cancer specific probe to fresh tissue

We obtained SNU-601 and NCI-N87 solid tumor from mouse xenograft model. Trastuzumab-IRDye800CW (80ug) and affibody-IRDye800CW (40ug) were sprayed to gastric cancer cell derived isolated tumor. We applied each molecule for 5, 30 and 45 min to tumors. After washing the probes, we took fluorescence images using Lumina II IVIS system.

Histology and immunohistochemistry

For histological analysis, the formalin-fixed paraffin-embedded (FFPE) tissues were sectioned at a thickness of about 4um, stained with hematoxylin and eosin (H&E) using Image Scope (Aperio, Leica Biosystems, USA), and immunohistochemistry (IHC) was carried out by the department of pathology at Seoul National University Hospital.

Statistical analysis

Student's t-test was used to compare gene expression and paired t-test was used to test cell proliferation. Wilcoxon signed-rank test and Mann-Whitney test was were performed using GraphPad PRISM version 8.0.0 (GraphPad Software, San Diego, CA, USA). All data were presented as means ± standard deviation (SD). Statistical significance was defined as two-sided p-values $P < 0.05$.

Results

Expression level of HER2 in gastric cancer

We examined gene expression of ErbB2 in four gastric cancer cell lines, NCI-N87, SNU-601, SNU-620, SNU-638 by quantitative real-time PCR (Fig. 1A). Significant differences in the expression of HER2 was quantified by normalizing to the expression of GAPDH housekeeping gene. NCI-N87 and SNU-601 showed remarkably high and low expression of ErbB2.

Expression of HER2 and GAPDH as determined by western blotting of cultured cell lysates. Based on this, we selected NCI-N87 and SNU-601 as HER2 positive and negative. (Fig.1B)

Fig. 1

(A)

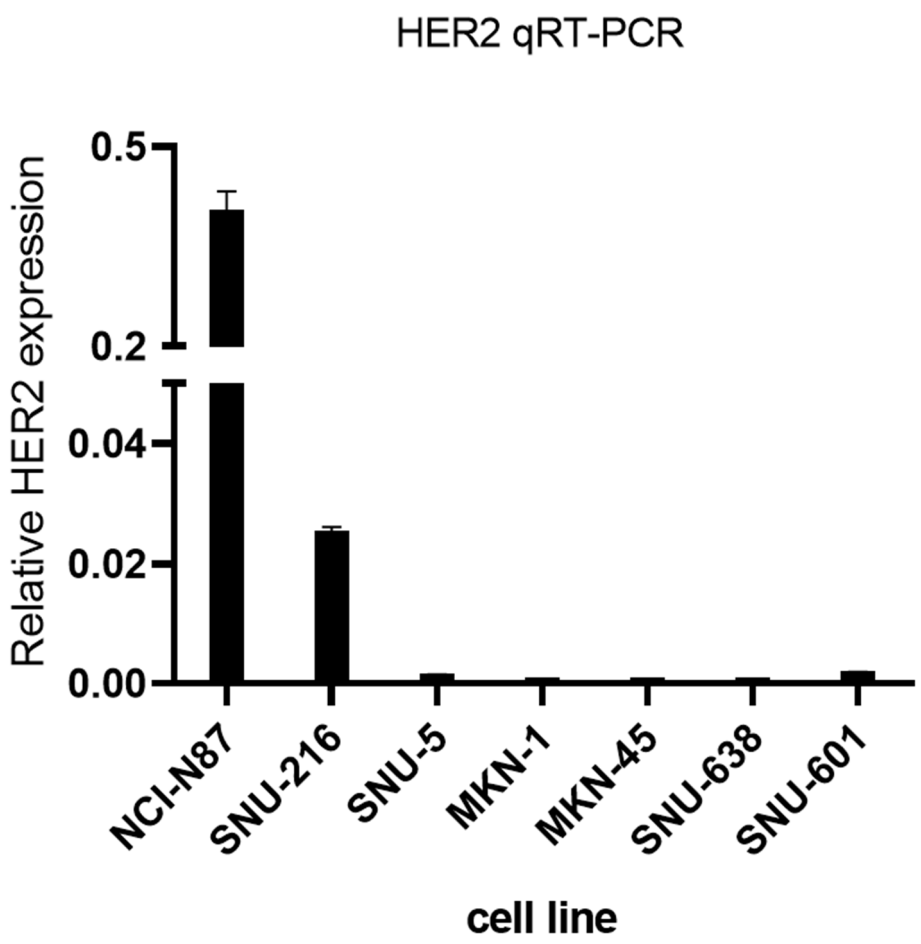

HER2 qRT-PCR
Relative HER2 expression
0.5
0.2
0.04
0.02
0.00
NCI-N87
SNU-216
SNU-5
MKN-1
MKN-45
SNU-638
SNU-601
cell line

Fig. 1

(B)

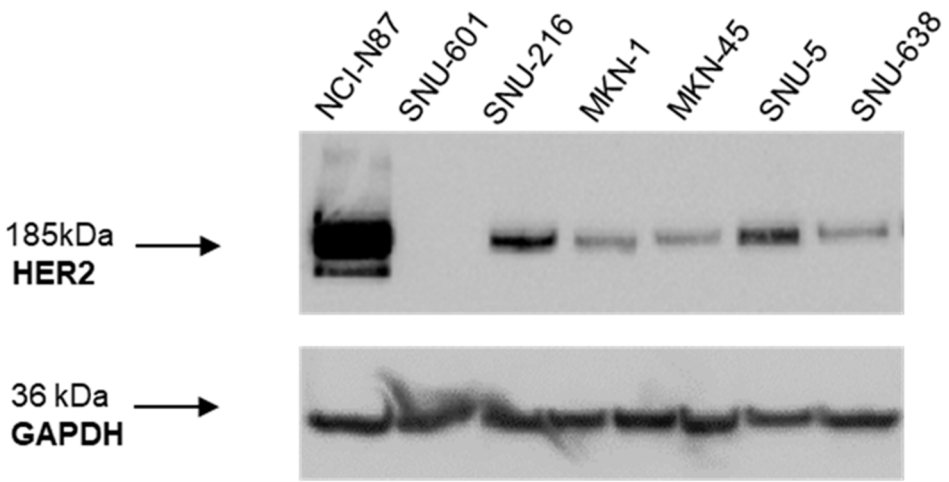


Figure 1. HER2 expression of gastric cancer cell line

A, HER2 mRNA level of gastric cancer cell line B, HER2 protein level of gastric cancer cell line

Fluorescence conjugation of the HER2-specific peptide

To ensure that the HER2-target peptide, trastuzumab and affibody, retained its ability to bind HER2/neu receptor, surface plasmon resonance (SPR) was performed.

Trastuzumab-IRDye800CW and affibody-IRDye800CW were successfully synthesized with click mechanism (Fig. 2A) The efficiency of conjugation of IRDye800CW to both trastuzumab and affibody were determined based on absorbance measurements using a Varioskan LUX (Thermofisher, USA) at a different emission wavelength spectrum. There were no big difference in absorbance between Trastuzumab-IRDye800 and Affibody-IRDye800. (Fig. 2B)

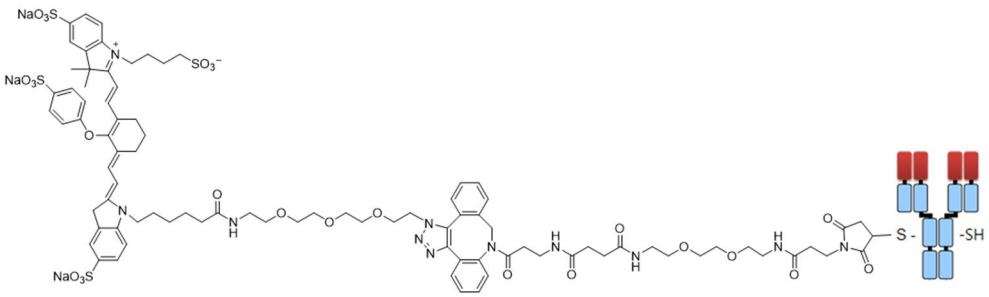
Trastuzumab molecules were analyzed by Reichert. (Fig. 2C, Supplementary Table. 1A) ErbB2 was immobilized at the 1,100 relative unit (RU) in the sample channel (Supplementary Table. 1B). Scrubber2 analysis software was used to obtain the equilibrium dissociation rate constant between ligand (ErbB2) and analyte (trastuzumab, trastuzumab-DBCO, trastuzumab-DBCO-

IRDye800CW). Data were entered into 1:1 kinetic binding model and the values of Ka($M^{-1}s^{-1}$), Kd(s^{-1}), KD (nM) were calculate (Table 2).

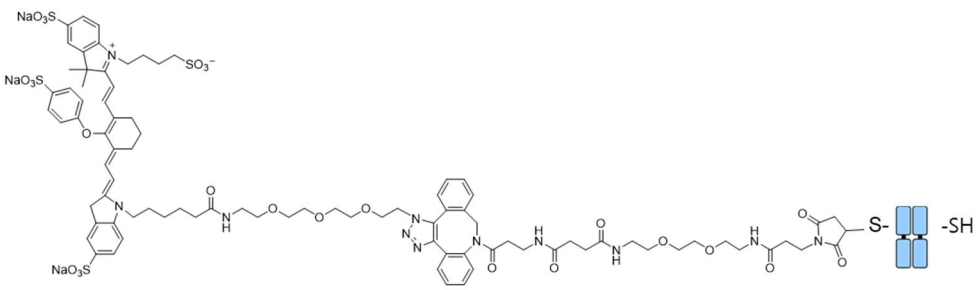
Binding affinity of Affibody molecules was performed with Biacore. (Fig. 2D, supplementary Table.1b) BIAevaluation analysis software was used to obtain the equilibrium dissociation rate constant between ligand (ErbB2) and analyte (affibody, affibody-DBCO, affibody-DBCO-IRDye800CW). Data were entered into 1:1 kinetic binding model and the values of Ka, Kd, KD were calculate (Table2). The binding affinity level of trastuzumab-dye is higher than trastuzumab itself. However, affibody-dye affinity of HER2 was less than the unlabeled affibody.

Fig. 2

(A)

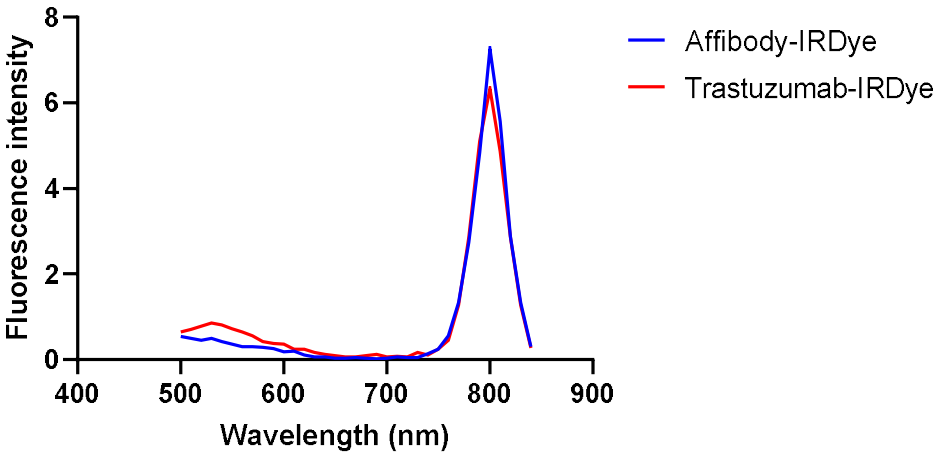


Trastuzumab-IRDye800CW

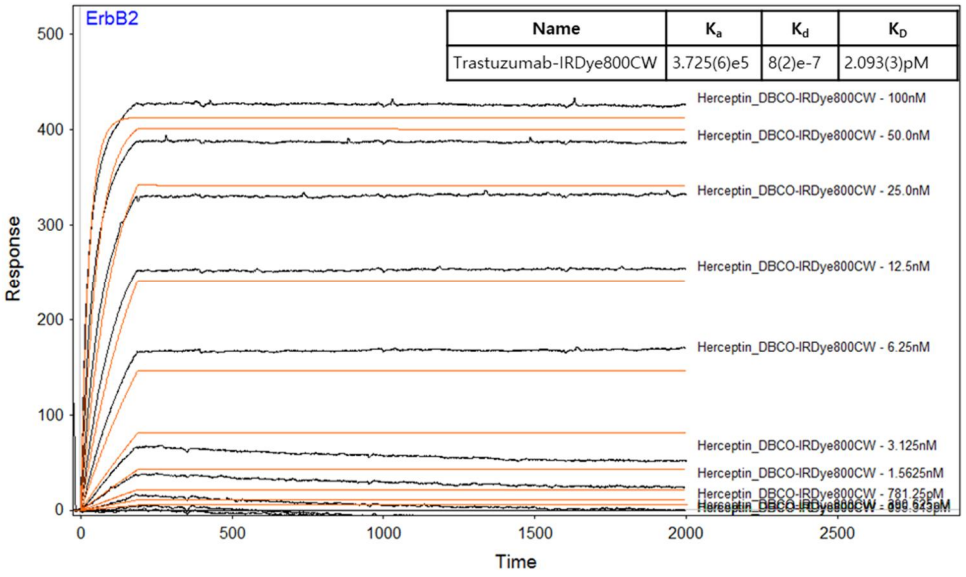


Affibody-IRDye800CW

(B)


Fluorescence intensity
8
6
4
2
0
400
500
600
700
800
900
Wavelength (nm)
Affibody-IRDye
Trastuzumab-IRDye

(C)


ErbB2
Name
K_a
K_d
K_D
Trastuzumab-IRDye800CW
3.725(6)e5
8(2)e-7
2.093(3)pM
Herceptin_DBCO-IRDye800CW - 100nM
Herceptin_DBCO-IRDye800CW - 50.0nM
Herceptin_DBCO-IRDye800CW - 25.0nM
Herceptin_DBCO-IRDye800CW - 12.5nM
Herceptin_DBCO-IRDye800CW - 6.25nM
Herceptin_DBCO-IRDye800CW - 3.125nM
Herceptin_DBCO-IRDye800CW - 1.5625nM
Herceptin_DBCO-IRDye800CW - 781.25pM
Response
Time
0
100
200
300
400
500
0
500
1000
1500
2000
2500

(D)

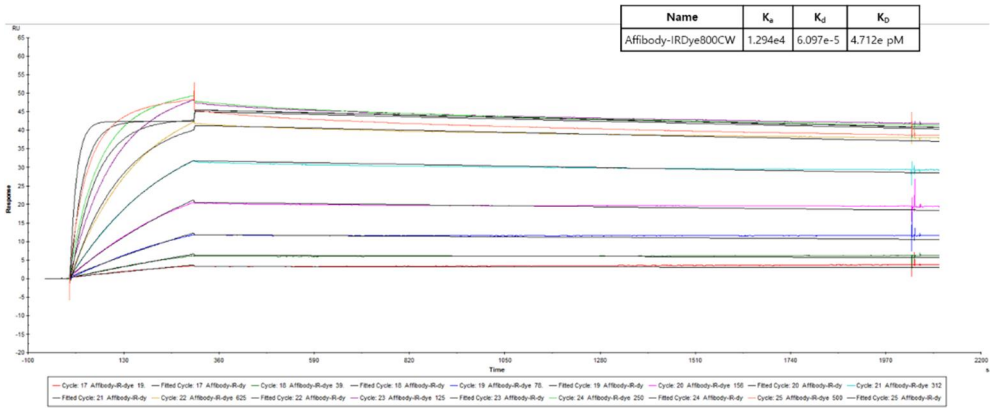


Figure 2. Binding affinity of the conjugates

A, Structure of Trastuzumab-IRDye800CW and Affibody-IRDye800CW B. Absorbance spectrum of Trastuzumab-IRDye800CW and Affibody-IRDye800CW C, Binding affinity result of trastuzumab conjugated with IRDye800CW D, SPR result of HER2-target-affibody conjugated with IRDye800CW

Supplementary Table 1A. Immobilization of ErbB2 for binding assay of trastuzumab molecules

Ligand	Concentration	Immobilization buffer	Flow rate	Immobilization level
ErbB2	3.3 ug/mL	10mM Sodium acetate, pH 5.5	20 uL/min	1,100RU

Supplementary Table 1B. Immobilization of ErbB2 for binding assay of affibody molecules

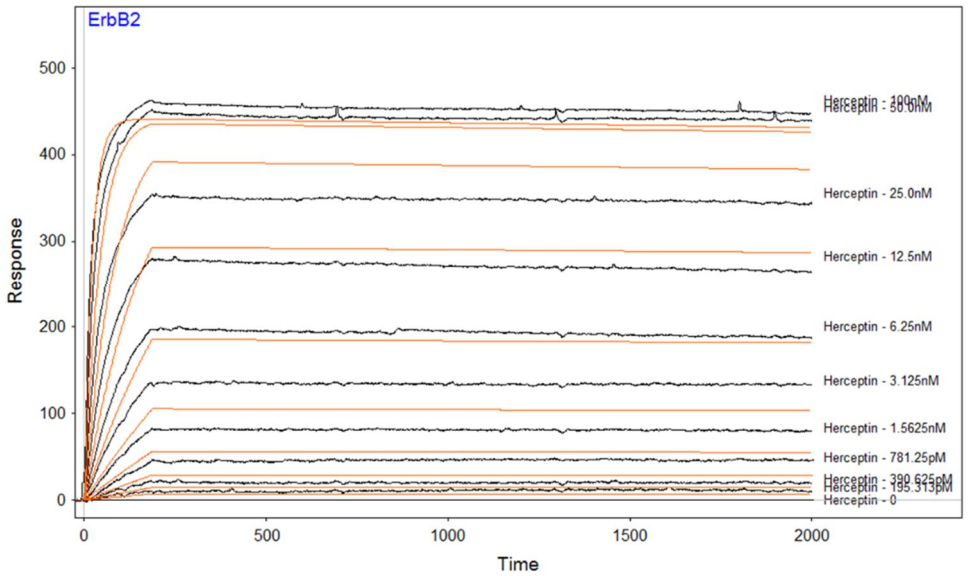
Ligand	Concentration	Immobilization buffer	Flow rate	Immobilization level
ErbB2	5 ug/mL	10mM Sodium acetate, pH 5.5	10 uL/min	1,136RU

Supplementary 1. Immobilization of ErbB2 for binding assay

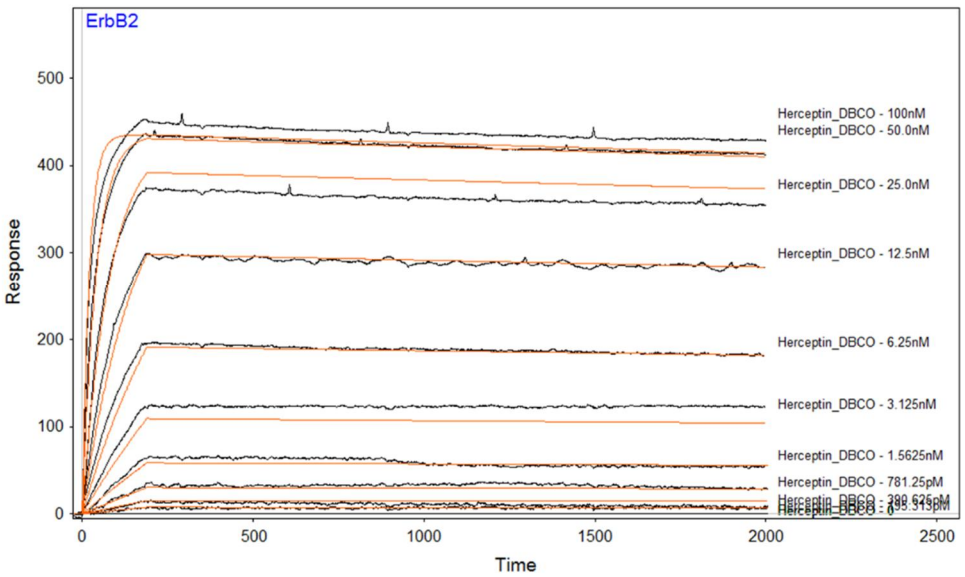
Immobilizing a Protein on a sensor chip for Surface Plasmon Resonance of **A,** trastuzumab conjugates and **B.** affibody conjugates

Supplementary Figure 1. Binding affinity of synthesized molecules

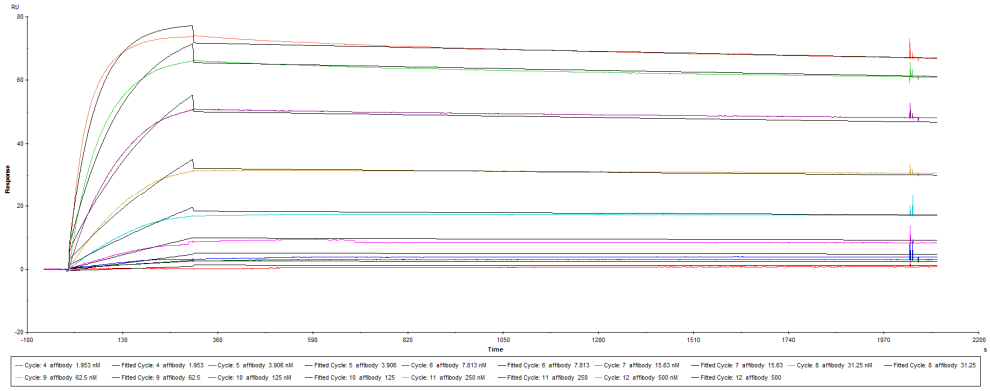
(A)



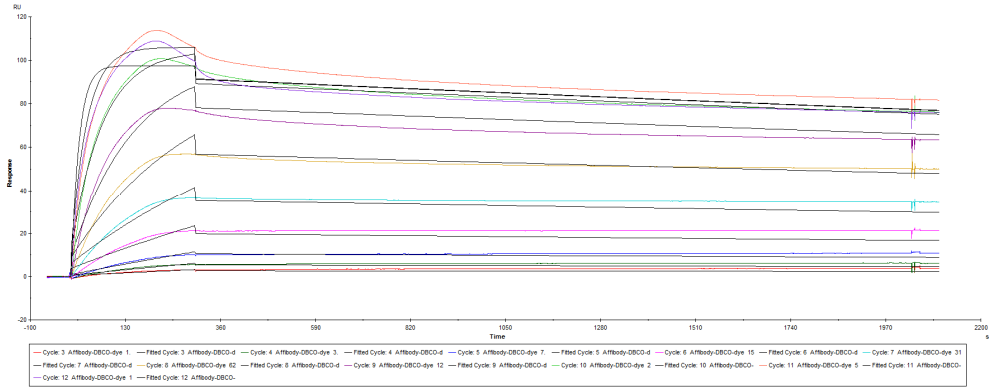
(B)


ErbB2
Response
Time
0
100
200
300
400
500
0
500
1000
1500
2000
2500
Herceptin_DBCO - 100nM
Herceptin_DBCO - 50.0nM
Herceptin_DBCO - 25.0nM
Herceptin_DBCO - 12.5nM
Herceptin_DBCO - 6.25nM
Herceptin_DBCO - 3.125nM
Herceptin_DBCO - 1.5625nM
Herceptin_DBCO - 781.25pM
Herceptin_DBCO - 390.625pM
Herceptin_DBCO - 0

(C)


RU
80
60
40
20
0
-20
Response
-100
130
360
590
820
1050
1280
1510
1740
1970
2200
Time
s
Cycle: 4 affibody 1.953 nM
Fitted Cycle: 4 affibody 1.953
Cycle: 5 affibody 3.906 nM
Fitted Cycle: 5 affibody 3.906
Cycle: 6 affibody 7.813 nM
Fitted Cycle: 6 affibody 7.813
Cycle: 7 affibody 15.63 nM
Fitted Cycle: 7 affibody 15.63
Cycle: 8 affibody 31.25 nM
Fitted Cycle: 8 affibody 31.25
Cycle: 9 affibody 62.5 nM
Fitted Cycle: 9 affibody 62.5
Cycle: 10 affibody 125 nM
Fitted Cycle: 10 affibody 125
Cycle: 11 affibody 250 nM
Fitted Cycle: 11 affibody 250
Cycle: 12 affibody 500 nM
Fitted Cycle: 12 affibody 500

(D)



Supplementary Figure 1. Binding affinity of the conjugates

SPR result of **A,** Trastuzumab **B.** Trastuzumab-DBCO **C,** Affibody **D,** Affibody-DBCO

Table2. Equilibrium dissociation constant

Ligand	Analyte	$K_a(M^{-1}s^{-1})$	$K_d(s^{-1})$	K_D (M)
HER2	Unlabeled Trastuzumab	4.69E+05	1.207E-05	25.75E-12
	Trastuzumab-DBCO	4.916E+05	2.720E-05	55.32E-12
	Trastuzumab-DBCO-IRDye800CW	3.725E+05	8E-07	2.093E-12
HER2	Unlabeled Affibody	3.10E+04	4.38E-05	1.42E-09
	Affibody-DBCO	5.27E+04	9.57E-05	1.82E-09
	Affibody-DBCO-IRDye800CW	1.36E+04	6.40E-05	4.71E-09

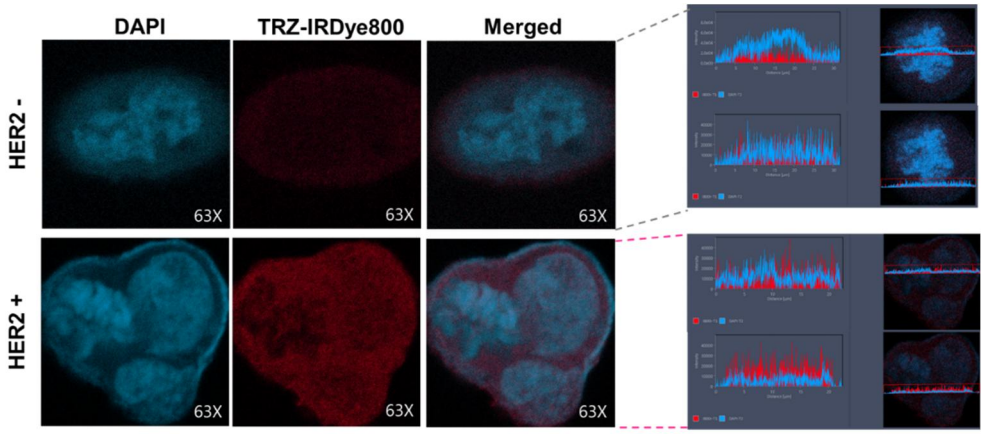
Table 2. Equilibrium dissociation constant of molecules

Binding assay result of modified molecules compared to unlabeled antibody and affibody

Confocal microscopy experiments were performed to further investigate the binding specificities and patterns of the conjugates. Remarkable HER2 expression was shown with trastuzumab-IRDye800CW in the cytoplasm together with cell surface was observed in NCI-N87 cells. (Fig.3A) On the other hand, affibody-IRDye800CW was attached only at the membrane of NCI-N87 cells (Fig. 3B)

Fig. 3

(A)



(B)

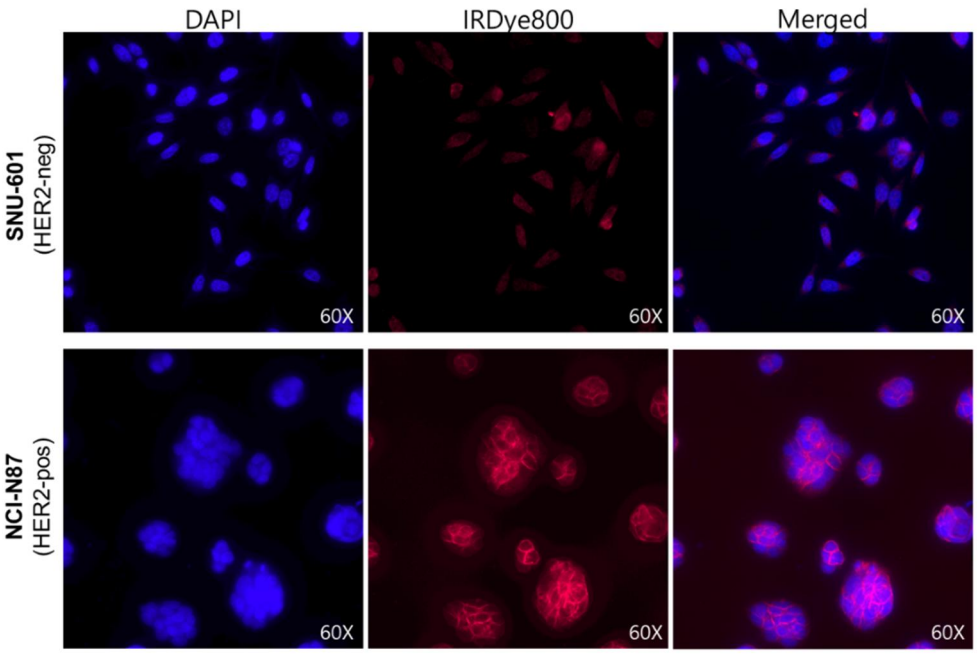


Figure 3. Confocal microscopy of gastric cancer cell line

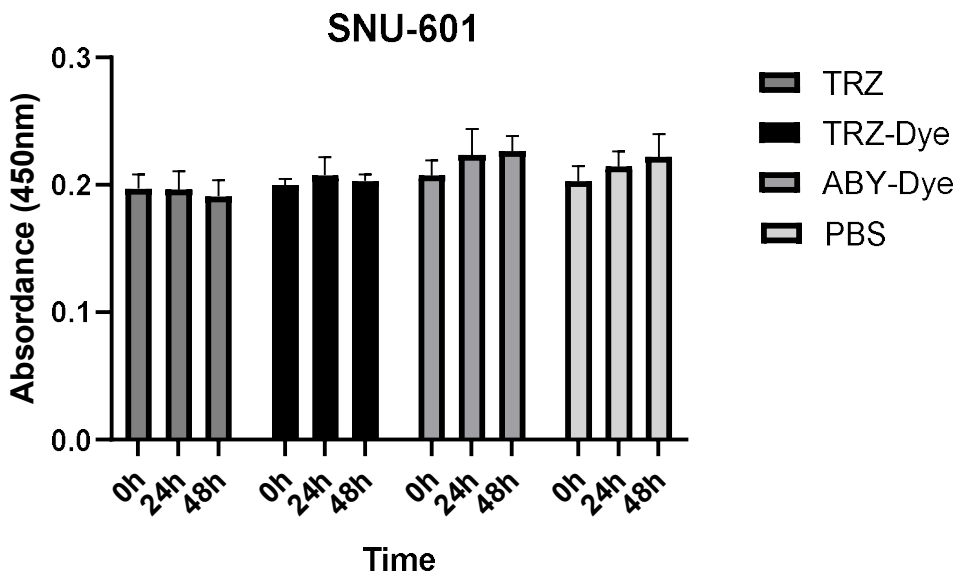
A, Intracellular image of gastric cancer cell with treating 1mM of Trastuzumab-IRDye800 and incubated 1 day imaged by LSM800(Zeiss) B, Intracellular image of gastric cancer cell with treating high dose of trastuzumab before applying 1mM of Trastuzumab-IRDye800 with HCS(Molecular Devices, USA)

Cellular toxicity of trastuzumab and affibody molecules

To evaluate the toxicity of HER2-specific probes, their effects on cell proliferation was assessed. Two cancer cell lines NCI-N87 and SNU-601 were grouped as (1)Trastuzumab treated (2) Trastuzumab-IRDye800CW treated (3) Affibody-IRDye800CW treated (4) Control and incubated for 48 hours. The conjugated molecules without Trastuzumab-IRDye800CW did not inhibit NCI-N87 cell growth and proliferation in any concentration of 10uM conjugates. Trastuzumab and trastuzumab-IRDye800CW as well as affibody did not induce any significant effect on HER2-negative SNU-601 cells as expected. (Fig.4A, 4B)

Fig. 4

(A)


SNU-601
Absordance (450nm)
0.3
0.2
0.1
0.0
0h
24h
48h
Time
TRZ
TRZ-Dye
ABY-Dye
PBS

(B)

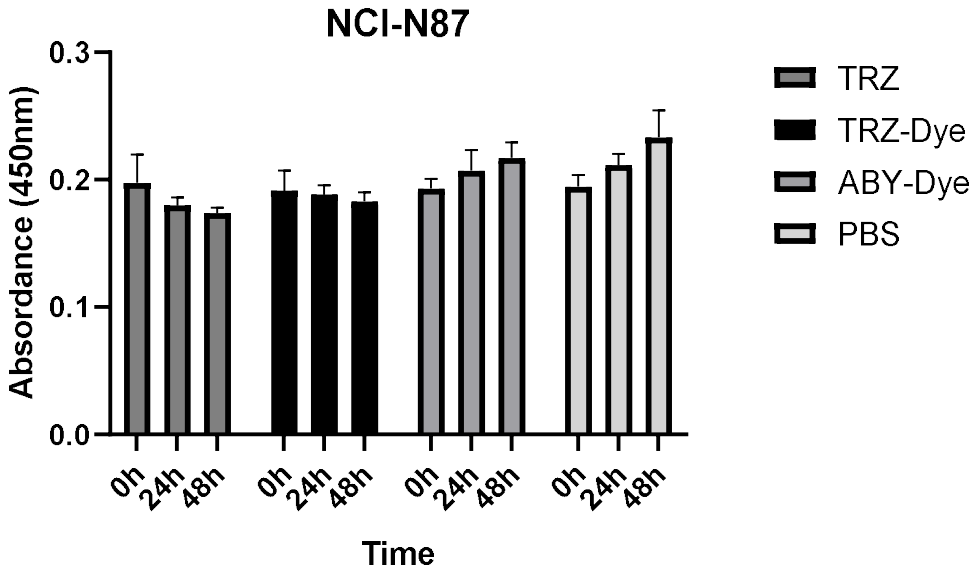


Figure 4. Cell Proliferation assay

Each cancer cell line was applied 10uM of substances, incubated 2 days and measured cell viability. The absorbance of 450nm was measured at 48hr in SNU-601 (A) and NCI-N87(B)

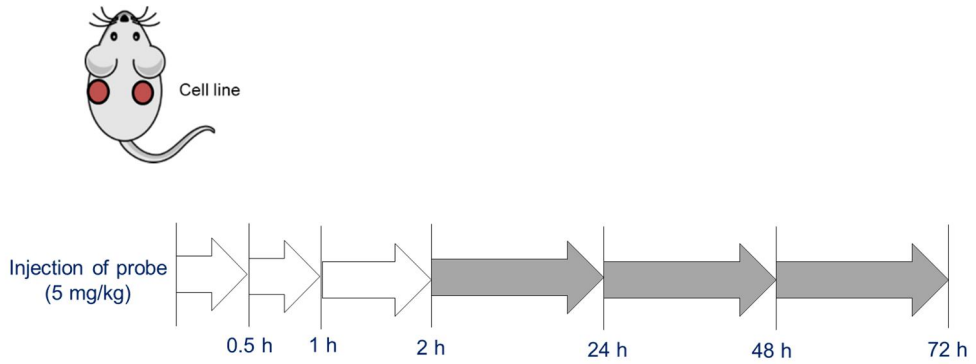
NIR fluorescent imaging of tumors

IRDye800CW conjugated trastuzumab and affibody were tested as NIR probe for *in vivo* optical imaging using mouse xenografts. Images of mouse whole body were measure by charge-coupled device camera Lumina II IVIS system and NIR-camera camera at different time points after tail vein injection of the trastuzumab-IRDye800CW, affibody-IRDye800CW (Fig. 5A). Mice injected with the trastuzumab-IRDye800CW showed less fluorescence intensities at first around the tumor and liver, until 24hr time point. Localization of the signal intensities after 24 hours and peaked around 48 hours. After that, the signals from the tumor area lasted up to 3 days, while slowly growing weaker (Fig. 5B). Signal-to-background ratios of each probe were calculated by normalizing signals from tumor area to those from normal tissue area. (Fig. 5C-5E) Specificity of HER2 imaging analysis shows by the experiments with HER2-nonexpressing SNU-601 tumors. The fluorescence intensities from each isolated tissue were also measured by Lumina II IVIS. (Fig. 5F)

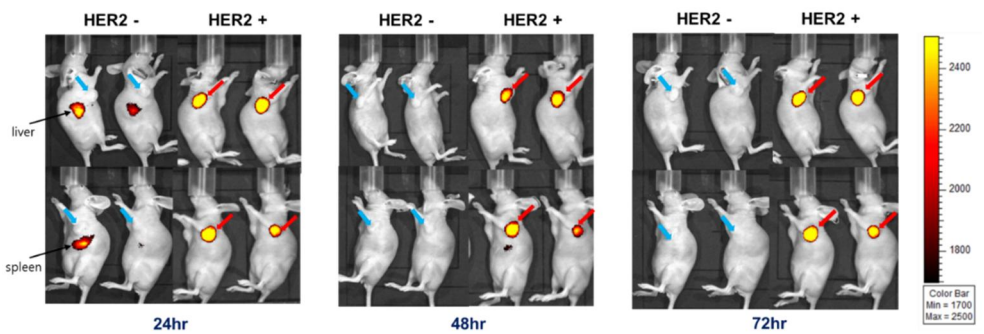
Daily imaging of the HER2 positive and negative tumors after administration of trastuzumab-IRDye800CW showed that peak tumor background ratio(TBR)s for NIR-camera camera occurred 2-day post injection. (Fig. 5G) Additionally, after 3 days, we sacrifice mice and harvest tumor, liver, kidney and spleen to how signal works in ex vivo. A statistically significant difference (24hr; P=0.002456, 48hr; P=0.000413, 72hr; P=0.000994, after sacrifice; P=0.000701) was noted between HER2 positive and negative tumor. (Fig. 5H) We measured gray scale of the signal by ImageJ software. After injecting trastuzumab-IRDye800CW intravenously, HER2 positive solid tumor showed significantly higher signal than those in HER2 negative tumor (24hr; P=0.023817, 48hr; P=0.000337, 72; P=001438) The peak time of the signal of HER2 positive tumor was 48 hours after injection as we expected. (Fig. 5I)

Fig. 5

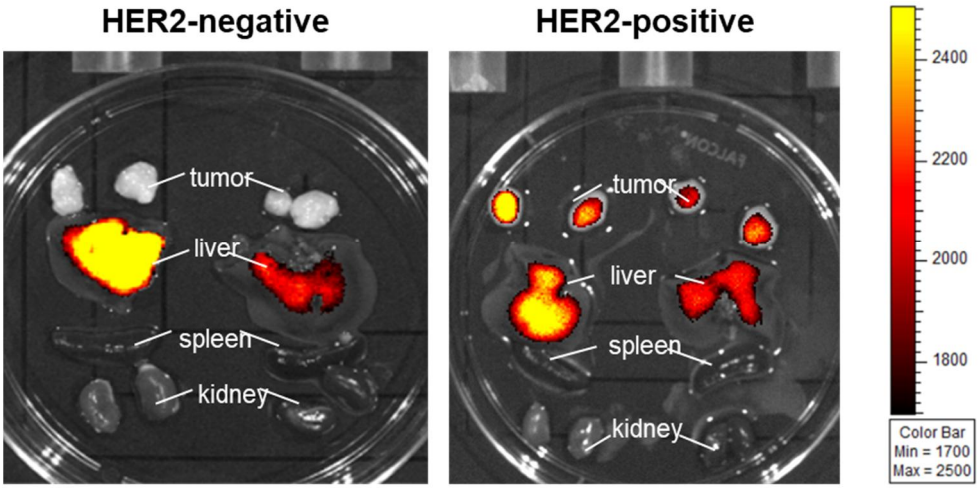
(A)


Cell line
Injection of probe
(5 mg/kg)
0.5 h
1 h
2 h
24 h
48 h
72 h

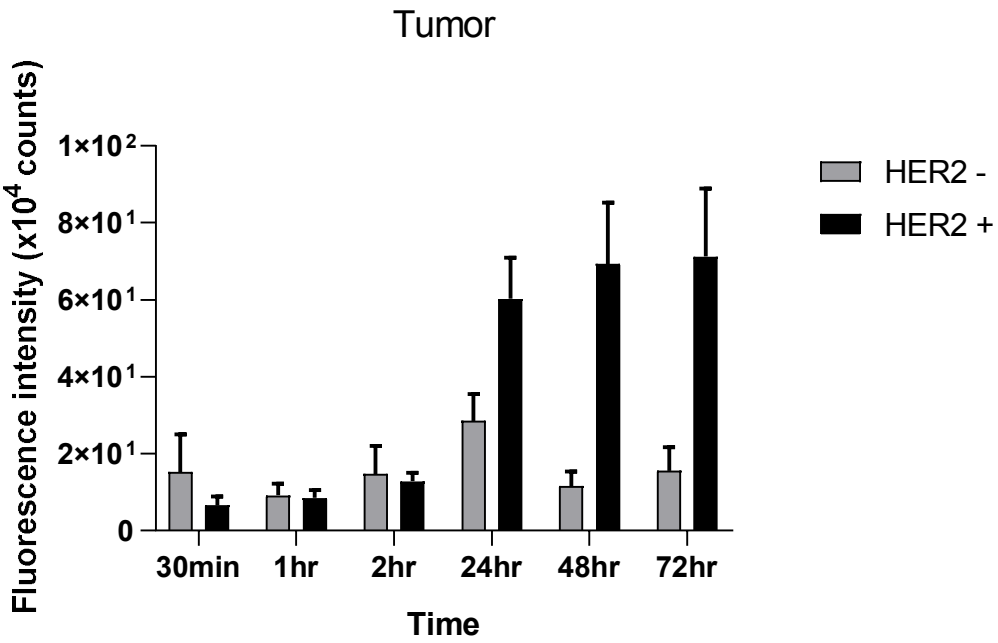
(B)


HER2 -
HER2 +
liver
spleen
24hr
48hr
72hr
2400
2200
2000
1800
Color Bar
Min = 1700
Max = 2500

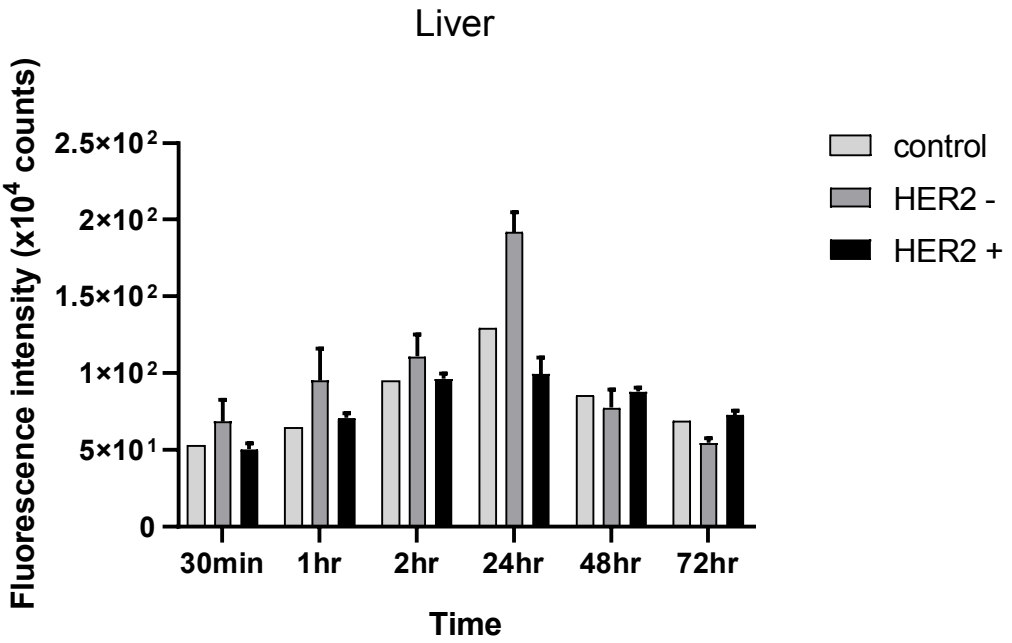
(C)


HER2-negative
HER2-positive
tumor
liver
spleen
kidney
tumor
liver
spleen
kidney
2400
2200
2000
1800
Color Bar
Min = 1700
Max = 2500

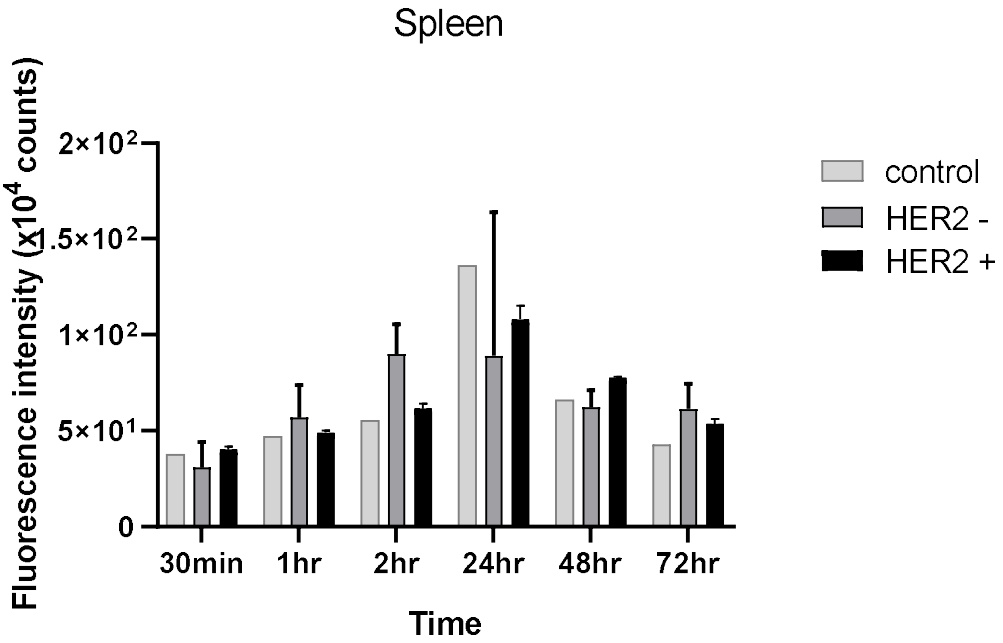
(D)


Tumor
Fluorescence intensity (x10^4 counts)
1×10^2
8×10^1
6×10^1
4×10^1
2×10^1
0
30min
1hr
2hr
24hr
48hr
72hr
Time
HER2 -
HER2 +

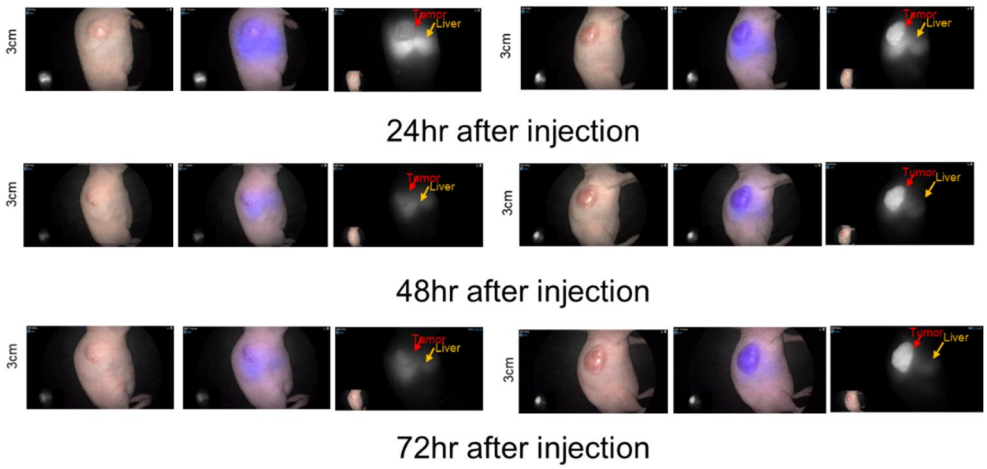
(E)


Liver
Fluorescence intensity (x10^4 counts)
2.5×10^2
2×10^2
1.5×10^2
1×10^2
5×10^1
0
30min
1hr
2hr
24hr
48hr
72hr
Time
control
HER2 -
HER2 +

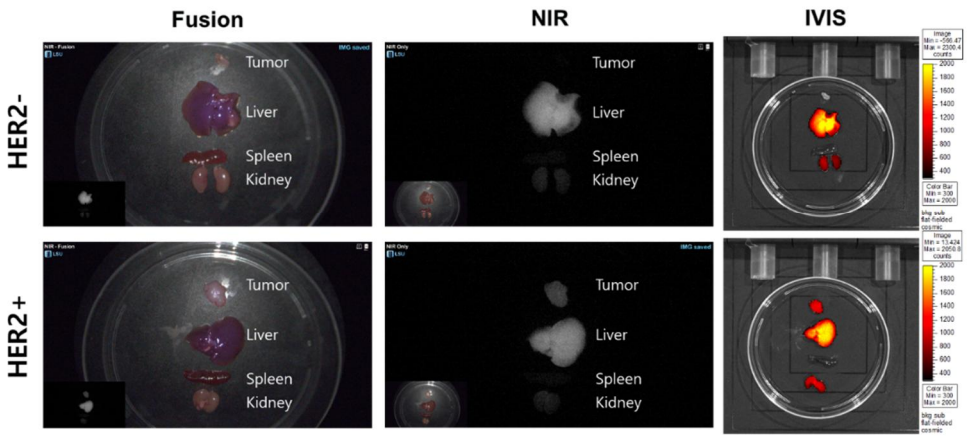
(F)


Spleen
Fluorescence intensity (x10^4 counts)
2×10^2
1.5×10^2
1×10^2
5×10^1
0
30min
1hr
2hr
24hr
48hr
72hr
Time
control
HER2 -
HER2 +

(G)


3cm
Tumor
Liver
24hr after injection
48hr after injection
72hr after injection

(H)



(I)

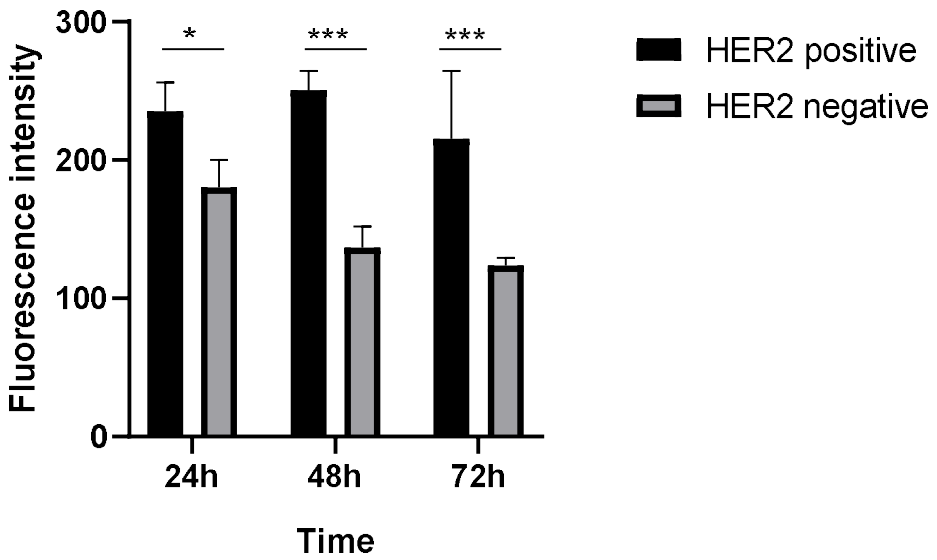
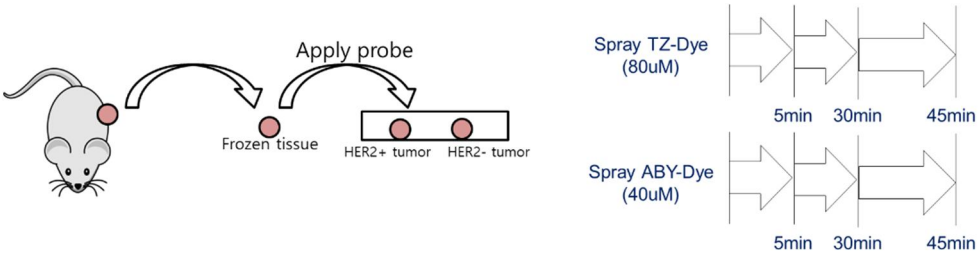


Figure 5. NIR fluorescence imaging of trastuzumab-IRDye800

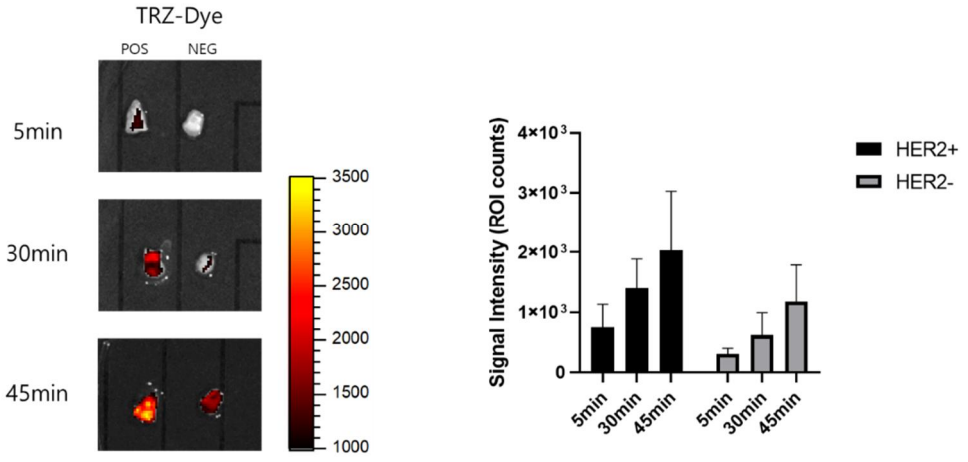
A, the overall scheme for imaging of mouse xenograft model. B, mouse image of 1-3days after injected with trastuzumab-IRDye800CW C, Ex vivo NIR image of sacrificed mice D-F, fluorescence intensity of tumor, liver, spleen (biodistribution) G, obtained image of NIR-camera camera at different time points H, NIR fluorescence of ex vivo image I, Quantified signal of intensity of gray scale of NIR camera by ImageJ software

Fig. 6

(A)


Apply probe
Frozen tissue
HER2+ tumor
HER2- tumor
Spray TZ-Dye
(80uM)
5min
30min
45min
Spray ABY-Dye
(40uM)
5min
30min
45min

(B)


TRZ-Dye
POS
NEG
5min
30min
45min
3500
3000
2500
2000
1500
1000
Signal Intensity (ROI counts)
4×10³
3×10³
2×10³
1×10³
0
5min
30min
45min
5min
30min
45min
HER2+
HER2-

(C)

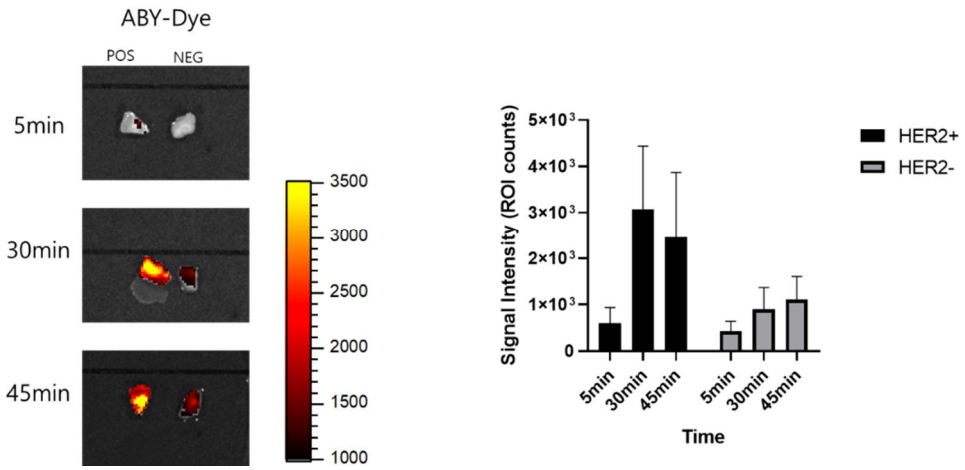
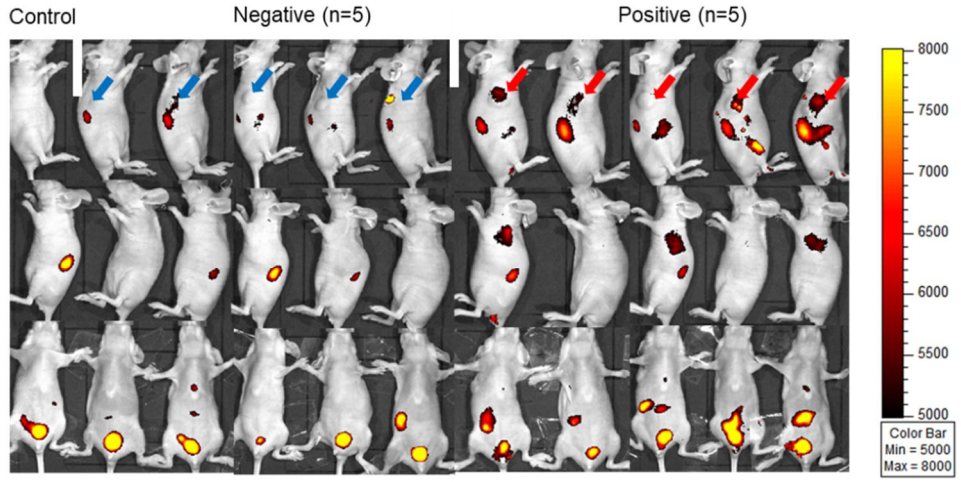


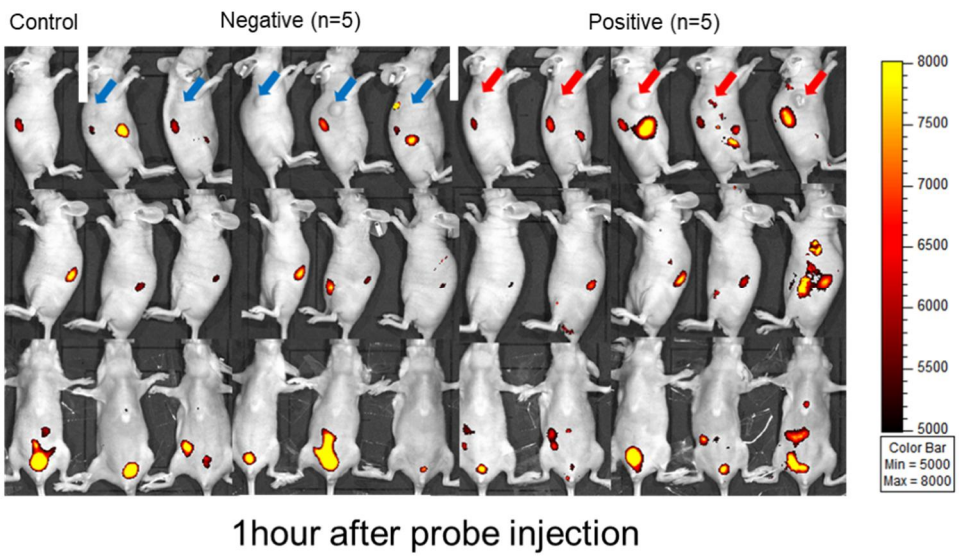
Figure 6. Staining of NIR fluorescence

A, the overall scheme for imaging of stained frozen tissue. B, IVIS image of trastuzumab-IRDye800CW stained tumor for 5min, 30min, 45min. C, IVIS image of affibody-IRDye800CW stained tumor for 5min, 30min, 45min.

Imaging of the HER2 positive and negative tumors was also performed after administration of affibody-dye. Data showed that peak TBRs for NIR- camera occurred in 30min post injection. (Fig. 7A) Additionally, after 1 hour, the signal of tumor was all gone, but high fluorescence intensity was shown at kidney and bladder. (Fig. 7A) After 2days, we sacrifice mice and harvest tumor, liver, kidney and spleen to how signal works in ex vivo. (Fig. 7B) To determine if the affibody-dye binds to HER2 protein and to see how long it will wash out, department of Nuclear Medicine Chemistry labeled ^{111}In to affibody and performed SPECT/CT of HER2 positive mouse. (Supplementary Fig. 2)

Fig. 7


Control
Negative (n=5)
Positive (n=5)
8000
7500
7000
6500
6000
5500
5000
Color Bar
Min = 5000
Max = 8000
30min after probe injection


Control
Negative (n=5)
Positive (n=5)
8000
7500
7000
6500
6000
5500
5000
Color Bar
Min = 5000
Max = 8000
1hour after probe injection

(A)

(B)

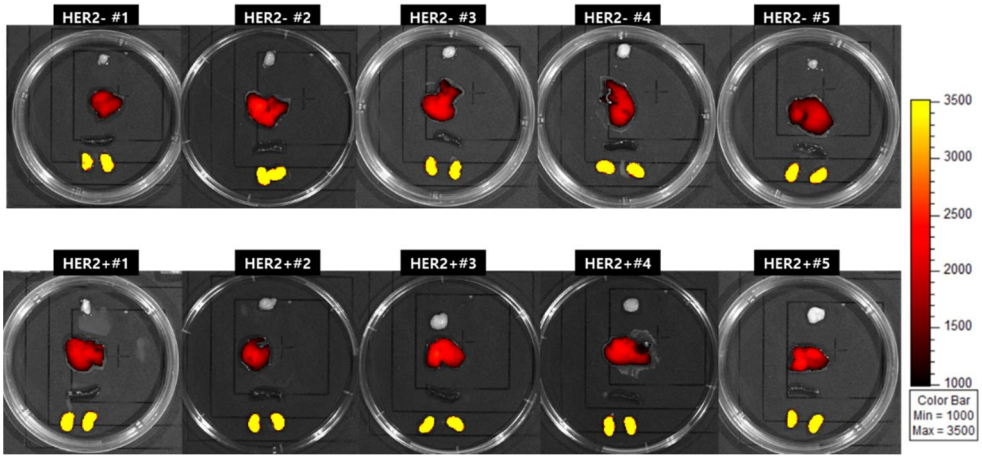
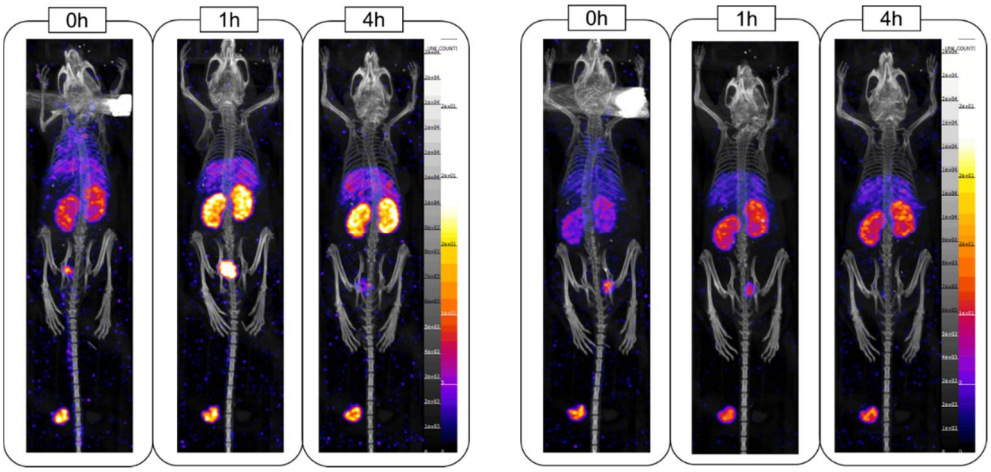


Figure 7. NIR fluorescence imaging of affibody-IRDye800CW

A, 30min and 1hour after injection of probe, slight signal is shown and washed out immediately B, Ex vivo NIR image of sacrificed mice

Supplementary Fig. 2



· **Supplementary Figure 2.** To validate circulation of affibody in mice, SPECT/CT was performed to follow up radioisotope simultaneously. However, no signal of HER2 positive tumor.

Histology and immunohistochemistry

To confirm HER2 expression level of extracted tumor from mice, we extracted tumor 72 hours after probe injection and performed immunohistochemistry analysis. Harvested tumor from mice bearing NCI-N87 showed positive membrane staining for anti-HER2 antibodies, while SNU-601 showed very weak or no membrane signal. (Fig. 8) This suggest that although tumor express high level of HER2, the very small molecule, affibody-dye, may not well-attached to protein.

Fig. 8

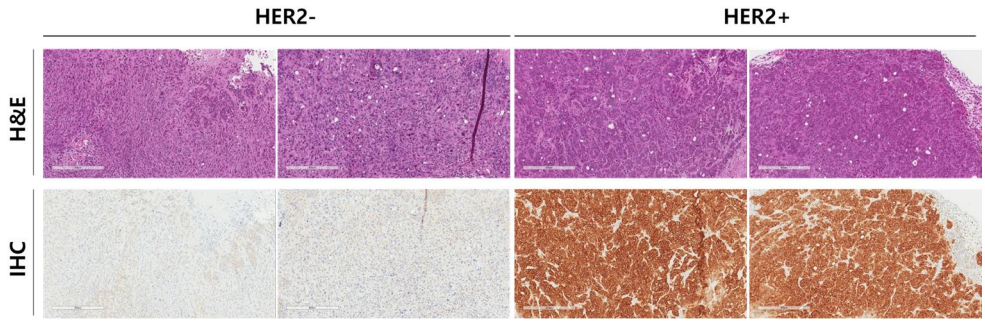


Figure 8. Immunohistochemistry and Haematoxylin & Eosin(H&E) staining
Histological observations of the SNU-601 and NCI-N87 tumors. (x100)

Discussion

Molecular imaging has been widely used as a tool for biological understanding, which focuses on the management of patients through the utilization of tracers in conjugation with imaging technologies. Recently people are trying to establish non-invasive, specific, sensitive, quantitative, and cost-effective real-time NIR-optical imaging techniques.(36) Applying fluorescence-labeled tumor targeting molecules to patient's tissue, surgeons can discriminate the lesion more safe and easily, so that they can detect tumor cells and will be well-guided to dissect margins of tumor.(37, 38)

There is growing evidence that HER2 is an important biomarker in gastric cancer, with studies showing amplification or overexpression in 7-34% of tumors. HER2 3+ at IHC and FISH amplified or HER2 score 2+ at IHC and FISH amplified are HER2 positive in gastric cancer.

Among various imaging agents, indocyanine green (ICG), which absorbs light at 780nm to 820nm, is used to evaluate blood flow, liver function assessment and map the lymph node vessels.(39)

However, ICG is rapidly dispersed and degraded in a aqueous conditions with low tissue penetration. IRDye800CW shows less non-specific binding, dispersion time and can bind biomolecules more than ICG. Although IRDye800CW has not been FDA approved, it has a drug master file with FDA and is safe at doses of 20mg/kg, IRDye800CW can be a promising future near-infrared fluorophore and alternative of ICG. (40, 41),

Since molecular imaging is a non-invasive procedure, it may have an advantage over biopsy-based approaches and has potential to help guide physicians to tailor treatment for patients. Various kinds of antibody or antibody fragments were now engineered. One of the antibody segments, affibody is very small proteins (6-7 kDa) while general IgG is 150kDa. This protein has been reported in many studies with high water-solubility, high stability α-helical proteins. Since the size of affibody is very small compare to antibody, we hypothesized that HER2-specific affibody conjugated with IRDye800CW can visualize tumor better than HER2-specific antibody conjugated with IRDye800CW. In this study, we aimed to visualize HER2 expression in gastric cancer and compare which fluorescent-labeled HER2 tracer work efficiently.

Many studies had shown HER2 expression of gastric cancer cell lines. (42, 43) The HER2 expressing human gastric cancer cell line NCI-N87 and SNU-601 has been selected and used for tumor xenograft in mice (Fig. 1) The fluorescence intensity of conjugated molecules were observed at different wavelength. The intensity was highest at a 800nm emission filter. (Fig. 2B) Before investigating *in vivo* binding to these cells, it was necessary to first study the *in vitro* binding. The kinetics observed was similar to both unlabeled HER2 target peptides. (Fig. 2C,D) Our data from confocal microscopy showed that trastuzumab-IRDye800CW is significantly internalized into HER2 positive cell. (Fig. 3) Through cell proliferation assay, attaching fluorescence or not had no significant effect on the cancer cells. (Fig. 4) Our results agree with previous findings on cellular toxicity and intracellular signaling, supporting that the probe does not affect the system to be studied. (Fig. 3 and Fig. 4)

It has been well documented that conjugation chemistry using an azide group to react with an alkyne is an efficient method of conjugation. As shown in Table2, modification of the trastuzumab with IRDye800CW azide dyes resulted in increased affinity, but

affibody modification is resulted in a slight reduction of the affinity of the conjugates. Affibody binds to domain III of HER2 while trastuzumab binds to domain IV of HER2(44).

There are several optical methods and systems in clinically use today where light can penetrate into the tissue. In this study, we used optical imaging system presented for preclinical studies on small animal, IVIS system and NIR-camera. (Fig. 5) The novelty of our study is that we used an actual NIR-camera camera to visualize HER2 in gastric cancer by the different distance between the tumor and camera. (Fig. 5G) As affibody does not access to the same epitope as trastuzumab, this molecule can be used to monitor HER2 receptor expression during Trastuzumab treatment. However, although the affibody was expected to be clinically usable in the future due to its small size and good circulation in the body, it seemed to be inadequate in mouse model because of rapid renal clearance (Fig. 7, Supplementary Fig. 2), suggesting the small size of the protein injected *in vivo* does not seem to visualize tumor successfully.

In a preclinical setting, NIR fluorescence-labeled antibodies targeting HER2 allowed to specificity and sensitivity of detection of

tumor in vivo. These findings strongly suggest that the NIR-fluorescence-labeled anti-cancer tracer can be used in a clinical application, including intraoperative image-guided surgery. This may give us new approaches in the diagnosis or treatment of HER2-positive in gastric cancer. Although further studies are needed to investigate efficacy of the synthesized molecules, non-invasive procedure for cancer specific imaging can provide a tool for optimization or characterization of HER2 in patients or guidance where to dissect.

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국문 초록

배경: 영상을 기반으로 하는 진단과 수술은 광범위하게 사용되고 있는데, 그 중 근적외선 (NIR) 기반의 형광 이미징이 수술 중 혹은 내시경 중에 그 활용도가 매우 높다. 표적 항체와 작은 펩타이드를 이용하여 종양을 영상화 하는 연구가 활발히 진행되고 있으며 이는 전임상 및 임상에서 적용되고 있다. 인체에 주입할 수 있는 형광물질인 indocyaninegreen(ICG)는 특이적으로 종양을 표지 할 수 없으며, 주입 후 금방 퍼지는 단점이 있다. 또한 ICG 는 액체 상태에서는 매우 불안정 하며, 금방 퍼지기 때문에 주변 형광 신호가 함께 표지된다. 반면, 근적외선 형광 IRDye800CW 는 ICG 보다 수용성에서 훨씬 안정적이며 주변 비특이적 형광신호가 적게 보인다는 장점이 있다. HER2 는 세포의 표면에 발현하고 있는 단백질로써 위암을 비롯한 많은 종류의 암에서 과발현시, 안 좋은 예후를 보인다. 특정 분자를 타겟하는 단백질 및 항체와 항체의 일부 조각들이 많이 발견되고 엔지니어링되었다. HER2 에 특이적으로 부착할 수 있는 단백질 중 HER2 의 서로 다른 위치에 붙는 antibody(trastuzumab)과 affibody 를 이용한 연구가 활발히 되어있다.

목적 : 본 연구는 IRDye800CW 와 HER2 에 단일 항체 trastuzumab 과 6-7kDa 정도의 사이즈인 affibody 를 부착하여 HER2 를 생체 내 정량 할 수 있는 도구로써의 분자 프로브를 개발하고 각각을 비교하며, 이를 소동물 근적외선 카메라와 실제 수술장에서 사용되고 있는 복강경 카메라를 사용하여 마우스 위종양모델에서 영상화하고 그 효용성을 확인하고자 한다.

방법: 본 연구팀에서는 HER2 발현 수준에 따라 인간 위암 세포주 NCI-N87, SNU601 을 사용하여 실험을 진행하였다. HER2 단일 항체인 trastuzumab 과 작은 단백질 구조를 가지고 있는 affibody 를 근적외선 형광인 IRDye800 에 합성하여 HER2 단백질에 부착하는 정도와 특성화도를 표면 플라즈몬 공명(SPR)을 통하여 확인했다. 합성된 표적 프로브로 세포주를 형광 촬영하였으며 Trastuzumab-dye 의 효용성을 보기 위하여 각 세포주는 암컷 BALB/c-nu 마우스 피하에 주입하여 2-3 주간 종양 형성을 관찰하였고 부피가 200-300(mm^3)에 이르렀을 때, 소동물 영상 장비인 IVIS 와 복강경 카메라를 이용하여 형광을 영상화 하였다. Affibody-dye 가 생체 내에서 HER2 를 효과적으로 영상할 수 있는지를 보기 위하여 trastuzumab-dye 모델과 마찬가지로 각 세포주를 암컷 BALB/c-nu 피하에 주입 후 종양의 부피가 200-300(mm^3)에 이르렀을 때, 소동물 영상장비로 형광을 확인하였다. Affibody-dye 를 마우스에 주입 후 해당 물질이 종양에 도달하기까지 얼마의 시간이 필요한지 확인하기 위하여 HER2 양성 세포주 마우스모델에 affibody-In 을 소동물 SPECT/CT 를 이용하여 보았다. 형광 영상이 끝난 모든 마우스에서 종양과 다른 부위의 장기를 적출하여 포르말린에 고정시켜 파라핀 블록으로 만들어 Hematoxylin & Eosin (H&E) 염색과 면역조직화학염색법을 진행하여 암 조직의 HER2 발현과 분자적 특징을 확인하였다.

결과: 표면 플라즈몬 공명에서 합성된 trastuzumab-dye 는 trastuzumab 보다 높은 친화도를 보였으며, affibody-dye 는 affibody 보다 낮은 친화도를 보였다. Trastuzumab-dye 를 각 HER2 양성 및 음성 세포주에 염색하였을 때, 둘 다 형광이 보이긴 하였으나 HER2 과발현 되어있는 NCI-N87 에서 그 신호가

매우 셌음을 확인할 수 있었다. IVIS 와 복강경 카메라를 이용한 trastuzumab-dye 영상은 HER2 음성 위암 세포 마우스 모델에서보다 양성 위암 세포 마우스 모델의 종양에서 유의하게 높은 신호를 보였으며, HER2 음성 그룹에서는 신호 저해가 보이지 않거나 상당히 낮았다. 반면, affibody-dye 영상은 마우스 꼬리 정맥에 주사 후 약 30 분 ~ 1 시간 내에 신장으로 물질이 갔으며 이는 IVIS system 에서 확인할 수 있었다. 체내 순환을 확인하기 위하여 affibody 에 방사선 동위원소인 In 를 부착하여 시간 별로 영상을 촬영해보았으나 신장으로 대부분의 형광이 갔음을 확인할 수 있었다.

결론: 본 연구를 통해 IRDye800CW 와 결합 된 trastuzumab 이 전임상적으로 과발현 되어있는 HER2 종양을 모니터링 할 수 있는 도구가 될 수 있음을 보였다. 크기가 매우 작은 affibody 는 체내 순환이 빠르고 HER2 단백질에서 trastuzumab 과 다른 위치에 부착한다는 이점을 바탕으로 전임상적인 효과가 뛰어날 것으로 예상하였지만 마우스에서는 그 효용성이 낮음을 확인하였다. 더 많은 연구가 진행되어야 할 것으로 보이나 두 물질은 향후 위암 환자에서 조직검사를 하기 앞서 HER2 발현을 평가하기위한 보완적인 수단을 제공할 수 있을 것이다. 또한 이 물질은 영상 유도 수술 중 HER2 양성 병변을 추가로 탐지하는 데 활용될 수 있을 것이다.

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주요어: 위암, HER2, Trastuzumab, Affibody, 종양 표적 추적자, 근적외선 형광, 마우스 이종이식 모델, 복강경 카메라

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