Master of Science

Cytotoxic effects of the novel recombinant immunotoxin, HER2(scFv)-PE26, on the HER2-expressing breast cancer cell lines

새로운 재조합 면역 독소 HER2(scFv)-PE26 의 HER2 발현 유방암 세포주에 대한 세포 독성 효과

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Cytotoxic effects of the novel recombinant immunotoxin, HER2(scFv)-PE26, on the HER2-expressing breast cancer cell lines

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Master's thesis

Submitted to
the Graduated School of the University of Ulsan
in partial Fulfillment of the Requirements
for the Degree of

Master of Science

by

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August 2018

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August 2018

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LIST OF ABBREVIATIONS

ABC	Ammonium bicarbonate
ACN	Acetonitrile
BSA	Bovine serum albumin
CV	Column volume
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
E. coli	*Escherichia coli*
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
$Furin_{cs}$	Furin cleavage site
GFP	Green fluorescence protein
GPC	Gel Permeation Chromatography
HC	Hill coefficient
HER2	Human epidermal growth factor receptor 2
HPLC	High performance liquid chromatography
IAA	Iodoacetamide
IC_{50}	50% inhibit concentration
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl 1-thio-β-d-galactopyranoside

LAL	Limulus Amebocyte Lysate
LB medium	Luria-Bertani medium
LC	Liquid chromatography
MBP	Maltose binding protein
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
OD	Optical density
PBS	Phosphate buffered saline
PE	*Pseudomonas* exotoxin A
PEG	Polyethylene glycol
PI	Isoelectric point
RIT	Recombinant immunotoxin
RPMI 1640	Roswell Park Memorial Institute 1640 Medium
scFv	Single chain variable fragment
SE	Standard Error
SEC	Size exclusion
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEV	Tobacco etch virus
TEVrs	Tobacco etch virus protease recognition site
TFA	Trifluoroacetic acid

Abstract

Cytotoxic effects of

the novel recombinant immunotoxin, HER2(scFv)-PE26,

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College of Medicine

Breast cancer is known to be mainly caused by the estrogen signal and the human epithelial growth factor receptor 2 (HER2) signal. Patients overexpressing HER2 have a significantly lower survival rate and overexpression of HER2 in breast tissue stimulates transformation into a malignant phenotype. In addition, HER2-overexpressing tumors are resistant to general chemotherapy treatments. The anti-HER2 antibody, trastuzumab, blocks HER2 signaling that concerned about growth of tumor cells. It has been approved for the treatment of HER2 positive early breast cancer and metastatic breast cancer. *Pseudomonas* exotoxin A (PE) is a bacterial toxin of *Pseudomonas aeruginosa*, consisting of an A-domain with enzymatic activity and a B-domain with cell binding activity. In this study, the novel recombinant immunotoxin composing of a single-chain of anti-HER2 (HER2(scFv)) from

trastuzumab and 26 kDa catalytic fragment (PE26) from PE was created. The recombinant immunotoxin was fused with the maltose binding protein (MBP) at the N-terminus to increase the expression and solubility of protein expression in *E. coli*. After cation exchange chromatography and removal of MBP, HER2(scFv)-PE26 was stable and further immobilized metal affinity chromatography and gel filtration chromatography allowed a purity of greater than 91%. Finally, 0.25 mg of pure HER2(scFv)-PE26 was successfully obtained from 500 mL flask culture. FACS analysis with HER2(scFv)-GFP showed the highest expression of HER2 on SKBR3 and BT-474 cell lines with low expression on MDA-MB-231 cell line and little expression on MCF7 cell line. The purified HER2(scFv)-PE26 showed cytotoxicity at much lower doses than the purified HER2(scFv) or PE26 alone. IC_{50}s of HER2(scFv)-PE26 were estimated to be 28.1 ± 2.5 pM (n = 9), 19 ± 1.4 pM (n = 9) with the Hill coefficient of 2.24 ± 0.16 and 1.87 ± 0.17, for the high HER2-expressing cell lines, SKBR3 and BT-474, respectively. On the other hand, the cytotoxicity of HER2(scFv)-PE26 on MDA-MB-231 and MCF7, was weaker than those on SKBR3 and BT-474. These results suggest the new immunotoxin, HER2(scFv)-PE26, could be a new drug candidate for the HER2-overexpressing cancer patients.

Keywords: Human epidermal growth factor receptor 2 (HER2), Breast cancer, Immunotoxin, Trastuzumab, *Pseudomonas* exotoxin A, Soluble overexpression and purification

Introduction

Immunotoxins are fusion proteins, including modified toxin fused with a tumor-specific antibody and it have been studied for cancer target therapy [1)]. Antibody region of these molecules specifically targets tumor cell surface receptor and then internalize to the endocytic compartment, subsequently resulting in cell death. With the above mechanism, toxin molecules delivered to the cytosol of target tumor cells effectively compared with single toxin molecules [2-4)].

The analysis of gene expression arrays has classified human breast cancer into subtypes depending on differences in their gene expression patterns, and overexpression of human epidermal growth factor receptor 2 (HER2) has been observed in 20–30% of all breast tumors [5, 6)]. HER2 is a 185 kDa transmembrane tyrosine kinase receptor and belongs to the epidermal growth receptor (EGFR) family 2. The phosphorylation of HER dimers results in activation of various downstream cell proliferation, survival, differentiation, angiogenesis, invasion and metastasis [7, 8)]. Patients with HER2 overexpression have a significantly poor prognosis and overexpression of HER2 in breast tissues stimulates malignant phenotypic transformation. In addition, HER2-overexpressing tumors are more resistant to general chemotherapy treatment [9)].

Trastuzumab have been used as a drug targeting the HER2 receptor and approved by FDA for the treatment of HER2 positive early stage breast cancer and metastatic breast

cancer [10, 11]. Trastuzumab blocks HER2 signaling by binding to its extracellular domain and attracts immune cells to tumor sites, resulting in inhibition of tumor growth, survival, differentiation [12, 13]. Because in many previous studies, trastuzumab showed weak cytotoxicity, it has been conjugated with various drug to enhance cytotoxicity and for active targeting against HER2 positive breast cancer cells [14-19]. Even though it showed clinical efficacy, there is challenge for trastuzumab resistance [20].

Pseudomonas exotoxin A (PE) is a bacterial toxin from *Pseudomonas aeruginosa*, which is expressed as a protein with 613 amino acids (a.a) and it is consist of several structural and functional domains [21]. In general, PE is composed of A domain with enzymatic activity and B domain as cell binding subunit. In detail, at the N-terminus of PE, there is a highly hydrophobic leader peptide of 25 a.a and it is removed during secretion. The leader sequence is followed by the receptor binding domain Ia (1–252 a.a). Translocation domain II (253–364 a.a) transfer the toxin into cell membranes. The last four residues (400–404 a.a) of domain Ib (365–404 a.a) with domain III (405–613 a.a) is a catalytic subunit of the toxin with ADP-ribosyltransferase activity [22]. The catalytic enzyme activity of domain Ib and domain III causes apoptotic cell death [23]. With this catalytic activity for the apoptosis, in some studies, the 40, 38, 24 kDa portion of the PE except for the cell binding domain was used and the cell binding domain was designed by replacing the antibody fragment that targets the cancer cell [24-27]. PE38 is the most common fragment of PE for recombinant immunotoxin [28]. However, the nonspecific toxicity and strong immunogenicity of the PE38

were the limit of PE based immunotoxins. For prevention of immunogenicity, most of domain II (253-364 a.a) as B-cell and T-cell epitopes except for furin cleavage site was removed. This new PE fragment significantly decrease immunogenicity and nonspecific toxicity of PE [27, 29-32]. Hence, new variant of PE called PE26 was fused with HER2(scFv).

In this study, the novel recombinant immunotoxin (RIT), which is composed of single-chain Fv (scFv) of anti-HER2 antibody fused with a 26 kDa fragment of PE (PE26) was created and it tagged with MBP tag at the N-terminus to improve solubility in *E. coli*. MBP fusion protein was compared expression and solubility level with 3 different strains, BL21 (DE3), SHuffle, Origami 2. From BL21 (DE3), HER2(scFv)-PE26 was produced successfully with soluble form. Despite the removal of MBP tag acts as a general molecular chaperone and promotes proper folding, HER2(scFv)-PE26 was stable and had a purity of greater than 91%. Furthermore, it shows dose-dependent cytotoxicity through MTT assay on high HER2-expressing cell lines, SKBR3, BT-474. IC_{50}s of HER2(scFv)-PE26 was calculated to 28.1 pM ± 2.5 pM (n = 9), 19 ± 1.4 pM (n = 9) on SKBR3 and BT-474, respectively. On the other hand, it shows less effect to low HER2-expressing cell line, MDA-MB-231 and MCF7.

Materials and Methods

1. Materials

All the chemicals were analytical grade. Dithiothreitol (DTT) and 1-thio-β-d-galactopyranoside (IPTG) were acquired from Anaspec (Fremont, CA). Ampicillin was acquired from Duchefa Biochemie (Haarlem, Netherlands), and NaCl, glycerol and Trifluoroacetic acid (TFA) from Samchun Chemical (Pyeongtaek, Korea). Coomassie brilliant blue R-250, and Tris-HCl were from Amresco (Solon, Ohio). Imidazole was from Daejung Chemicals (Siheung, Korea). All the columns and ÄKTA Explorer, ÄKTA Prime, ÄKTA Start for purification were purchased from GE healthcare (Piscataway, NJ). Gateway™ BP Clonase™ II Enzyme mix was from Thermo Fisher Scientific (MA, USA). Overlap cloner, Lambda integrase/excisionase and Lambda integrase were from Elpis Biotech (Daejeon, Korea). Dialysis membranes from Viskase (Darien, IL). Amicon Ultra was from Merck Millipore (Billerica, MA). PDVF membrane, Acrodisc Syringe Filters and Supor Membrane, were from Pall Corporation (Ann Arbor, MI). *E. coli* BL21 (DE3), SHuffle and Origami 2 (DE3) cells were acquired from Novagen (Madison, WI). Silver Stain Plus kit was from Bio-Rad Laboratories (Hercules, CA). Ammonium bicarbonate from Junsei chemical (Tokyo, Japan). Ultrasonic cell disruptor JY99-IIDN from Ningbo Scientz Biotechnology (Guangdong, China). Acetonitrile from Honeywell Burdick & Jackson (MI, USA). Waters 600 Controller, Waters 486 Tunable Absorbance Detector, Waters 717 Plus

Autosampler and Protein-pak 300SW SEC 7.5 x 300 mm column was from Waters Corporation (Milford, MA). HER2(scFv) from trastuzumab was synthesized by Bioneer (Daejeon, Korea). PE26 from PE was synthesized by GenScript (Piscataway, NJ). RPMI-1640 Medium, 0.25 % trypsin-EDTA, fetal bovine serum (FBS), and penicillin-streptomycin from GIBCO (Carlsbad, CA). SKBR3, BT-474, MDA-MB-231 and MCF7 cell lines from Korea Cell line Bank (Seoul, Korea). Sequencing grade modified trypsin from promega (Madison, WI). Toxin Sensor Chromogenic LAL Endotoxin Assay Kit from GenScript (Piscataway, NJ). Triton X-114, 4',6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) from Sigma-Aldrich (St. Louis, MO).

2. Construction of recombinant immunotoxin, MBP-HER2(scFv)-PE26

The multisite gateway cloning method was used to obtain expression plasmid. To facilitate expression of HER2(scFv)-PE26 in *E. coli*, a codon optimized oligonucleotide encoding 243 amino acids for HER2(scFv) from trastuzumab and 234 amino acids for PE from PE. HER2(scFv) was synthesized heavy and light chain separately and attached together by overlap cloning. The tobacco etch virus protease recognition site (TEVrs), ENLYFQ/G, was placed at the N-terminus of the HER2(scFv) polynucleotide. Before BP reaction, PCR was performed to attach attB1 and attB5r at the end of both sides of HER2(scFv); attB5 and attB2 was attached to PE26. After that, BP reaction was

accomplished to make entry clones. With 2 entry clones and destination vector, LR reaction was performed to get the expression clone. The correct sequence of expression construct was confirmed by sequencing analysis (Macrogen, Daejeon, Korea).

3. Expression and solubility test of MBP-HER2(scFv)-PE26 in *E. Coli*

E. coli BL21 (DE3), SHuffle and Origami 2 were transformed with expression plasmid to get single colonies and then inoculated into Luria-Bertani (LB) medium containing 50 μg/mL ampicillin at 37°C overnight. The overnight-cultured cells were then transferred to a fresh LB medium containing ampicillin at a 1:100 ratio, and the cells allowed to grow at 37°C and 200 rpm. To induce expression of MBP-HER2(scFv)-PE26, 0.5 mM IPTG was added into the culture batch when the OD_{600} reached 0.4–0.6. In this step, the cells were induced at 37°C for 4 h or at 18°C for 18 h. Finally, the cells were harvested and analyzed by SDS-PAGE using a 10% tricine gel.

4. Purification and tag removal of HER2(scFv)-PE26 in BL21 (DE3)

After 18 h post-induction at 18°C with 0.5 mM IPTG and 50 μg/mL ampicillin, a cultured cells were harvested from 500 mL cultivation by centrifugation at 3,800 × g for 20 min at 4°C. The cell pellets were achieved for next step or stored at -20°C until use. Cell

pellets were resuspended in 200 mL of buffer A (20 mM Tris-HCl, pH 8.0, 5% glycerol (v/v)) and disrupted by ultrasonic cell disruptor until the lysate was completely homogenized. The cell lysate was then centrifuged at 23,000 × g for 30 min at 4ºC to remove cell debris, and a supernatant containing MBP-TEVrs-HER2(scFv)-PE26 was filtered through a 0.4-μm-pore membrane before purification. The filtered supernatant was applied to a 10 mL cation exchange column, HiTrap SP HP that was equilibrated with buffer A for 10 column volumes (CVs) using an ÄKTA Explorer. An NaCl gradient (0–200 mM, 250 mM-450 mM) was used to elute impurities and MBP-TEVrs-HER2(scFv)-PE26, respectively. To remove MBP tag, TEV protease and 1 mM DTT were added to the eluate fractions (MBP fusion protein : TEV protease = 10 : 1, w/w) and cleavage reaction was performed at 18℃ for 18 h. The cleaved mixture was dialyzed against buffer B (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5% glycerol (v/v)) before applied to 5 mL IMAC column, HisTrap FF that pre-equilibrated with buffer B. In this step, HER2(scFv)-PE26 and some impurities passed the column. IMAC flow through was concentrated and centrifuged at 23,000 × g for 20 min at 4ºC before inject to GPC column, Hiload 16/600 Superdex 75 pg, which is equilibrated with 1X PBS, pH 7.4. On the GPC column, HER2(scFv)-PE26 was separated from the other impurities and then was stored at -20℃. The purification steps were checked using SDS-PAGE with 10% tricine gels. Protein concentrations were measured using the Bradford method with BSA as the standard.

5. Purification and tag removal of HER2(scFv)-GFP in BL21 (DE3)

MBP-TEVrs-HER2(scFv)-GFP plasmid was obtained by multisite gateway cloning method and transformed into *E. coli* BL21 (DE3). A single colony inoculated into LB medium containing 50 μg/mL ampicillin at 37℃ for overnight. The overnight-cultured cells inoculated into fresh LB medium containing 50 μg/mL ampicillin, then when the OD_{600} reached 0.5~0.7, 0.5 mM of IPTG was added and incubated at 18℃ for 18h. After induction, cultured cells were harvested from 500 mL cultivation by centrifugation at 3,800 × g for 20 min at 4°C. Cell pellets were resuspended in 100 mL of buffer B (20 mM Tris-HCl, 0.5 M NaCl pH 8.0, 5% glycerol (v/v)) and disrupted by ultrasonic cell disruptor until the lysate was completely homogenized. To remove cell debris, the cell lysate was centrifuged at 23,000 × g for 30 min at 4°C, and a supernatant containing MBP-TEVrs-HER2(scFv)-GFP was filtered through a 0.4-μm-pore membrane before purification. The filtered supernatant was passed through a 10 mL IMAC column, HisTrap FF that pre-equilibrated with 5 CVs of buffer B using an ÄKTA Prime. After binding, the column was washed by buffer B for 5 CVs. Then, the column was washed more than 10 CVs with buffer B containing 100 mM imidazole to remove any nonspecific bound proteins. The bound MBP-TEVrs-HER2(scFv)-GFP was then eluted by buffer B containing 500 mM imidazole for 5 CVs. To remove MBP tag, TEV protease was added to eluate fractions with 1 mM DTT (MBP fusion protein : TEV protease = 10 : 1, w/w) and incubated at 18℃ for 18 h. After TEV cleavage, cleaved mixture was dialyzed against buffer B, then it passed through a 5 mL MBPTrap HP MBP column

that was equilibrated with buffer B for 5 CVs. In this step, cleaved HER2(scFv)-GFP from MBP-HER2(scFv)-GFP and TEV protease passed column and MBP-HER2(scFv)-GFP MBP tag were eluted by buffer B containing 20 mM maltose. Flow through that passed MBP column was concentrated and centrifuged at 23,000 × g for 20 min at 4ºC before inject to GPC column, Hiload 16/600 Superdex 75 pg, which is equilibrated with 1X PBS, pH 7.4. HER2(scFv)-GFP and TEV protease were separated on the GPC column. All of purification steps analyzed by SDS-PAGE using 10% tricine gel. Protein concentrations were measured using the Bradford method with BSA as the standard. HER2(scFv)-GFP was stored at -20℃ for next experiments.

6. Purification and tag removal of HER2(scFv) and PE26 in BL21 (DE3)

Purification of HER2(scFv) and PE26 followed below same methods. The MBP-TEVrs-HER2(scFv) and His8-TEVrs-PE26 plasmids were made by gateway cloning method and transformed into *E. coli* BL21 (DE3), respectively. A single colony was cultured in LB medium containing 50 μg/mL ampicillin at 37℃ for overnight. Overnight-cultured cells were then inoculated into fresh LB medium containing 50 μg/mL ampicillin and induced by 0.5 mM IPTG at OD_{600} 0.4~0.6. After induced cells, it harvested from 500 mL cultivation by centrifugation at 3,800 × g for 20 min at 4ºC. Centrifuged pellets were resuspended in 100 mL of buffer B (20 mM Tris-HCl, 0.5 M NaCl pH 8.0, 5% glycerol (v/v)) and disrupted by

ultrasonic cell disruptor until the lysate was completely homogenized. Cell debris removed from cell lysate by centrifugation (23,000 × g for 30 min at 4°C). Supernatant containing tag fusion protein was filtered through a 0.4-µm-pore membrane before purification. The filtered supernatant was passed through a 10 mL IMAC column, HisTrap FF that pre-equilibrated with 5 CVs of buffer B using an ÄKTA Start. The IMAC column then washed by buffer B for 5CVs. After that, nonspecific bound proteins were washed by more than 10 CVs with buffer B containing 50 mM imidazole. The tag fusion protein which bound to column was eluted by buffer B containing 500 mM imidazole. To remove fused tag, TEV protease was added to eluate fractions with 1 mM DTT (Tag fusion protein : TEV protease = 10 : 1, w/w) and incubated at 18℃ for 18 h. Cleaved mixture then dialyzed against buffer B. After dialysis, it passed through a 5 mL IMAC column that was equilibrated with buffer B for 5 CVs. The target protein passed IMAC column and other impurities bound to column and washed by buffer B containing 1 M imidazole. IMAC flow through which are containing just target protein dialyzed against 1 X PBS, pH 7.4 and measured concentrations using the Bradford method with BSA as the standard. Finally, final products were stored at -20℃ for next experiments. All of purification steps analyzed by SDS-PAGE using 10% tricine gel.

7. Electrophoresis and quantification of protein expression and solubility level

Protein fractions were mixed with 5X sample buffer (312.5 mM Tris-HCl, pH 6.8, 50% glycerol, 5% SDS, 0.05% bromophenol blue, 300 mM DTT) and boiled for 10 minutes before they were runned with a 10% tricine SDS-PAGE gel. The protein bands were visualized with Coomassie brilliant blue R-250 solution. The expression and solubility levels of the fusion proteins and the purity of the target proteins were measured by ImageJ image software (http://imagej.nih.gov/ij) as in the previous studies [33-37].

8. Determination of HER2(scFv)-PE26 purity by HPLC and silver staining

To determine HER2(scFv)-PE26 purity, the final product was analyzed by HPLC using a Protein-pak 300SW SEC 7.5 x 300 mm column. The column was equilibrated with 1X PBS buffer, pH 7.4 with more than 10 CVs using Waters 600 Controller that was connected with Waters 486 Tunable Absorbance Detector and Waters 717 Plus Autosampler. The protein was injected to the column at a flow rate of 1 mL/min for 25 min. The protein elution peaks were detected at 280 nm and were checked on an SDS-PAGE gel. HER2(scFv)-PE26 purity was also evaluated using the Silver Stain Plus kit. The reaction was completed by adding 5% acetic acid (v/v) for 15 minutes when the bands were obviously visible.

9. Endotoxin removal and endotoxin assay

To remove endotoxins, Triton X-114 was used in the purified sample following a previously described method [38]. In order to measure the endotoxin level, the Toxin Sensor Chromogenic LAL Endotoxin Assay Kit was used following the manufacturing instruction. HER2(scFv)-PE26 was diluted with endotoxin free water to adjust concentration to 1 μg/mL. 100 μL of a 1 μg/mL sample, or an endotoxin standard (1, 0.1, 0.05, 0.025, and 0.01 EU/mL) were dispensed into endotoxin free vials. 100 μL of LAL reagent was added and mixed well by swirling gently and incubated at 37°C using heating blocks for 50 min. After incubation, 100 μL of chromogenic substrate solution was added to each vials and mixed by swirling and incubated at 37°C using heating blocks for 6 min. 500 μL of stop solution (color-stabilizer #1) was added to each vials and swirled. Then 500 μL of color-stabilizer #2 was added to each vials and mixed well. Finally, 500 μL of color-stabilizer #3 was added to each vials and mixed well for 3 seconds. After transferred 150 μL of reacted mixture into each two well of 96 well plate, spectrophotometer was used to measure absorbance at 545 nm wavelength. After analyzing the absorbance, the units of endotoxin were calculated using the standard curve obtained from the standard solution.

10. Mass analysis of purified HER2(scFv)-PE26

For mass analysis to identify HER2(scFv)-PE26, the SDS-PAGE gel containing HER2(scFv)-PE26 band was cut and destained by 100 mM ammonium bicarbonate (ABC) in 50% acetonitrile (ACN) solution, then the gel was washed by 100 mM ABC. 100% ACN was added to the gel for dehydration and the gel was dried at room temperature. The dried gel was incubated at 50℃ for 1 h in 50 mM DTT/50 mM ABC. After incubation, the gel was washed by 50 mM ABC and then added 55 mM iodoacetamide (IAA)/50 mM ABC and incubated at room temperature for 1 h with darkness. For trypsinization, the gel was washed by 100% ACN, then 20 μL trypsin (0.1 μg/μL) with 2 mM $CaCl_2$ to the gel and incubated for 1 h on the ice. Thereafter, 50 mM ABC was added and incubate at 37℃ for 16 h. Supernatant was moved to other tube and the gel was washed with 40% ACN (+0.1% trifluoroacetic acid (TFA)) and low vortexing. Supernatant was combined with the supernatant obtained in the previous step. The supernatant was dried using speed vacuum. After desalting the sample, LC-MS/MS was performed on a Ultimate 3000 liquid chromatography (Thermo, MA, USA) system connected to a Q Exactive plus biopharm spectrometer (Thermo, MA, USA). Each peptides digested by trypsin were analyzed using Proteome Discoverer 2.2 (Thermo, MA, USA) and identified using Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi). The LC-MS/MS data searches (SEQUEST) were performed using Proteome Discoverer 2.2 (Thermo, MA, USA).

11. Flow Cytometric Analysis

For flow cytometric analysis, trypsinized cells (2×10^6) were centrifuged and resuspended in 1 mL PBS (pH 7.4). Then the cells were incubated with a concentration of HER2(scFv)-GFP (5 μg/mL) for 30 min at 4℃ and then stained by DAPI for 10 min at 4℃. After incubation, the cells washed with PBS for 3 times. Finally, intensity of GFP was determined using FACSCanto II flow cytometer (BD Biosciences, San Diego, CA). Data was analyzed using FlowJo_V10 (FlowJo LLC, Ashland, OR).

12. MTT assay

Cell viability was measured by MTT assay. For MTT assay, 5×10^4 cells were seeded in 24-well plate and incubated at 37□C for 18 h. Thereafter, HER2(scFv)-PE26, HER2(scFv), PE26 were treated to high HER2-expressing cell lines, SKBR3 and BT-474 or low HER2-expressing cell line, MDA-MB-231, MCF7. After 72 h proteins were treated, the cell viability was calculated by means of the MTT assay. All the protein concentrations were tested in triplicate.

All data are presented as the mean ± standard error (SE) of $n \geq 3$ of 3 independent experiments. The data was processed using following equation and Microsoft Excel software:

$$V = top - (top-bottom) / (1 + (IC_{50} / conc.) \text{ ^ } HC)$$

where V is cell viability, top is the highest cell viability, bottom is the lowest cell viability, conc is concentration of the proteins, HC is Hill coefficient of inhibition.

13. Statistics

All data are presented as the mean ± standard error (SE) of $n \geq 3$ of 3 independent experiments. The data was analyzed by Graphpad Prism 7 software (GraphPad, San Diego, CA) and $P \leq 0.05$ was considered significant.

Results

1. Construction of recombinant immunotoxin, MBP-HER2(scFv)-PE26

To obtain expression vector, multisite gateway cloning method was performed. For multisite gateway cloning, first of all, the DNA sequence that encodes TEVrs-HER2(scFv) was amplified by PCR to attach attB1 and attB5r at the end of both sides. Also, the DNA sequence of GGSG_Furin$_{CS}$_GGSG-PE26 that contained attB5 and attB2 was gotten by PCR. After that, TEVrs-HER2(scFv) and GGSG_Furin$_{CS}$_GGSG-PE26 was performed BP reaction with pDONR221-P1P5r and pDONR221-P5P2, respectively. As a results of BP reaction, pENTR-HER2(scFv) constructed attL1-TEVrs-HER2(scFv)-attL5r and pENTR-PE26 constructed attL5-GGSG_Furin$_{CS}$_GGSG-PE26-attL2 called entry clones were generated. attL1, attL5r and attL5, attL2 were then recombined with attR1 and attR2 in destination vector, pDEST-HMGWA. In this step, ccdb and Cm (R) in destination vector were exchanged for TEVrs-HER2(scFv)-GGSG_Furin$_{CS}$_GGSG-PE26 (Figure 1. A, B). Finally, expression plasmid is controlled by T7 promotor and induced by IPTG.

2. Expression and solubility of MBP-HER2(scFv)-PE26

The plasmid was transformed into *E. Coli* BL21 (DE3), SHuffle, Origami 2 cells, respectively, and induced by 0.5 mM of IPTG at 37℃ or 18℃. Then, proteins in the total-

cell extracts, soluble fraction and insoluble fraction were analyzed using SDS-PAGE, and expression, solubility levels were quantified. As shown in Fig. 2, 40~50% expression was observed in BL21 (DE3), SHuffle strain. Origami 2 showed 40~50% expression level at only 37℃. At 18℃, solubility level was improved compared to 37℃ in all strains.

3. Purification of HER2(scFv)-PE26

The total cell lysate of *E. Coli* expressing HER2(scFv)-PE26 tagged with MBP were applied to Hitrap SP HP cation exchange chromatography. MBP fusion protein starting to elute from 250 mM to 450 mM NaCl. As shown in figure 3, lane 3, 97.4 kDa of MBP fusion protein band was visualized on the gel. Subsequently, a TEV protease added to the purified MBP-HER2(scFv)-PE26 to cleave MBP tag from MBP fusion protein. Half of MBP fusion protein was cleaved by TEV protease after overnight incubation at 18℃ (Figure 3, lane 4). Cleaved sample was then dialyzed against buffer B (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5% glycerol (v/v)) and passed through Histrap FF IMAC column. On the IMAC column, 53.5 kDa of cleaved target protein was passing the column, while more than half of undigested MBP fusion protein and MBP tag, TEV protease bound to the column and washed out by imidazole. (Figure 3, lane 5). To separate HER2(scFv)-PE26 from other impurities, IMAC flow through sample was injected to Hiload 16/600 Superdex 75 pg GPC column. Finally, impurities were removed and the pure HER2(scFv)-PE26 was gotten (Figure 3, lane 6). After

final step of purification, 0.25 mg of HER2(scFv)-PE26 was obtained from 500 mL of cell culture (Table 1). After purification, for endotoxin removal, Triton X-114 was added to the target protein, and the endotoxin level was estimated. After endotoxin removal, the endotoxin level in final purified HER2(scFv)-PE26 was lower than 0.01 EU/μg, which is in the range (<1 EU/μg) of commercially and clinically available protein product. To determine the purity of the final HER2(scFv)-PE26 product, SEC-HPLC and silver staining were performed. In the result of SEC-HPLC, major peak appeared at 12.239 min. The chromatogram indicated that the purity of HER2(scFv)-PE26 was approximately 91% (Figure 4. A), and fraction was analyzed using SDS-PAGE (Figure 4. B). Silver staining result showed the purity of final product contained the bands corresponding to 53.5 kDa HER2(scFv)-PE26 with a purity of 93% (Figure 4. C).

4. Purification of HER2(scFv)-GFP

The total cell lysate of MBP-HER2(scFv)-GFP expressed in *E. Coli* were applied to Histrap FF IMAC column. 98.3 kDa of MBP-HER2(scFv)-GFP was bound to column and eluted by buffer B (20 mM Tris-HCl, 0.5 M NaCl pH 8.0, 5% glycerol (v/v)) containing 500 mM imidazole (Date was not shown). Subsequently, a TEV protease added to the purified MBP-HER2(scFv)-GFP to cleave MBP tag from MBP-HER2(scFv)-GFP. ¼ of MBP fusion protein was cleaved by TEV protease after TEV protease was added 18 h at 18℃ (Data was

not shown). Cleaved mixture was then dialyzed against buffer B and passed through MBPtrap HP MBP column. On the MBP column, 54.4 kDa of cleaved HER2(scFv)-GFP and TEV protease were passing the column, while MBP fusion protein and MBP tag bound to the column and eluted by buffer B containing 20 mM maltose (Data was not shown). To separate HER2(scFv)-GFP and TEV protease, MBP flow through was injected to Hiload 16/600 Superdex 75 pg GPC column. Finally, 0.06 mg of pure HER2(scFv)-GFP was gotten from 500 mL of cell culture with a purity of greater than 98% (Figure 6, A). After final purification step, Triton X-114 was added to remove endotoxin. After endotoxin removal, the endotoxin level in final purified HER2(scFv)-GFP was lower than 0.1 EU/μg, which is in the range (<1 EU/μg) of commercially and clinically available protein product.

5. Identification of HER2(scFv)-PE26 by mass analysis

To identify HER2(scFv)-PE26, LC-MS/MS was performed using sample that was digested by trypsin. As shown in figure 5, HER2(scFv)-PE26 was identified with 77.18% sequence coverage. HER2(scFv)-PE26 cleaved into 36 peptides. 18 of the 36 peptides were consistent with the predicted peptide and identified as trastuzumab or PE through BLAST (Data was not shown).

6. HER2 expression of breast cancer cell lines and binding capacity of HER2(scFv) to high HER2-expressing or low HER2-expressing cell lines

To investigate the binding capacity of HER2(scFv) and HER2, PE26 of HER2(scFv)-PE26 replaced with GFP. HER2(scFv)-GFP was incubated with high HER2-expressing cell lines, SKBR3 and BT-474, or low HER2-expressing cell lines, MDA-MB-231 and MCF7. Figure 6, B, C indicates HER2(scFv)-GFP bound to SKBR3 and BT-474 cells. However, most of HER2(scFv)-GFP didn't bind to MDA-MB-231 and MCF7. Just a small amount of HER2(scFv)-GFP bound to MDA-MB-231 and MCF7 (Figure 6, D, E).

7. Cytotoxicity of HER2(scFv)-PE26 to high HER2-expressing or low HER2-expressing cell lines

To measure cell viability, MTT assay was performed with HER2(scFv)-PE26, HER2(scFv), PE26 on high HER2-expressing cell lines, SKBR3, BT-474 and low HER2-expressing cell lines, MDA-MB-231, MCF7. HER2(scFv)-PE26 showed strong cytotoxicity to the SKBR3 cell line that expressed HER2 with a high level, while HER2(scFv) and PE26 didn't affect to same cell line (Figure 7, A). Another high HER2-expressing cell line, BT-474 was killed by HER2(scFv)-PE26 and PE26 only, but HER2(scFv)-PE26 showed stronger cytotoxicity than PE26 only (Figure 7, B). HER2(scFv)-PE26 killed low HER2-expressing cell lines, MDA-MB-231 and MCF 7 also at higher concentration than high

HER2-expressing cell lines. Besides, more than 50% low HER2-expressing cells were killed by 200 nM of PE26 only (Figure 7 C, D). Moreover, in all cell lines, HER2(scFv) didn't affect to cell viability. In summarize, HER2(scFv)-PE26 showed stronger cytotoxicity to high HER2-expressing cell lines, SKBR3 and BT-474 than low HER2-expressing cell lines, MDA-MB-231 and MCF7. IC_{50}s of HER2(scFv)-PE26 was calculated to 28.1 ± 2.5 pM (n=9), 19 ± 1.4 pM (n=9), 5.8 ± 0.28 nM (n=3) and 0.28 ± 0.046 nM (n=3) on SKBR3, BT-474, MDA-MB-231 and MCF7 with the Hill coefficient of 2.24 ± 0.16, 1.87 ± 0.17, 0.65 ± 0.21 and 3.22 ± 1.87, respectively (Figure 8).

8. Correlation between HER2 expression and IC_{50} of HER2(scFv)-PE26

The relationship between HER2 expression and IC_{50} of HER2(scFv)-PE26 is revealed. As shown in table 2, HER2 expression level is; BT-474 > SKBR3 > MCF7 > MDA-MB-231 and IC_{50} of HER2(scFv)-PE26 is; BT-474 < SKBR3 < MCF7 < MDA-MB-231. Figure 9 shows these correlation as a graph. IC_{50} of HER2(scFv)-PE26 decreased with increasing HER2 expression level and they associated with a strong negative correlation ($r = -0.68$, $P \leq 0.05$).

Discussion

Trastuzumab targeting human epidermal growth factor receptor 2 (HER2) have been used for HER2-positive breast cancer patients [10, 11]. In addition, it have been tried to conjugate the antibody with a number of drug, resulting in increase of cytotoxicity and specificity [18, 19]. The bacterial toxin, *Pseudomonas* exotoxin A (PE) from *Pseudomonas aeruginosa* and its varied truncated forms, have been used in many immunotoxin studies [24-32]. Hence, the goal of this present study was to construct and produce a new recombinant immunotoxin, HER2(scFv)-PE26 as a promising drug for targeting and killing HER2-positive cancer cells.

For testing cytotoxicity of purified HER2(scFv)-PE26, four kinds of breast cancer cell lines expressing HER2 in different amounts were used. First of all, expression level of HER2 on the breast cancer cell lines was analyzed by using FACS and purified HER2(scFv)-GFP (Figure 6. A). Figure 6. B, C, D, E showed the highest expression of HER2 on SKBR3 and BT-474, and low expression of HER2 on MDA-MB-231 and little expression of HER2 on MCF7. These results are in agreement with previous studies that SKBR3 and BT-474 are well known as high HER2-expressing cell lines whereas MDA-MB-231 and MCF7 are low HER2-expressing cell lines [17, 39-41]. Besides, it also indicates synthesized HER2(scFv) from trastuzumab specifically bound to HER2. Second, cytotoxicity of HER2(scFv)-PE26 on four kinds of breast cancer cell lines was measured by MTT assay. In many studies, trastuzumab shows stronger cytotoxicity when it was combined with other drugs [14-17]. In figure 7,

HER2(scFv)-PE26 shows stronger cytotoxicity than HER2(scFv) or PE26 only. Furthermore, HER2(scFv) didn't reduce cell viability significantly. It seems because of absence of Fc region. The original trastuzumab recruit immune effector cells and leads to tumor-lysis, however, HER2(scFv) doesn't have Fc region [11]. Hence, HER2(scFv) prevent differentiation and proliferation without cell death. Moreover, even though domain I and II of PE except for furin cleavage site were removed and replaced with HER2(scFv), it still shows cytotoxicity. It means combination of HER2(scFv) and PE26 carries out positive effect to enhance cytotoxicity. Theoretically, immunotoxin bind to antibody specific receptor and internalize by endocytosis. Then antibody and toxin is cleaved by protease. The antibody is digested and the toxin induce cell death [42]. With these mechanism, HER2(scFv)-PE26 induce apoptosis. As shown in figure 8, HER2(scFv)-PE26 shows the most strong cytotoxicity to high HER2-expressing cell lines, SKBR3 and BT-474 than low HER2-expressing cell lines, MDA-MB-231 and MCF7. On SKBR3 and BT-474 cell lines, IC_{50}s were calculated to 28.1 ± 2.5 pM (n = 9) and 19 ± 1.4 pM (n = 9), with the Hill coefficient of 2.24 ± 0.16 and 1.87 ± 0.17 respectively. On MDA-MB-231 and MCF7 cell lines, IC_{50}s were calculated to 5.8 nM ± 0.28 nM (n = 3) and 0.28 nM ± 0.046 nM (n = 3) with the Hill coefficient of 0.65 ± 0.21 and 3.22 ± 1.87 respectively. Approximately 33-fold of IC_{50} value of HER2(scFv)-PE26 on high HER2-expressing cell lines was observed compared to MDA-MB-231 which are low HER2-expressing cell line. However, the IC_{50} value of HER2(scFv)-PE26 on MCF7 that also known as low HER2-expressing cell line was only 10-fold lower than on high HER2-

expressing cell lines. According to these results, the safety margin is too small, so there would be a difficulty to adjust dose. In previous study, anti-HER2-PE38 immunotoxin was produced and it showed around 4-fold of IC_{50} value on SKBR3, BT-474 compared to MCF7 [43]. Compared with this study, the safety margin of HER2(scFv)-PE26 is improved. Moreover, figure 9 indicates strong negative correlation between HER2 expression and IC_{50} of HER2(scFv)-PE26. As shown in table 2, the HER2 expression level is; BT-474 > SKBR3 > MCF7 > MDA-MB-231 and as the HER2 expression level is higher, IC_{50} of HER2(scFv)-PE26 is decreased. It suggesting that HER2(scFv)-PE26 specifically targets HER2. Due to specificity of HER2(scFv) to HER2 (Figure 6) and cytotoxicity of PE26 to four breast cancer cell lines (Figure 7), the recombinant immunotoxin, HER2(scFv)-PE26 synergized compared to HER2(scFv) or PE26 only.

Despite immunotoxins which are based on small recombinant antibody molecules, such as single-chain Fv (scFv) fragments showed encouraging effect in clinical or preclinical animal trials, short half-life hampers the therapeutic efficacy [44-46]. There is several strategies to improve the pharmacokinetic properties of protein-based therapeutics such as chemical modification with long-chain polyethylene glycol (PEGylation) [47] and genetic fusion with human serum albumin (HSA) [48] or human IgG Fc domain [49]. However, these strategies have some problem. PEGylation of immunotoxin reduced cytotoxicity, while it increase half-life [50]. In addition, polyethylene glycol (PEG) leads to unwanted mono or multi conjugation randomly with lysines and cysteins that commonly found in proteins [51]. To avoid this

situation, the N-terminal methionine residue of protein could be PEGylated by the reductive alkylation method at low pH [52]. Nevertheless, for recovery of the PEGylated products, additional purification steps were usually required [53]. Since fusion with IgG FC domain or HSA increases the molecular size more than the renal clearance threshold, proteins are protected from intracellular degradation, but expression of proteins with FC domain or HSA in bacteria is difficult due to their huge size [54]. In some study, albumin binding domain (ABD) which are short peptide consisting of 46 a.a fused to N-terminus of the immunotoxin anti-HER2-PE38 which has similar construct with HER2(scFv)-PE26 to increase half-life [43]. According to this study, although fusion of ABD with immunotoxin carried out extending 24.4-fold of half-life, there remained challenge for immunogenicity. The novel recombinant immunotoxin, HER2(scFv)-PE26 designed using PE26 which are reduced immunogenicity and nonspecific toxicity of PE and it showed cytotoxicity to breast cancer cell lines. (Figure 7, 8). Therefore, it could be a good candidate for the HER2-overexpressing cancer therapy after fusing with a ABD or conjugated with PEG to the N-terminal methionine residue.

In many studies, *E. coli* was used to overexpress and produce human proteins, but it misfolded and aggregated forming inclusion body [55, 56]. To address this problem, several tag systems that increase solubility have been established for the highly soluble expression of the proteins in prokaryotic cytoplasm [33-37]. For this study, MBP tag that have chaperone activity [57] was chosen and attached to N-terminus of HER2(scFv)-PE26.

Because cytoplasmic reducing environment in *E. coli* have been proven not to facilitate the formation of correct disulfide bonds of foreign proteins, it's very hard to obtain high yield of disulfide rich proteins [56, 58, 59]. SHuffle and Origami 2 which are genetically engineered E. coli host strains have been generated to fix out those challenges by its oxidative cytoplasmic environment [56, 58, 59]. Hence, MBP-HER2(scFv)-PE26 was tested expression and solubility level in three kinds of *E.coli* hosts, BL21 (DE3), Origami 2 and SHuffle at 18℃ and 37℃ because the induction temperature is also important factor to enhance solubility of target proteins. As shown in figure 2, expression level of SHuffle strain was similar to BL21 (DE3) and Origami 2 showed the lowest expression level between three kinds of host strains. Moreover, all of host strains enhanced soluble production of MBP-HER2(scFv)-PE26 at 18℃. Even though BL21 (DE3) and SHuffle showed similar expression level, BL21 (DE3) was selected for the host strain to purify the target proteins because BL21 (DE3) has advantage to grow the bacteria cell fast and 18℃ was chosen for the induction.

For the purification of HER2(scFv)-PE26, cation exchange, IMAC, GPC column were used. MBP fusion target protein was purified by cation exchange column, but as shown in Table 1, just 19.41% of HER2(scFv)-PE26 was gotten. Half of MBP fusion protein was cleaved by TEV protease (Figure 3. lane 3, lane 4). After second purification using IMAC to remove MBP fusion protein, MBP tag and TEV protease, 6.11% of HER2(scFv)-PE26 was gotten. Finally, 0.25 mg of pure HER2(scFv)-PE26 was purified on the GPC column. It

means just 1.07% of final product was obtained from 23.23 mg of starting target protein (Table 1). The reason why got the poor yield is low binding capacity of MBP-HER2(scFv)-PE26 to cation exchange column and low efficiency of TEV cleavage. To purify MBP fusion protein, IMAC and MBP column have been tried to use as a first column because of its low binding capacity to cation exchange column. However, it was not bind to IMAC or MBP column both, even though it has a MBP tag and hexa-histidine at the N-terminus (Data was not shown). One possibility is MBP tag and hexa-histidine exist inside of MBP fusion protein. In previous study, low buffer pH was used to facilitate target protein binding [33)]. Therefore, adjustment of pH to change the PI of MBP fusion protein could be one of the strategy to enhance the yield. Another way is denaturation of MBP fusion protein by urea before apply to IMAC or MBP column. Usually, urea which are chaotropic agent have been used to facilitate a reversible protein denaturation [60)]. After denaturation, MBP tag and hexa-histidine might be exposed to outside and improve binding capacity to IMAC or MBP column. Low TEV cleavage efficiency is also problem to obtain high yield of target protein. In many studies, TEV protease cleavage has been used as a very common method to remove tag successfully [33-37)] and seemed quite important since its low efficiency might be a hampers for the next purification step [61, 62)]. Therefore, finding suitable condition for TEV cleavage to remove MBP tag could be increase the yield of final product.

In conclusion, the present research data represent the novel recombinant immunotoxin, HER2(scFv)-PE26 produced successfully as soluble form using MBP tag in *E.*

Coli. The HER2(scFv)-PE26 was purified with a high purity and showed stronger cytotoxicity compared to HER2(scFv) or PE26. Interestingly, its cytotoxicity followed HER2 expression level. The HER2 expression and the IC_{50} of HER2(scFv)-PE26 were associated with a strong negative correlation ($r = -0.68$, $P \leq 0.05$). These results suggest that production of immunotoxin HER2(scFv)-PE26 in *E. Coli* would be approached cost-effective technique and could be a great candidate for HER2-overexpressing cancer therapy.

Tables

Table 1. Purification of HER2(scFv)-PE26 expressed in BL21 (DE3)

Purification step	Total protein (mg)	Purity (%)	HER2(scFv)-PE26 (mg)	Yield (%)
Bacterial culture (500 mL)	1400			
Supernatant	103.2	41.07	23.23	100
1st Cation exchange	12.07	68.18	4.51	19.41
2nd IMAC	4.96	28.72	1.42	6.11
3rd GPC	0.2	100	0.25	1.07

Table 2. HER2 expression of four cell lines and IC_{50} of HER2(scFv)-PE26

Cell lines	HER2 expression (%)	IC_{50} (pM, n ≥ 3)	Hill coefficient
BT-474	97.5	19 ± 1.4	1.87 ± 0.17
SKBR3	93.8	28.1 ± 2.5	2.24 ± 0.16
MCF7	11.8	280 ± 46	3.22 ± 1.87
MDA-MB-231	0.4	5800 ± 280	0.65 ± 0.21

Figures

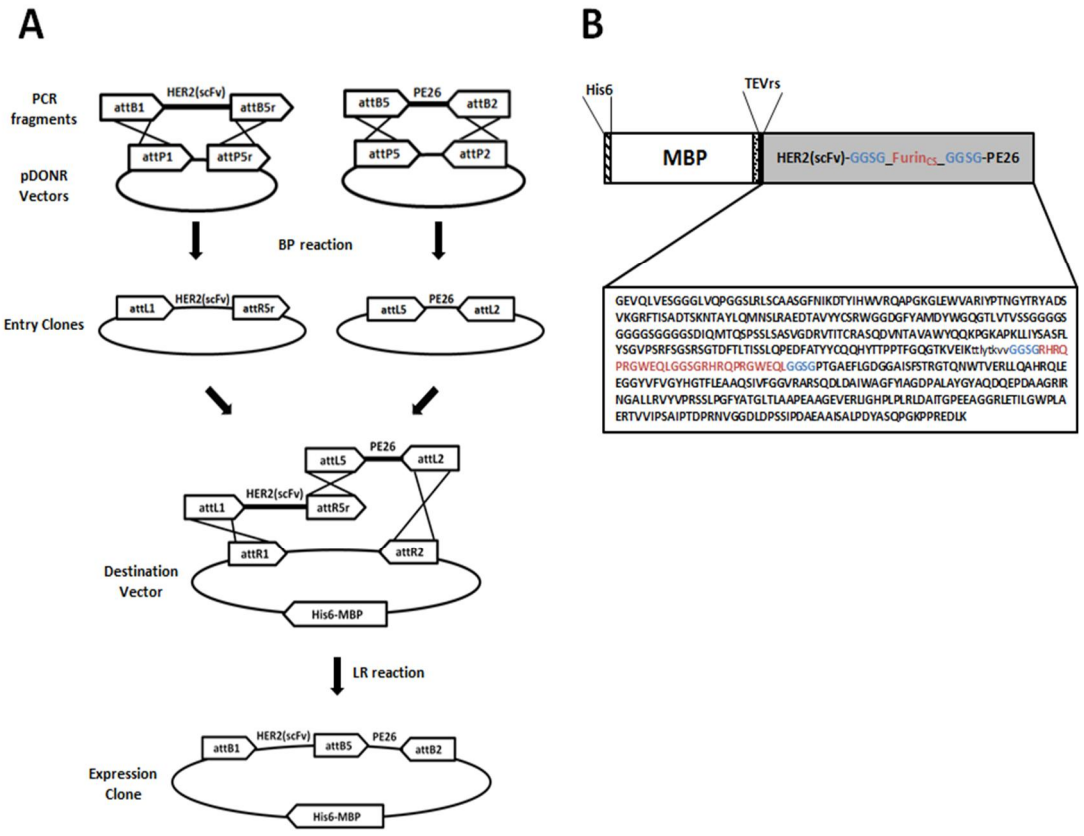

Figure 1. Construction of the MBP-HER2(scFv)-PE26 expression vector, and a schematic representation of MBP-HER2(scFv)-PE26 fusion protein.

(A) The MBP-HER2(scFv)-PE26 was designed by multisite gateway cloning method. (B) Schematic structure of MBP-HER2(scFv)-PE26. The expression of MBP-HER2(scFv)-PE26 is driven by the IPTG-inducible T7 promoter with ampicillin as the selection marker.

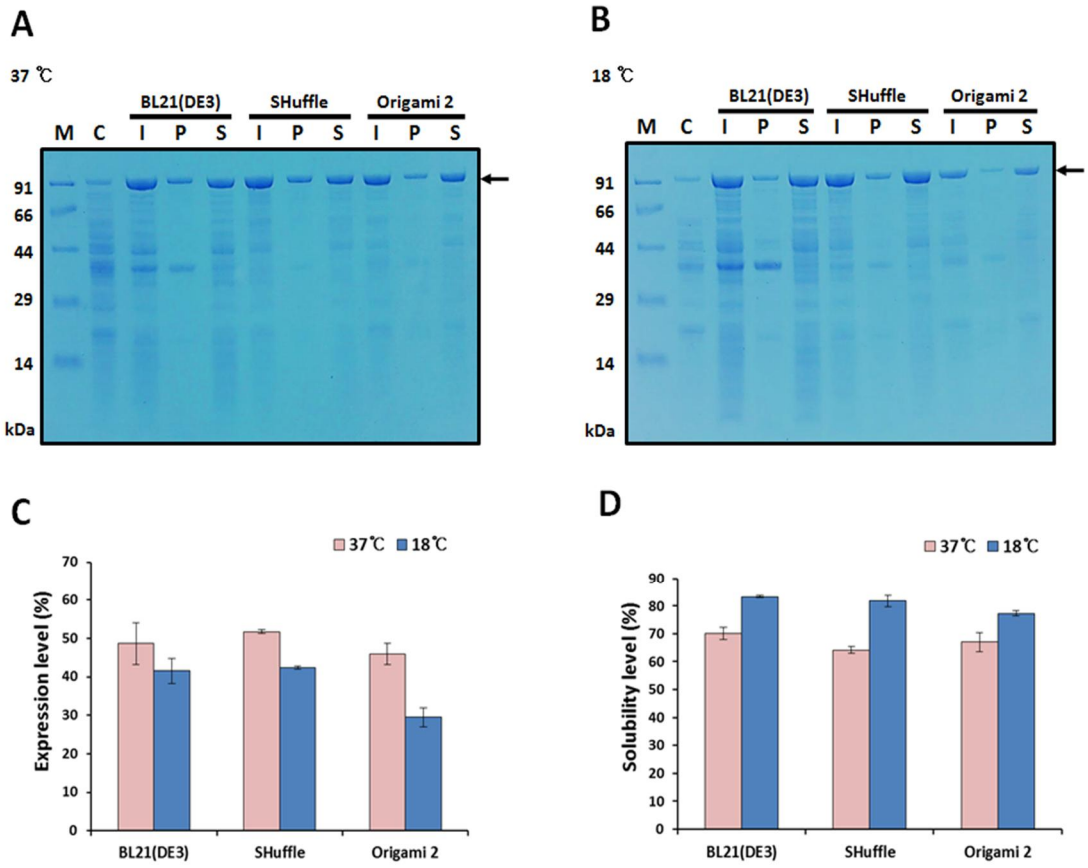

Figure 2. Expression and solubility levels of MBP-HER2(scFv)-PE26 in different *E. coli* hosts. Expression of the MBP fusion protein was induced by 0.5 mM IPTG at 37℃ (A), and 18℃ (B). The arrows indicates MBP-HER2(scFv)-PE26 (97.4 kDa). M, molecular weight size marker; C, total proteins before IPTG induction as negative control; I, total proteins after IPTG induction; P, insoluble pellet fraction after cell sonication; S, soluble fraction after cell sonication. (C) indicates expression level and (D) shows solubility level at 37℃, and 18℃ in three kinds of *E. coli* hosts. The expression and solubility level were analyzed using densitometry method with three repeats of experiment. Expression level (%) of MBP-HER2(scFv)-PE26 was calculated based on the density ratio of MBP-HER2(scFv)-PE26 to

the total *E. coli* expressed proteins. The solubility level (%) was calculated based on the density ratio of soluble-expressed MBP-HER2(scFv)-PE26 to the total-expressed MBP-HER2(scFv)-PE26.

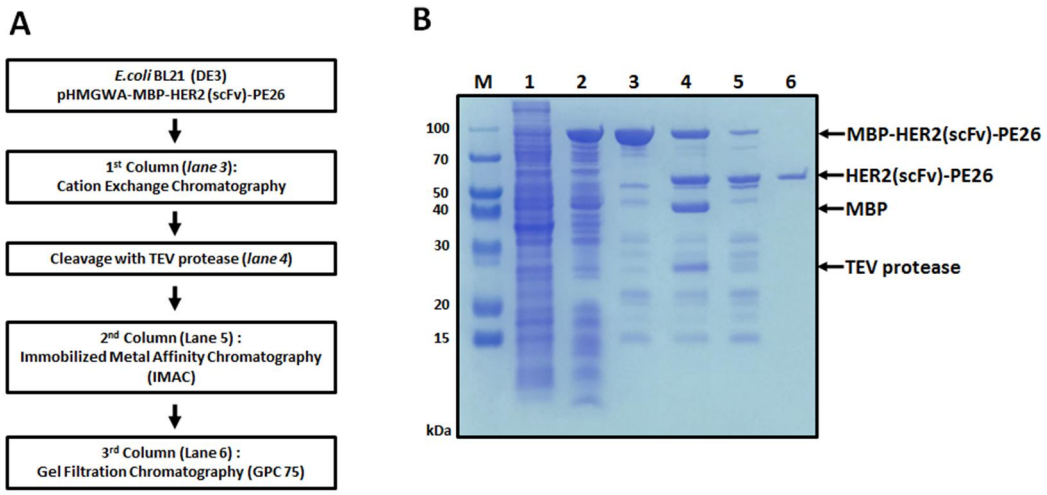

Figure 3. Purification of HER2(scFv)-PE26 in BL21 (DE3). (A) Flow chart of the purification steps. (B) MBP fusion-derived HER2(scFv)-PE26 was purified from BL21 (DE3) by Cation exchange chromatography, IMAC and Gel filtration chromatography. M, molecular weight size marker; lane 1, total cell proteins before IPTG induction as negative control; lane 2, soluble proteins after cell sonication from total cell proteins induced by IPTG; lane 3, MBP-HER2(scFv)-PE26 fusion protein (97.4 kDa) purified with cation exchange chromatography; lane 4, MBP tag cleavage with TEV protease (28.6 kDa): MBP tag (43.9 kDa) and HER2(scFv)-PE26 (53.5 kDa); lane 5, IMAC purification of HER2(scFv)-PE26 after TEV cleavage; lane 6, HER2(scFv)-PE26 (53.5 kDa) was purified with gel filtration chromatography.

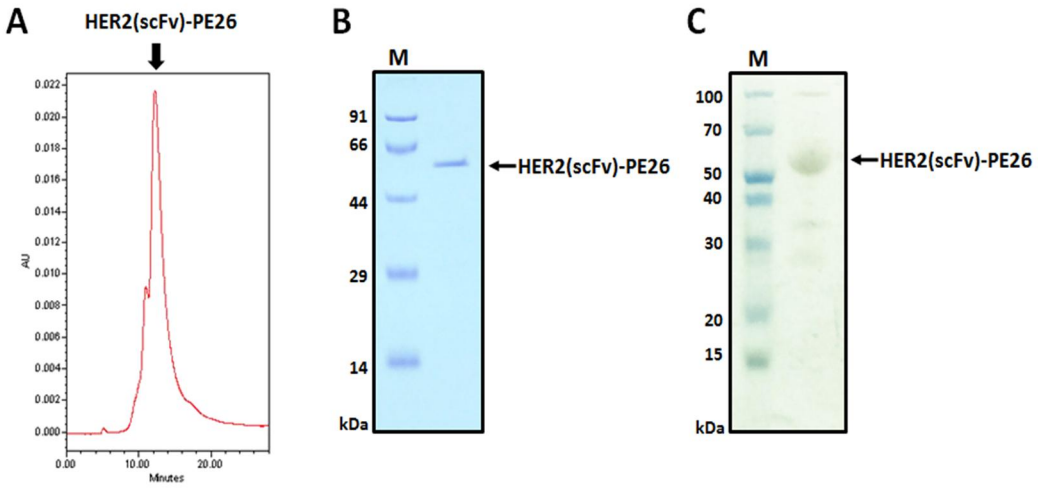

Figure 4. Determination of the purity of HER2(scFv)-PE26. (A) The purified HER2(scFv)-PE26 was analyzed by HPLC using a protein-pak 300SW SEC 7.5 × 300 mm column to evaluate the purity. The x-axis shows retention time (min) and the y-axis indicates the absorbance at 280 nm (arbitrary units, AU). The main peak of HER2(scFv)-PE26 appeared at 12.239 min. (B) fraction from (A) were analyzed using SDS-PAGE. (C) Silver stained SDS-PAGE gel used to assess the purity of the recombinant immunotoxin, HER2(scFv)-PE26 (53.5 kDa).

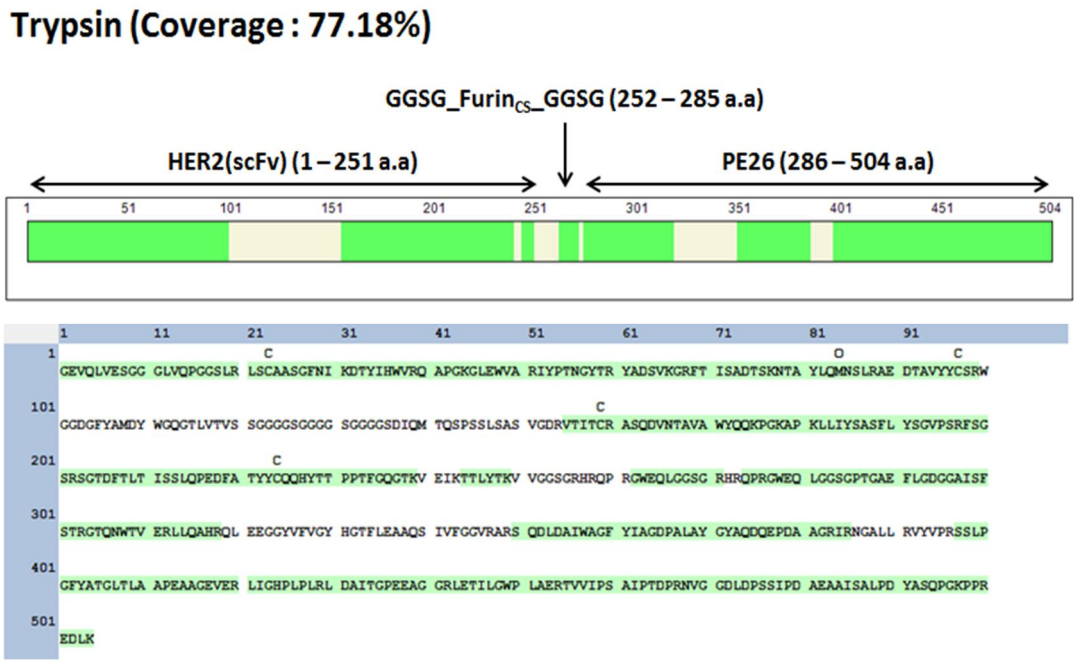

Figure 5. LC-MS/MS sequencing for identification of HER2(scFv)-PE26. HER2(scFv)-PE26 was digested by trypsin before sequencing. Percentage of coverage for protein identification is represented to 77.18%. Confidence of each sequence is represented by peptide spectrum matches as illustrated by high (green: p-value < 0.01) Percolator confidence scores.

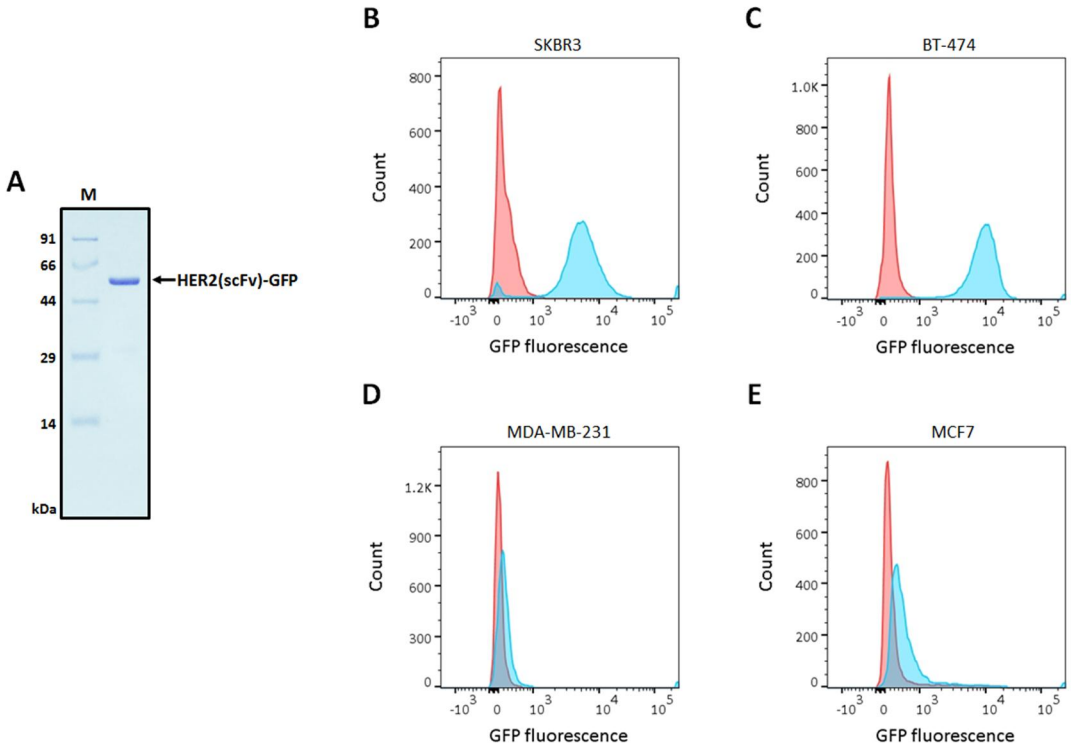

Figure 6. HER2 expression of breast cancer cell lines and binding capacity of HER2(scFv)-GFP to high HER2-expressing or low HER2-expressing cell lines. (A) purified HER2(scFv)-GFP (54.4 kDa) from E. coli was used for FACS analysis. High HER2-expressing cell lines (B) SKBR3 and (C) BT-474 and low HER2-expressing cell lines (D) MDA-MB-231 and (E) MCF7 were incubated with DAPI and HER2(scFv)-GFP. Red color shows HER2(scFv)-GFP untreated cells and blue indicates HER2(scFv)-GFP treated cells.

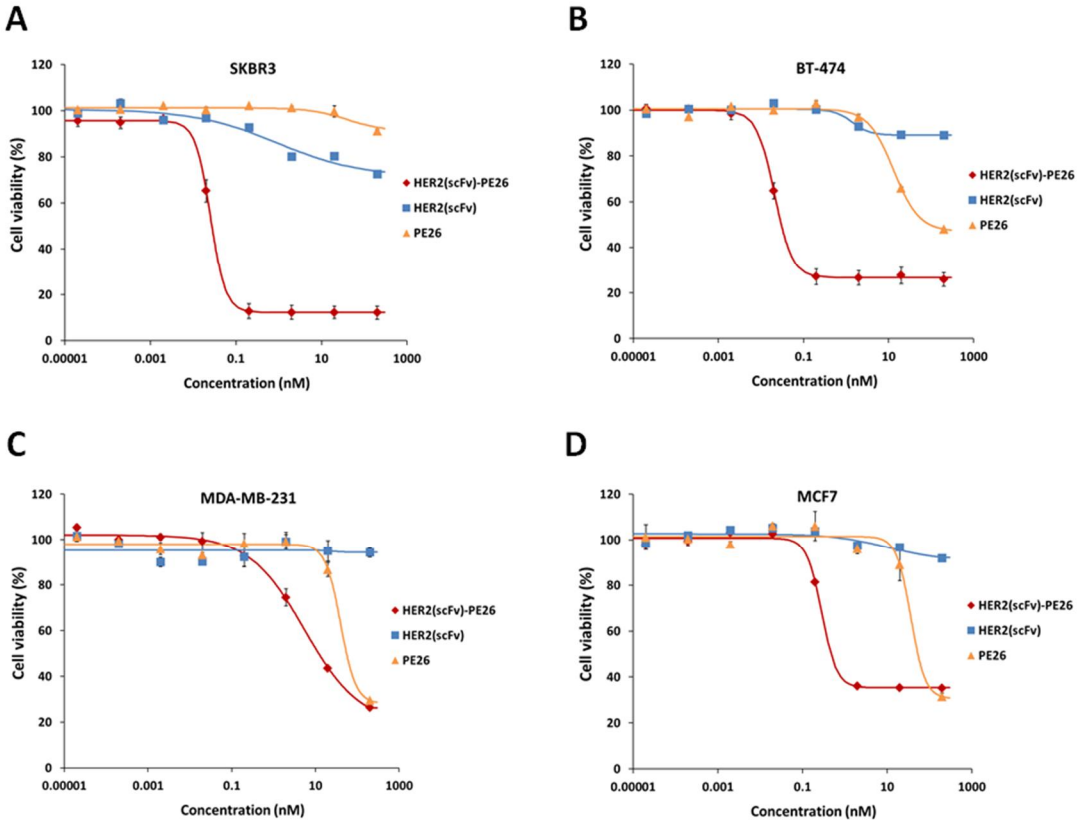

Figure 7. Cytotoxicity of HER2(scFv)-PE26, HER2(scFv) and PE26 to high HER2-expressing or low HER2-expressing cell lines. HER2(scFv)-PE26, HER2(scFv) and PE26 were added to high HER2-expressing cell lines (A) SKBR3 and (B) BT-474 or low HER2-expressing cell lines (C) MDA-MB-231 and (D) MCF7 and incubated for 72 h. For all cell lines, HER2(scFv)-PE26 showed stronger cytotoxicity than HER2(scFv) and PE26 only. Untreated cells used as controls. Cell viability was determined by more than 3 independent experiments of MTT assay and calculated as the absorbance ratio of treated to control groups. Data were presented as mean ± standard error (SE).

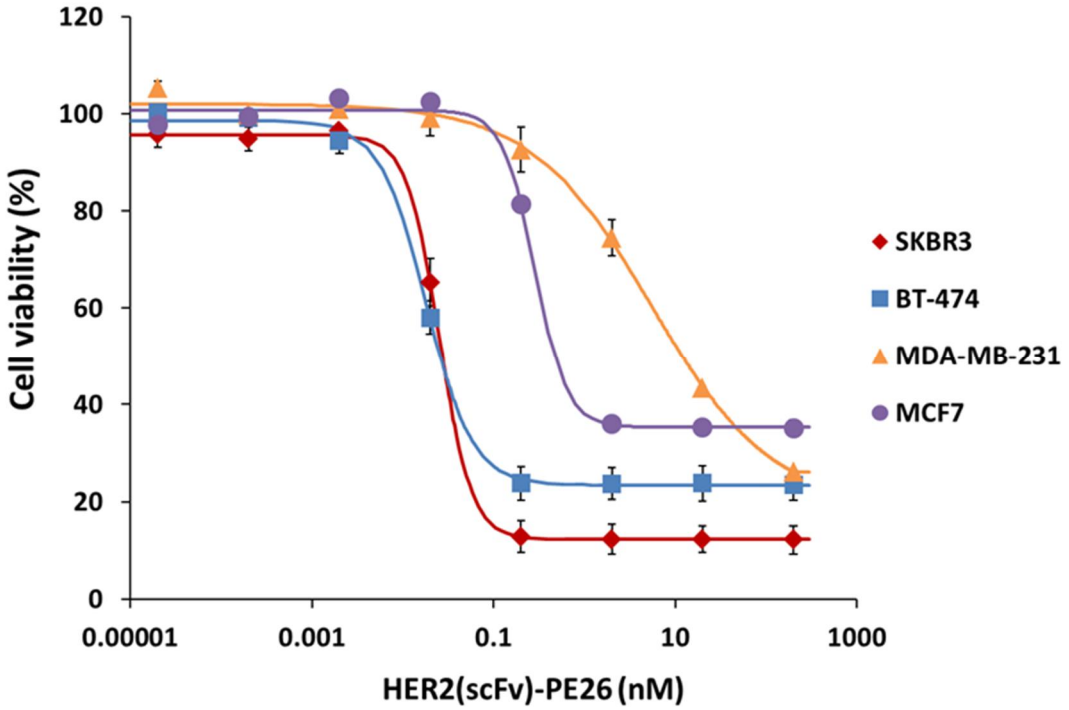

Figure 8. Comparison of cytotoxicity of HER2(scFv)-PE26 between high HER2-expressing cell lines and low HER2-expressing cell lines and IC_{50} of HER2(scFv)-PE26 to HER2-expressing cell lines. On high HER2-expressing cell lines, SKBR3 and BT-474, IC_{50} of HER2(scFv)-PE26 was calculated to 28.1 ± 2.5 pM (n = 9) and 19 ± 1.4 pM (n = 9), with the Hill coefficient of 2.24 ± 0.16 and 1.87 ± 0.17 respectively. On low HER2-expressing cell lines, MDA-MB-231 and MCF7, IC_{50} of HER2(scFv)-PE26 was calculated to 5.8 nM ± 0.28 nM (n = 3) and 0.28 nM ± 0.046 nM (n = 3) with the Hill coefficient of 0.65 ± 0.21 and 3.22 ± 1.87 respectively. Data were presented as mean ± standard error (SE).

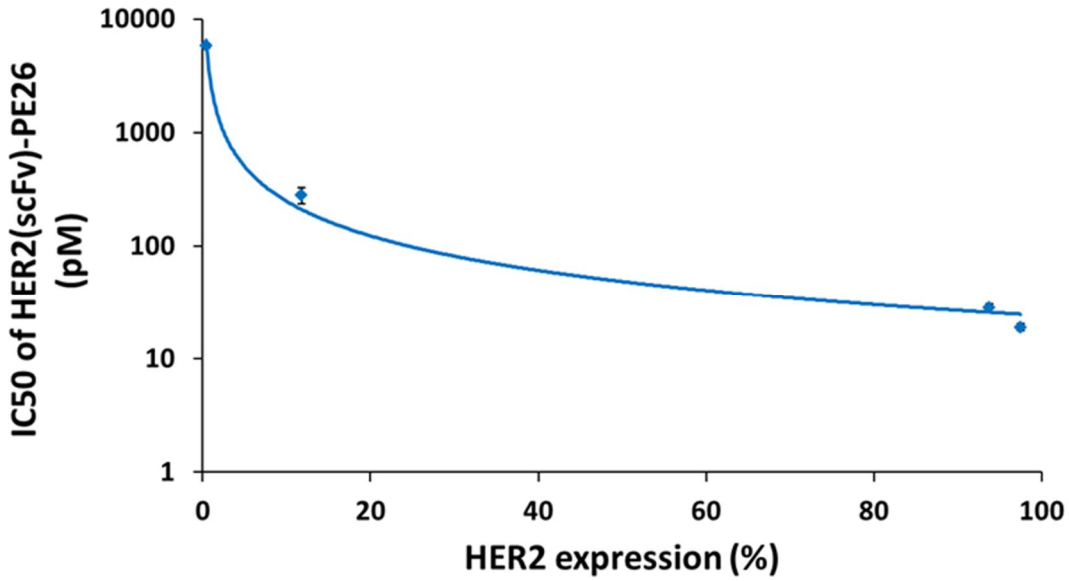

Figure 9. Correlation between HER2 expression and IC_{50} of HER2(scFv)-PE26. The IC_{50} of HER2(scFv)-PE26 and HER2 expression are associated with a strong negative correlation. As the HER2 expression higher, the IC_{50} of HER2(scFv)-PE26 tends to decrease. ($r = -0.68$, $P \leq 0.05$)

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국문요약

새로운 재조합 면역 독소 HER2(scFv)-PE26 의 HER2 발현 유방암 세포주에 대한 세포 독성 효과

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의학과

유방암은 주로 에스트로겐 신호와 인간 상피 성장 인자 수용체 2 (HER2) 신호에 의해 발병한다고 알려져 있다. 인간 상피 성장 인자 수용체 2 를 과발현하고 있는 환자들은 생존률이 유의하게 낮고, 유방 조직에서의 인간 상피 성장 인자 수용체 2 의 과발현은 악성 표현형으로 변형을 자극한다. 또한, 인간 상피 성장 인자 수용체 2 과발현 종양은 일반적인 화학 요법 치료에 내성이 있다. 트라스투주맙은 인간 상피 성장 인자 수용체 2 신호 전달을 차단하여 종양 세포의 성장을 예방한다. 트라스투주맙은 인간 상피 성장 인자 수용체 2 를 표적으로 삼는 약물로 사용되어 인간 상피 성장 인자 수용체 2 양성 조기 유방암과 전이성 유방암 치료제로 FDA 의 승인을 받았다. 슈도모나스 외독소 A (PE)는 녹농균의 박테리아 독소로, 효소 활성을 갖는 A 도메인과 세포와 결합하는 부위인 B 도메인으로 이루어져 있다. A 도메인에서 도메인 Ib (365-

404 a.a)의 마지막 4 개의 잔기 (400-404 a.a)와 도메인 III (405-613 a.a)는 ADP-리보 실 트랜스퍼라제 활성을 갖는 독소의 촉매 소단위이다. 도메인 Ib 와 도메인 III 의 촉매 효소 활성은 세포 자멸사를 일으킨다. 따라서 PE 는 항암 요법의 후보가 될 수 있다. 이 연구에서는 항 인간 상피 성장 인자 수용체 2 항체의 단일 사슬 (HER2(scFv))과 퓨린 인식 부위를 제외한 B 세포 및 T 세포 에피토프가 제거된 26 kDa 의 PE 조각(PE26)으로 구성된 새로운 재조합 면역독소 (HER2(scFv)-PE26)를 만들었다. 이 재조합 면역독소의 N- 말단에 말토스 결합 단백질 (MBP)을 융합시켜, 대장균에서의 단백질 발현 용해성을 증가시켰고, 500 mL 세포 배양으로부터 0.25 mg 의 순수한 HER2(scFv)-PE26 을 성공적으로 정제하였다. 말토스 결합 단백질 (MBP)제거 후 HER2(scFv)-PE26 은 안정하고, 순도는 91% 이상이었다. 말토스 결합 단백질이 제거된 HER2(scFv)-PE26 는 세포 증식 및 사멸도 변화 평가를 통해 인간 상피 성장 인자 수용체 2 를 과발현하는 세포주인 SKBR3 과 BT-474 에서 농도에 의존적인 세포 독성을 가지는 것으로 확인되었으며, 각각 28.1 pM ± 2.5 pM (n = 9), 19 pM ± 1.4 pM (n = 9) 의 IC_{50} 값이 측정되었다. 반면에 인간 상피 성장 인자 수용체 2 를 낮게 발현하는 세포주인 MDA-MB-231 과 MCF7 에서는 세포독성이 미미하게 나타났다.

중심단어: 인간 상피 성장 인자 수용체 2, 유방암, 면역독소, 트라스투주맙, 슈도모나스 외독소 A, 수용성 과발현과 정제