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[UCI]I804:48009-200000177101

Master of Science

Cytotoxic effects of an immunoconjugate, HER2(scFv)–PE24, on the HER2-overexpressing breast cancer cells

HER2 과발현 유방암 세포에서의
면역접합체 HER2(scFv)–PE24 의 세포독성 효과

The Graduate School
of the University of Ulsan

Department of Medicine
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Cytotoxic effects of an immunoconjugate, HER2(scFv)–PE24, on the HER2-overexpressing breast cancer cells

Supervisor Han Choe

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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December 2018

Cytotoxic effects of an immunoconjugate, HER2(scFv)–PE24, on the HER2-overexpressing breast cancer cells

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December 2018

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ADA	Anti-drug antibodies
ADCC	Antibody-dependent cellular cytotoxicity
BSA	Bovine serum albumin
CD340	Cluster of differntiation 340
CV	Column volume
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ERBB2	Erythroblastic oncogene B-2
E. coli	*Escherichia coli*
Fc	Fragment crystallizable region
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
GFP	Green fluorescence protein
GPC	Gel Permeation Chromatography
HC	Hill coefficient
HER2	Human epidermal growth factor receptor 2
IC50	50% inhibit concentration
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl 1-thio-β-d-galactopyranoside
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
RIT	Recombinant immunotoxin
RPMI 1640	Roswell Park Memorial Institute 1640 Medium
scFv	Single chain variable fragment
SE	Standard Error
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPDP	Succinimidyl 3-(2-pyridyldithio)propionate
TEV	Tobacco etch virus
TEVrs	Tobacco etch virus protease recognition site

Abstract

Cytotoxic effects of

an immunoconjugate, HER2(scFv)–PE24,

on the HER2-overexpressing breast cancer cells

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College of Medicine

Conventionally, immunotoxins have been produced as a single polypeptide from fused genes of an antibody fragment and a toxin. In this study, we adopted a unique approach of chemical conjugation of a toxin protein and an antibody fragment. The two genes were separately expressed in *Escherichia coli* and purified to high levels of purity. The two purified proteins were conjugated using a chemical linker. The advantage of this approach is its ability to overcome the problem of low expression and production of recombinant immunotoxin observed in some immunotoxins. Another advantage is that various combinations of immunotoxins can be prepared with fewer efforts, because the chemical conjugation of components is relatively simpler than the processes involved in cloning, expression, and purification of multiple immunotoxins. As a proof of concept, the scFv of trastuzumab and the PE24 fragment of *Pseudomonas* exotoxin A were separately produced using *E. coli* and then chemically crosslinked. The new immunotoxin was tested on four breast cancer cell lines variably expressing HER2. The chemically crosslinked immunotoxin exhibited cytotoxicity in proportion to the expression level of HER2. In conclusion, the present study revealed an alternative method of generating an immunotoxin

that could effectively reduce the viability of HER2-expressing breast cancer cells. These results suggest the effectiveness of this method of chemical crosslinking as a suitable alternative for producing immunotoxins.

Keywords: scFv, HER2, exotoxin, Pseudomonas, breast cancer, immunoconjugate, crosslinking, PEGylation

Introduction

Most anticancer monoclonal antibodies exhibit weak antibody-dependent cytotoxic activity. A recombinant immunotoxin is a genetically engineered antibody fragment conjoined to a protein toxin that reduces the tumor tissues [1)]. The antibody region of these molecules specifically targets tumor cell surface receptors and then internalizes toward the endocytic compartment. Toxin molecules delivered to the cytosol of the target tumor cells destroy the target cells effectively [2, 3)].

Human breast cancers are classified into subtypes depending on their gene expression patterns [4)]. The human epidermal growth factor receptor 2 (HER2) is a 185-kDa transmembrane tyrosine kinase receptor, belonging to the epidermal growth receptor (EGFR) family 2 [5, 6)]. It is also referred to as HER2/neu, ERBB2, or CD340. Breast cancers with overexpression of HER2 are called as HER2-positive and HER2-positive cancer has been observed in 20–30% of all breast tumors [5)]. The signaling pathway induced by phosphorylation of HER2 dimers promotes cell proliferation, survival, differentiation, angiogenesis, invasion and metastasis [6)]. The overexpression of HER2 induces malignant transformation in breast cancer and confers more resistant to general chemotherapeutic treatment. As a result, patients with HER2 overexpression have a significantly poor prognosis [7)].

Trastuzumab, an anti-HER2 monoclonal antibody, has been approved by FDA for the treatment of HER2-positive breast cancer [8)]. Trastuzumab blocks HER2 signal by binding to its extracellular domain. It inhibits HER2 dimer formation and triggers HER2 internalization and degradation. Also, it attracts immune cells to tumor site and the tumor cells were killed by antibody-dependent cellular cytotoxicity (ADCC) [9, 10)]. By these mechanisms of Trastuzumab, tumor growth is inhibited. But, Trastuzumab itself showed

weak cytotoxicity, so it has been conjugated with various chemical drugs to enhance cytotoxicity for active targeting against HER2-positive breast cancer cells [11].

Pseudomonas exotoxin A (PE) is a bacterial exotoxin from *Pseudomonas aeruginosa* that is expressed as a protein with 613 amino acids (a.a.), and comprises three functional domains [12]. The receptor-binding domain Ia (1–252 a.a.) is followed by the translocation domain II (253–364 a.a.). 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 [13]. The catalytic enzyme activity of domain Ib and domain III ADP-ribosylates the elongation factor of the host ribosome, causing apoptotic cell death [14]. The 40-, 38-, or 24-kDa portions of the PE without the cell binding domain, designated as PE40, PE38, and PE24, respectively, was fused to the antibody fragment that targets the cancer cells [15].

Antibody fragments have advantages, including higher mobility and tissue penetration, ability to bind antigen monovalently and reduced immunogenicity. But, it associated with their short half-life *in vivo,* because it doesn't have the Fc region [16]. The one of solution to increase half-life is chemical conjugation of polyethylene glycol (PEG).

Polyethylene glycol (PEG) is biocompatible material and has several pharmacological advantages [17]. PEGylation, the process of chemical conjugation of PEG to drug and biomedical molecule, improves drug solubility and stability and reduces toxicity and immunogenicity [18, 19]. It also reduces renal clearance by increasing hydrodynamic size of drug and prevents proteolytic degradation.[17, 20] It has been used for extending *in vivo* half-life of many therapeutic biomedical molecules [21].

In this study, we adopted a unique approach of chemical conjugation between an antibody fragment and a toxin instead of the traditional immunotoxins that are recombinant fusion proteins of the two proteins. An advantage of this approach is that it can overcome the problem of low recombinant immunotoxin production that is observed in some immunotoxins. As a proof of concept, the scFv of trastuzumab and the PE24 protein were

produced separately using *E. coli* and then chemically crosslinked. The new immunotoxin was tested on the breast cancer cell lines that express HER2. And to improve *in vivo* retention time of HER2–PE24 conjugate, PEGylation was performed.

Materials and Methods

1. Materials

Sulfosuccinimidyl 6-[3'(2-pyridyldithio)-propionamido] hexanoate (sulfo-LC-SPDP) was acquired from CovaChem (Loves Park, IL, USA) and tris(2-carboxyethyl)phosphine (TCEP) was obtained from Thermo Fisher Scientific Korea (Seoul, Korea). Ampicillin was purchased from Duchefa Biochemie (Haarlem, Netherlands) and kanamycin sulfate was obtained from Biosesang (Seungnam, Korea). NaCl, glycerol, sodium cyanoborohydride and sodium phosphate dibasic were acquired from Samchun Chemical (Pyongtaek, Korea). PEG-aldehyde-20K was purchased from Nanocs (New York. USA). Overlap cloner DNA cloning kit, lambda integrase and excisionase were acquired from Elpis Biotech (Daejeon, Korea). Dithiothreitol (DTT) and 1-thio-β-d-galactopyranoside (IPTG) were acquired from Anaspec (Fremont, CA, USA). The 0.45 μm pore size filter was purchased from Hyundai Micron (Seoul, Korea). All columns for purification were purchased from GE Healthcare Korea (Seoul, Korea). Coomassie brilliant blue R-250 and Tris-HCl were from Amresco (Solon, OH, USA). Imidazole was obtained from Daejung Chemicals (Siheung, Korea). Dialysis membranes were purchased from Viskase (Darien, IL, USA), and Amicon Ultra was purchased from Merck Millipore (Billerica, MA, USA). Acrodisc syringe filters were acquired from Pall Korea (Seoul, Korea). *E. coli* BL21(DE3) cell was acquired from Novagen (Madison, WI, USA). 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium (MTT) was obtained from Sigma-Aldrich Korea (Yongin, Korea). SKBR3, BT474, MDA-MB-231 and MCF-7 cell lines were acquired from Korea Cell Line Bank (Seoul, Korea). RPMI-1640 Medium, 0.25% trypsin-EDTA, fetal bovine serum (FBS), and penicillin–streptomycin were acquired from Thermo Fisher Scientific Korea (Seoul, Korea). All chemicals were analytical grade.

2. Construction of expression vector

An overlap cloning was performed to make the entry clone for anti-HER2(scFv). Genes encoding V_H and V_L of trastuzumab were synthesized by Bioneer (Daejeon, Korea). The V_H and V_L regions of trastuzumab were amplified using primers: 5'-GGCTTCGAAAA CCTGTATTTTCAGGGCGAAGTACAATTGGTTGAAAGCGGGGGTGG and 5'-CCCG AGCCACCGCCACCTGAGCCACCGCCACCGGAGCTTACAGTTACCAGA GTACCCT GG for V_H, 5'-GCTCAGGTGGCGGTGGCTCGGGTGGCGGTGGCTCAGATATTCAAA TGACACAAAGCCCCTCTAG and 5'-GCCTTTGTACAAGAAAGCTGGGTTTAGCATT TAATTTCAACCTTCGTTCCCTGACCAAATG for V_L. Primers were designed for PCR products to have homologous sequence at both ends. attL1 and attL2 sites at each end of V_H and V_L were added, and scFv was formed via the linker $(G_4S)_4$ between V_H and V_L. In additional, the tobacco etch virus protease recognition site (TEVrs) at the N-terminus of HER2(scFv) and Cys residue at the C-terminus were inserted for tag removal and conjugation reaction, respectively. After overlap cloning with amplified donor vector (pDONR207), entry clone was formed. MBP–HER2(scFv) expression vector was created by multisite gateway cloning (LR reaction) with the destination vector (pDEST-HMGWA) and entry clone. In case of PE24, the PE24 sequence [27] was codon-optimized for *E. coli* expression and synthesized. The gene was amplified by PCR with the primers designed to include attB1 and attB2 sites at both ends of PE24: 5'-GGGGACAAGTTTGTACAAAAAA GCAGGCTTCGAGAATCTGTATTTCCAAGG and 5'-GGGGACCACTTTGTACAAGAA AGCTGGGTTTACTTCAGATCCTCACG. Then, the PCR product was inserted to the vector containing attP1 and attP2 sites by multisite gateway cloning (BP reaction). With the newly formed entry clone (pENTR-PE24) and destination vector (pDest-His8), LR reaction was performed. As a result, the expression vector for His8–PE24 was created. Correct constructs were confirmed by sequencing analysis (Macrogen, Daejeon, Korea).

3. Expression and solubility analysis of recombinant fusion protein in *E. coli,* BL21

The transformation of expression plasmid into *E.coli*, BL21 strain was performed by the heat shock method. Single colony was inoculated in the Luria–Bertani (LB) medium containing 50 μg/mL ampicillin or kanamycin and incubated overnight at 37°C. The cultured cells were diluted into 4 mL of fresh LB medium at a ratio of 1:50, and the cells were grown at 37°C. When the value of OD_{600} reached 0.6–0.7, 0.5 mM IPTG was added and cells were inducted at 37°C for 3 h or 18°C overnight. Cells were harvested and resuspended with sonication buffer containing 20 mM Tris, pH 8.0, 1 mM EDTA, and 20% glycerol (v/v). Resuspended cells were sonicated by ultrasonic cell disruptor. To obtain soluble fraction and pellet fraction, sonicated cell was centrifuge at 23,000 g for 10 min. The fusion protein expression and solubility was analyzed with SDS-PAGE and assessed by an ImageJ image analyzer (http://imagej.nih.gov.ij)

4. Purification of HER2(scFv)-Cys and PE24

Expression plasmids were transformed into *E. coli* BL21 strain. For 500 mL of culture, one colony was inoculated in 5 mL of LB with 50 μg/mL of ampicillin or kanamycin and grown at 37°C overnight. The cultured cells were diluted with 500 mL of LB containing ampicillin at a 1:100 ratio and grown at 37°C until OD_{600} = 0.6–0.7. At this stage, 0.5 mM IPTG was added to the culture and incubated at 18°C overnight to induce protein expression. Cultured and induced cells were harvested by centrifugation at 3,800 g for 20 min at 4°C. After centrifugation, some medium was removed and pellets were resuspended with the remaining medium. Resuspended pellets were collected to 50 mL falcon tube and centrifuged at 3,800 g for 30 min at 4°C. After centrifuge, supernatant was removed and cell pellets were stored at –20°C until used. For the purification of fusion protein, MBP–HER2(scFv) or His8–PE24, cell pellets were resuspended with 100 mL of buffer A containing 20 mM Tris, pH 8.0, 500 mM NaCl and 5% glycerol(v/v). Suspended pellets

were sonicated and homogenized by ultrasonic cell disruptor JY99-IIDN. The cell lysate was centrifuged at 23,000 g for 20 min at 4°C. After centrifugation, the supernatant was filtered with 0.45 μm filter and was applied to 10 mL HiTrap Ni HP that was already equilibrated with buffer A. For purification of MBP–HER2(scFv), after applying the cell lysate, 30 mM imidazole was allowed to flow in the column for 5 CV to wash off unbounded proteins. When 100 mM imidazole had flowed in the column for 3–4 CV, MBP–HER2 fusion protein was eluted and collected. In case of his8–PE24, unbounded proteins were washed by 50 mM imidazole and fusion protein was eluted at 500 mM imidazole. TEV protease was treated to remove the MBP tag of MBP–HER2(scFv) (fusion protein:TEV = 5:1, w/w) or His8 tag of His8–PE24 (fusion protein:TEV = 20:1, w/w). Tag cleavage by TEV protease was performed at 18°C overnight. The TEV-treated mixture was dialyzed against buffer A and applied to 10 mL HiTrap Ni HP that was equilibrated with the same buffer. The tag-free HER2(scFv) or PE24 did not bind to the column and was eluted from flow through (FT). The His-MBP tag or His8 tag and His7-TEV protease were eluted at 500 mM imidazole. The purified HER2(scFv) or PE24 was concentrated with Amicon Ultra from Merck Millipore by centrifuging at 3,800 g and dialyzed against PBS at pH 7.4. Protein concentrations were measured by the Bradford assay using BSA as a standard.

5. Crosslinking of HER2(scFv) and PE24

In this process, 12.5 μL of 20 mM sulfo-LC-SPDP was added to 1 mg of PE24 in PBS (pH 7.4) and 1 mM EDTA. The reaction mixture was incubated at room temperature for 40 min and dialyzed against PBS (pH 7.4) and 5 mM EDTA to remove excess unreacted SPDP reagent. Then, 1 mg/mL of HER2(scFv)-Cys was reduced by 10 mM TCEP to form monomer and was incubated at room temperature for 40 min. Unreacted TCEP was removed by dialysis against PBS (pH 7.4) and 5 mM EDTA. SPDP-modified PE24 was added to

reduced HER2(scFv) at a 5:1 molar ratio (PE24:HER2(scFv)) and incubated at room temperature for 30 min or at 4°C overnight.

6. Purification of GFP and HER2(scFv)–GFP conjugate.

pET28a-sfGFP plasmid was purchased from Addgene (Massachusetts, USA). At the C-terminal of Superfolder GFP (sfGFP), His6-tag was included. sfGFP was purified with IMAC chromatography as mentioned in the purification of MBP–HER2(scFv) fusion protein. HER2(scFv) and GFP were conjugated and purified as described in crosslinking of HER2(scFv) and PE24.

7. Flow Cytometric Analysis

SKBR-3, BT-474, MDA-MB-231, and MCF-7 cells were trypsinized and 2×10^6 cells were centrifuged and resuspended in 1 mL PBS. Then, 5 μg of HER2(scFv)–GFP conjugate was added to each tube and incubated at 4°C for 25 min. The cells were washed with PBS three times, and 1 μg DAPI was added to each tube and incubated at 4°C for 10 min. After washing three times, the cells were analyzed by FACS Canto II flow cytometer (BD Biosciences, San Diego, CA). FlowJo_V10 (FlowJo LLC, Ashland, OR) was used to analyze FACS data.

8. *In vitro* cytotoxicity assay

SKBR-3, BT-474, MDA-MB-231, and MCF-7 cells were grown in RPMI 1640 medium supplemented with 10% FBS. The cells were seeded into 24-well plate at a density of 0.5-1 $\times 10^5$ cells per well. After culture for 24 h, HER2(scFv)–PE24 conjugate, HER2(scFv) or PE24 was treated to seeded cell at different concentration (0.002, 0.02, 0.2, 2, 20 and 200 nM) in triplicate. At 72 h of incubation with the protein, the media was removed, and cells were washed with PBS. Then, 0.5 mL of the MTT solution (0.04 mg/mL in serum-

free medium) was added into the wells, and the plate was incubated for 2 hr. Then, the MTT solution was removed, and 0.5 mL of DMSO was added. The plate was incubated for 1 h and absorbance at 595 nm was measured. Cell viability was calculated using the following equation and Microsoft Excel :

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

Where V means cell viability, top is the highest value of cell viability, bottom is the lowest value of cell viability, conc. is the treated protein concentration and HC is the Hill coefficient of inhibition.

9. PEGylation of HER2(scFv)

For PEGylation at N-terminal of HER2(scFv), 1-1.5 mg/mL HER2(scFv)-Cys was reduced by 10 mM TCEP and dialyzed against 100 mM sodium phosphate, pH6.0. Aldehyde–PEG–20K was added to HER2(scFv) at a 10:1 molar ratio (PEG : HER2(scFv)) with 20 mM sodium cyanoborohydride. This mixture was incubated at 18 degree for O/N. The PEGylated HER2(scFv) was purified with gel filtration chromatography using Hiload 16/600 Superdex 75 in PBS, pH 7.4 with 5 mM EDTA.

10. Crosslinking and purification of PEGylated HER2(scFv)–PE24 conjugate.

Purified PEGylated HER2 was added to SPDP-modified PE24 at a 5:1 molar ratio (PE24: PEGylated HER2(scFv)) and incubated at room temperature for 30 min. And then, this mixture was dialyzed against the buffer containing 20 mM Tris-HCl, 0.5 mM EDTA, pH 7.0. Dialyzed reaction mixture was applied to a 5 mL cation exchange column, HiTrap SP HP that was equilibrated with the buffer same with dialyzed buffer. To elute the proteins that bind to the column, concentration of NaCl was increased from 0 M to 1 M with linear gradient for 10 column volume (CV). PEG–HER2(scFv) conjugate was eluted at 100-200 mM NaCl and was dialyzed against PBS (pH 7.4)

11. *In vitro* cytotoxicity assay of PEGylated HER2(scFv)–PE24

BT-474 and MCF-7 cells were grown in RPMI 1640 medium supplemented with 10% FBS. The cells were seeded into 24-well plate at a density of 0.5-1 × 10^5 cells per well. After culture for 24 h, PEGylated HER2(scFv)–PE24 conjugate was treated to seeded cells at different concentration (0.001, 0.01, 0.1 and 1 nM) in triplicate. At 72 h of incubation, MTT assay was performed as described at *in vitro* cytotoxicity assay of HER2(scFv)–PE24.

12. Statistics

Data are presented as mean ± standard error (SE) of n≥3 samples. The experiments were performed independently in triplicate.

Results

1. Cloning the constructs

For creating the MBP–HER2(scFv) expression vector, an overlap cloning and a gateway cloning method were used. To fuse three PCR products (i.e., V_H, V_L, and donor vector [pDONR207]) and create pENTR-HER2(scFv), an overlap cloning method was used. The primers were designed for PCR products to have a homologous sequence at both the ends. By overlap cloning, the TEV cleavage site was added at the N-terminal of HER2(scFv), and cysteine residue was added at the C-terminal for crosslinking reaction. A linker was inserted between V_H and V_L. The attL1 or attL2 site was added at each terminal for the next cloning step. The expression vector for MBP–HER2(scFv) was obtained using the LR reaction of the gateway cloning method with pENTR–HER2(scFv) and pDEST–HMGWA with MBP tag (Figure 1A, B). For making the PE24 expression vector, a multisite gateway cloning method was used. PE24-encoding gene was amplified by PCR. The attB1 and TEVrs sequence at the N-terminal and attB5 at the C-terminal of PE24 were added. attB site-flanked PE24 was inserted to the donor vector (pDONR221) by BP reaction and pENTR–PE24 was formed. The expression vector for His8–PE24 was created by LR reaction with His8 tag containing pDEST–His8 and pENTR–PE24 (Figure 2A, B).

2. Expression and solubility analysis of HER2(scFv) and PE24

The expression vector for MBP–HER2(scFv) or His8–PE24 was transformed to *E. coli,* BL21. The protein expression and solubility level were determined at different induction temperatures of 37°C or 18°C.

E. coli was grown at 37°C until $O.D_{600}$ = 0.6–0.7. When the O.D value reached the optical value, 0.5 mM IPTG was added and the protein expression was induced at 37°C for 3 h or 18°C for overnight. Then, the cells were sonicated. The total cell fraction, pellet, and soluble fraction were analyzed using SDS-PAGE (Figure 3). MBP–HER2(scFv) and His8–PE24

fusion proteins were expressed at both the temperatures. However, when the proteins were induced at 18°C, protein solubility was increased as compared with that at 37°C (Table 1).

3. Purification of HER2(scFv) and PE24

The *E. coli* cells expressing MBP–HER2(scFv) were sonicated, and the soluble fraction of the cell lysate was applied to the HiTrap FF immobilized metal affinity chromatography (IMAC) column. The MBP–HER2(scFv) fusion protein was eluted at 100 mM imidazole, and TEV protease was added to the elution containing MBP–HER2(scFv) at a ratio of 5:1 (fusion:TEV). After the MBP tag cleavage, HER2(scFv) was purified by the 2nd HiTrap FF IMAC column. The tag-free HER2(scFv) was collected from the flow through (FT) fraction, and the purified HER2(scFv) was dialyzed against phosphate-buffered saline (PBS) (Figure 4B). The yield of the final product was 31% (Table 2). PE24 also was purified by IMAC chromatography. However, the His8–PE24 fusion protein was eluted at 500 mM imidazole. TEV protease was treated to the eluted His8–PE24 at a ratio of 20:1 with 1 mM DTT addition. After the TEV protease digestion, PE24 was purified from the FT fraction of the 2nd IMAC column in the same manner as HER2(scFv) (Figure 4C). After dialysis against PBS, the final yield was 39% (Table 3).

4. Chemical conjugation of HER2(scFv) and PE24

To generate an anti-HER2 immunoconjugate, anti-HER2(scFv) and PE24 were chemically conjugated via N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), a disulfide bond-containing linker. The amino groups of PE24 were modified with SPDP (Figure 5B, lane 1), and HER2(scFv)-Cys was reduced by TECP to make the sulfhydryl group available for conjugation (Figure 5B, lane 2). After a reaction at a ratio of 5:1 (PE24:HER2(scFv)), the HER2(scFv)–PE24 conjugate was formed (Figure 5B, lane 3). Then, this conjugate was purified by size exclusion chromatography using the Hiload 16/600 Superdex 75 pg (Figure

5B, lane 4). The purity of the purified HER2(scFv)–PE24 conjugate was verified by SDS-PAGE under non-reducing condition (10% Tricine gel). The highest yield and purity of the HER2(scFv)–PE24 conjugate was 58% and 93%, respectively (Table 4).

5. HER2 expression of breast cancer cells

To evaluate the binding capacity of the HER2(scFv)–PE24 conjugate, the HER2(scFv)-GFP conjugate was prepared and flow cytometry analysis was performed. After incubation with HER2(scFv)–GFP, a shift of the fluorescence histogram to the right was observed in the HER2-overexpressing cell lines, SKBR3 and BT-474 (Figure 6A, B). On the contrary, the fluorescence histogram shifted slightly in HER2 low-expressing cells (MDA-MB-231 and MCF 7) as compared with that in HER2-overexpressing cells (Figure 6C, D). From the fluorescence-activated cell sorting (FACS) analysis data, we determined that the HER2(scFv)–PE24 conjugate strongly binds to HER2-expressing cells.

6. Cytotoxicity of HER2(scFv)–PE24 conjugate *in vitro*

To determine the cytotoxicity of the HER2(scFv)–PE24 conjugate, HER2-overexpressing and low-expressing cells were treated with HER2(scFv)–PE24, HER2(scFv), and PE24 at various concentrations. At 72 h of treatment, an MTT assay was performed to measure the cell viability. The HER2(scFv)–PE24 conjugate revealed high toxicity in HER2-overexpressing cell lines (SKBR-3 and BT-474) at the picomolar level. The IC_{50} value of SKBR-3 and BT-474 is 43 pM ± 8 (n = 9) and 6.7 pM ± 3 pM (n = 9), respectively (Figure 7A, B). In contrast, the HER2 low-expressing cell lines (MDA-MB-231 and MCF-7) were 20- to 1.4 × 10^3-fold less affected by the HER2(scFv)–PE24 conjugate. The obtained IC_{50} values of MDA-MB-231 and MCF-7 were 9.44 nM ± 3 nM (n = 9) and 1.01 nM ± 0.38 nM (n = 9), respectively (Figure 7C, D). These data indicate that the cytotoxicity in cells is correlated with the HER2 expression on the cell surface. Treatment with HER2(scFv) alone

did not inhibit the proliferation. Meanwhile, PE24 alone reduced cell viability at high concentration, except for the SKBR-3 cell line.

7. PEGylation of HER2(scFv)–PE24 conjugate

To increase the half-life of HER2(scFv)-PE24 conjugate in vivo, N-terminal specific PEGylation was performed with PEG-aldehyde. After incubation with PEG aldehyde for overnight, some HER2(scFv) was conjugated with PEG at pH 6.0. The PEGylated HER2(scFv) was purified by size exclusion chromatography. After adding SPDP-modified PE24 to PEGylated HER2, formation of PEGylated HER2(scFv)–PE24 was verified by SDS-PAGE under non-reducing condition. More than half of PEGylated HER2(scFv) was conjugated with PE24 via SPDP linker (Figure 8B, lane 2). And then, formed PEGylated HER2(scFv)–PE24 was purified using cation exchange chromatography (Figure 8B, lane 3).

8. Cytotoxicity of PEGylated HER2(scFv)–PE24 conjugate *in vitro*

MTT assay was performed to confirm the cytotoxic effect of HER2(scFv)–PE24 remains unchanged after PEGylation. At 72 h incubation with PEGylated HER2(scFv)–PE24, In HER2-overexpressing cell line, BT-474, cell viability was decreased as the concentration increased. But, cytotoxicity activity of PEGylated conjugate decreased. At picomolar level, it did not affect to cell viability, compared to un-PEGylated form (Figure. 9).

Discussion

In this study, an antibody fragment and a toxin were produced separately from *E. coli,* and the two proteins were chemically conjugated using a chemical linker. An advantage of this approach is that it could overcome the low recombinant immunotoxin production problem observed in some immunotoxins. Another advantage is that various combinations of immunotoxins can be made with fewer efforts, because the chemical conjugation of the two components is simple. This was attempted previously for anti-CTLA-4 scFv and saporin [22], but has not been explored further.

Inside the cancer cell, the traditional recombinant immunotoxin is digested by the intracellular protease, furin, giving rise to dissociated antibody fragment and the toxin that interferes with intracellular function [23]. Since our immunotoxin is in a chemically conjugated form, furin cannot be applied. Instead, the chemical crosslinker in our experiment creates a disulfide-containing linkage between the HER2(scFv) and PE24 such that the link would be cleaved inside the cell because of the reducing intracellular environment.

Four types of breast cancer cell lines were used to test the cytotoxicity of the chemically conjugated HER2(scFv)–PE24. SKBR-3 and BT-474 expressed HER2 higher than MDA-MB231 and MCF-7 did, as per the FACS analysis using HER2(scFv)–GFP (Figure 6). As expected, the cytotoxicity of the chemically conjugated immunotoxin was higher in SKBR-3 and BT-474 with IC_{50} of the picomolar range, whereas the other two cell lines showed IC_{50} of the nanomolar range (Figure 7). This cytotoxicity of the chemically conjugated HER2(scFv)–PE24 on the four breast cancer cells was comparable to that of the conventional HER2(scFv)–PE24 (unpublished result), demonstrating the feasibility of the chemically conjugated immunotoxin. The efficacy of immunotoxins can be augmented by a hundred- or thousand-fold, but more than a million fold by endosomal escape enhancers in exceptional cases, such as lysosomotropic amines, carboxylic ionophores, calcium channel antagonists, various cell-penetrating peptides, other organic molecules, and light-induced

techniques [24]. Our immunotoxin already demonstrated IC_{50} of picomolar range, and it remains to be decided how much these endosomal escape enhancers could increase the efficacy.

In patients with solid tumors that have normal immune systems, PE is highly immunogenic because it is a bacterial protein. Anti-drug antibodies (ADA) were detected in all mesothelioma patients who were treated with recombinant immunotoxins (RIT) that contained PE [25]. The ADA neutralized the RIT and prevented further treatment. Because immunocompromised hematological patients do not show a strong ADA response, a combination therapy of a RIT with an aggressive immunosuppression regimen was evaluated in patients with advanced chemo-resistant mesothelioma. ADA formation was delayed so that more cycles could be provided, and 40% of the patients showed dramatic tumor responses that substantially increased survival [26]. This result demonstrated that RITs could induce major regressions in mesothelioma once immunogenicity was resolved and highlights the need to control immunogenicity to make therapy more effective.

PE38 was usually used to create recombinant immunotoxin [27]. However, there were limitations for PE-based immunotoxins due to the nonspecific toxicity and strong immunogenicity of PE38 [28]. To reduce immunogenicity, most of domain II (253–364 a.a.) except for furin cleavage site was removed and B-cell and T-cell epitopes were eliminated by point mutations. This truncated form, PE24, significantly lower immunogenicity and nonspecific toxicity of PE [29]. In addition, six amino acids of PE were identified as the B-cell epitope [30, 31]. Six other amino acids of PE were mutated to decrease the immunogenicity of T-cell response [29, 32]. Two amino acid positions were overlapped so that 10 amino acids were mutated for dual B- and T-cell de-immunization [33]. Our PE24 was derived from 8 amino acid mutations to remove the B-cell epitope [34]. Obviously, our PE toxin requires improvements in de-immunization.

Despite immunotoxins showing encouraging effects in clinical or preclinical animal

trials, its short *in vivo* half-life hampers its therapeutic efficacy [35]. There are several strategies to improve the pharmacokinetic properties of protein-based therapeutics, such as chemical modification with polyethylene glycol (PEGylation) [36] or fatty acid, recombinant fusion with human serum albumin [37], albumin binding domain, or the Fc domain of the immunoglobulin [38]. We tried PEGylation and successfully PEGylated HER2(scFv)-PE24 and purified PEGylated form. However, the N-terminal specific PEGylation of scFv virtually eliminates in vitro cytotoxic activity. Because scFv usually has target binding site at N-terminal and PEGylation affects binding capabilities and reduce bioactivity [39, 40]. So, other approaches to improve *in vivo* half-life need to be considered. For sustained release, protein or peptide drugs have been encaptured in the poly lactic-co-glycolic acid (PLGA) microsphere [41], phospholipid gel [42], or PEG gel [43]. PLGA or PEG has been approved by the US FDA so that these methods may be applied to immunotoxins as well, in order to increase their *in vivo* half-lives.

In conclusion, the present study represents another method of generating an immunotoxin. HER2(scFv) and PE24 were produced separately with high purities from *E. coli*. Then, the two proteins were chemically crosslinked. This immunotoxin effectively reduced the viability of HER2-expressing breast cancer cells. Our results suggest that this method of immunotoxin crosslinking is a good alternative to produce immunotoxins.

Tables

Table 1. Expression and solubility level of MBP–HER2(scFv) and His8–PE24

Protein	Expression level (%)		Solubility (%)	
	37°C	18°C	37°C	18°C
HER2(scFv)	38.1	34.5	64.3	70.1
PE24	53.9	33.3	57.4	91.2

Table 2. Production yields of HER2(scFv)

Purification step	Total protein (mg)	Purity (%)	HER2(scFv) (mg)	Yield (%)
Bacterial culture (500 mL)	1400 (pellet)			
Supernatant	107.6	40	15.89	100
First IMAC*	21.15	79	6.2	39.02
Second IMAC	4.99	98.01	4.89	30.77

*IMAC, immobilized metal affinity chromatography

Table 3. Production yields of PE24

Purification step	Total protein (mg)	Purity (%)	PE24 (mg)	Yield (%)
Bacterial culture (500 mL)	1100 (pellet)			
Supernatant	91.4	31.46	24.78	100
1st IMAC	23.76	97.06	19.88	80.23
2nd IMAC	9.8	99.8	9.78	39.46

Table 4. Production yields of the HER2(scFv)–PE24 conjugate.

SPDP-PE24 (mg)	TCEP-treated HER2(scFv) (mg)	HER2(scFv)-PE24 conjugate (mg)	Yield* (%)
1.2	0.24	0.12	25
2.5	0.45	0.34	37
2.84	0.56	0.65	58

*Yield (%) = mole of conjugate / mole of HER2(scFv)×100 (%)

Figures

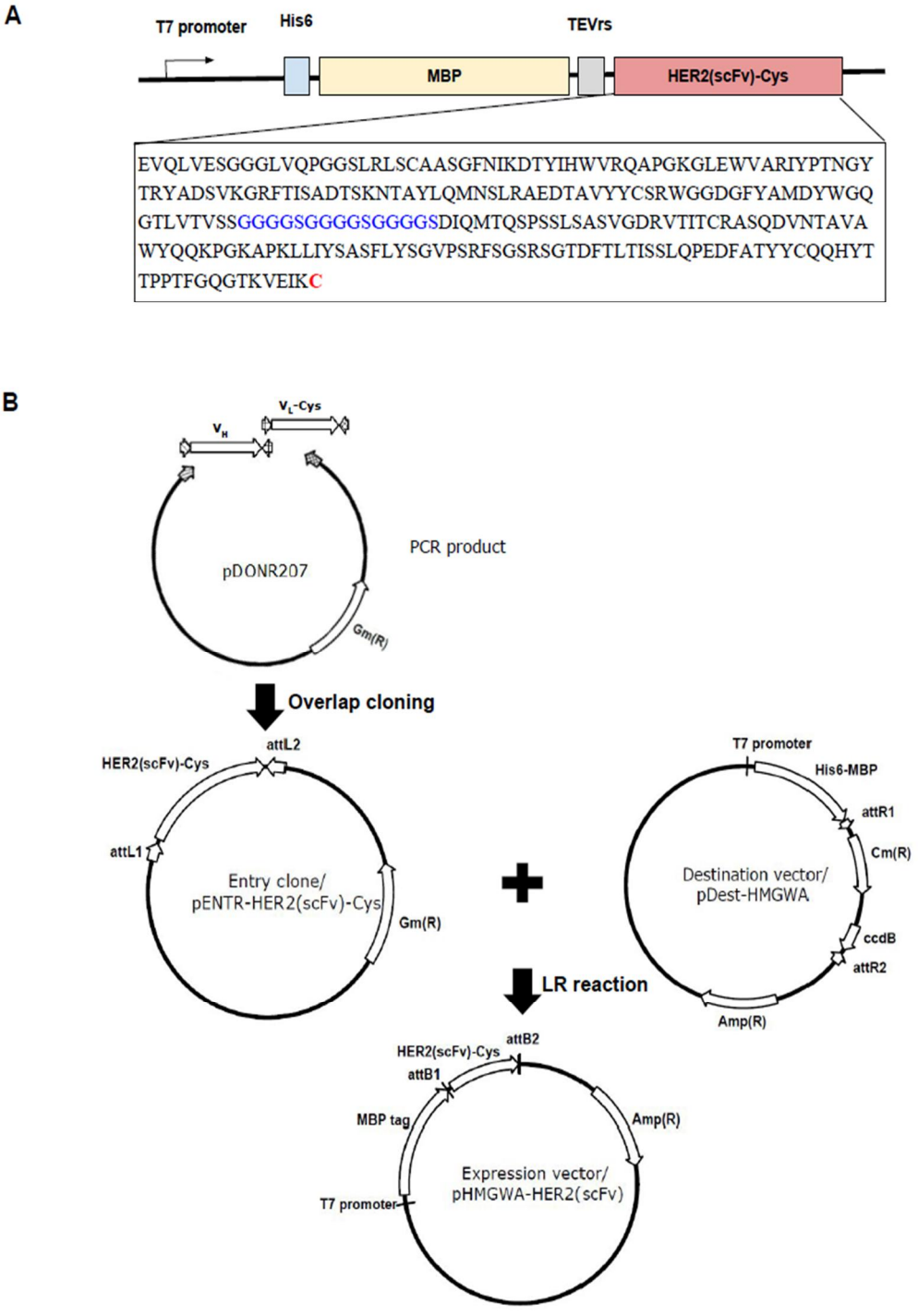

Figure 1. Construct design and cloning strategy of the MBP–HER2(scFv) expression vector.

(A) Designed constructs of MBP–anti-HER2(scFv). Cysteine residue was added at the C-terminal of anti-HER2(scFv) for crosslinking reaction. The TEV protease cleavage site was included at the N-terminal of HER2(scFv) for tag removal. (B) MBP–HER2(scFv) expression vector was created by an overlap cloning and a gateway cloning method.

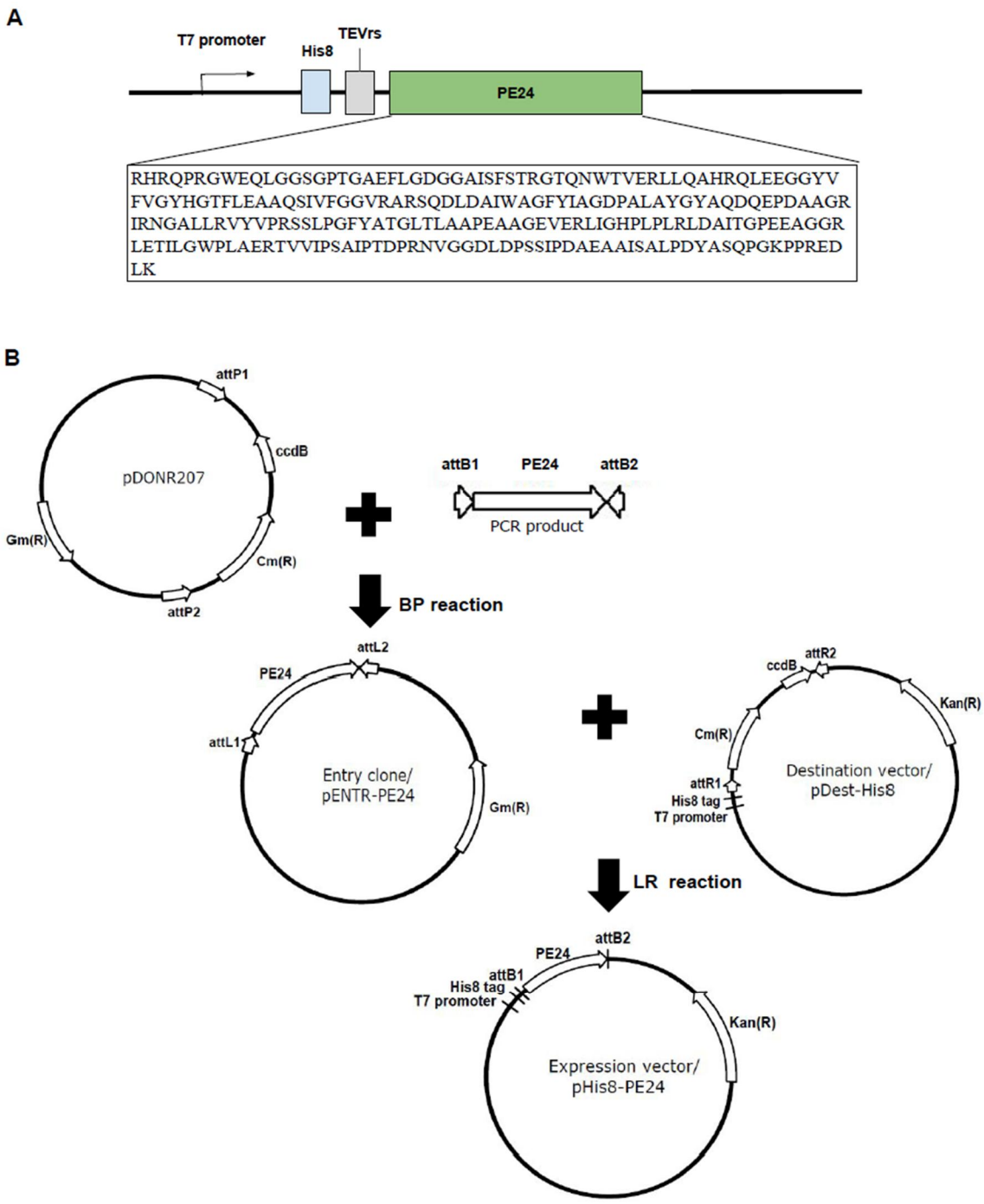

Figure 2. Construct design and cloning strategy of the His8–PE24 expression vector.

(A) Designed constructs of His8–PE24. The TEV protease cleavage site was included at the N-terminal of PE24 for tag removal. (B) The His8–PE24 expression vector was created by the gateway cloning method.

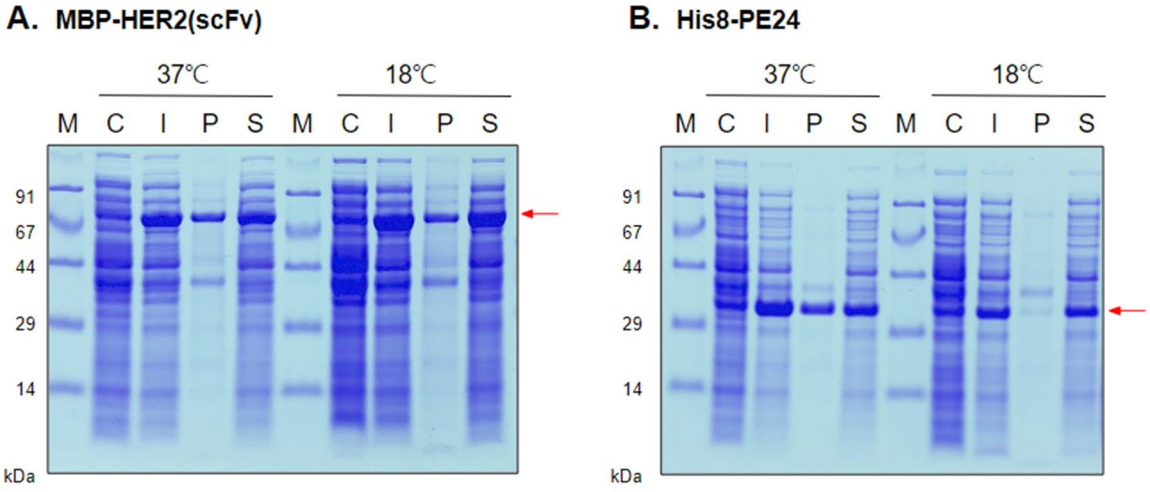

Figure 3. Expression and solubility analysis of MBP–HER2(scFv) and His8–PE24 in *E.coli*, BL21 strain.

Expressions of (A) MBP–HER2(scFv) and (B) His8–PE24 were induced at different induction temperatures of 18°C or 37°C. The arrows indicate MBP–HER2(scFv) (70 kDa) or His8–PE24 (29 kDa). M, molecular weight marker; C, IPTG not added-total protein as negative control; I, total cell fraction after induction; P, Pellet fraction after induction; S, soluble supernatant after induction.

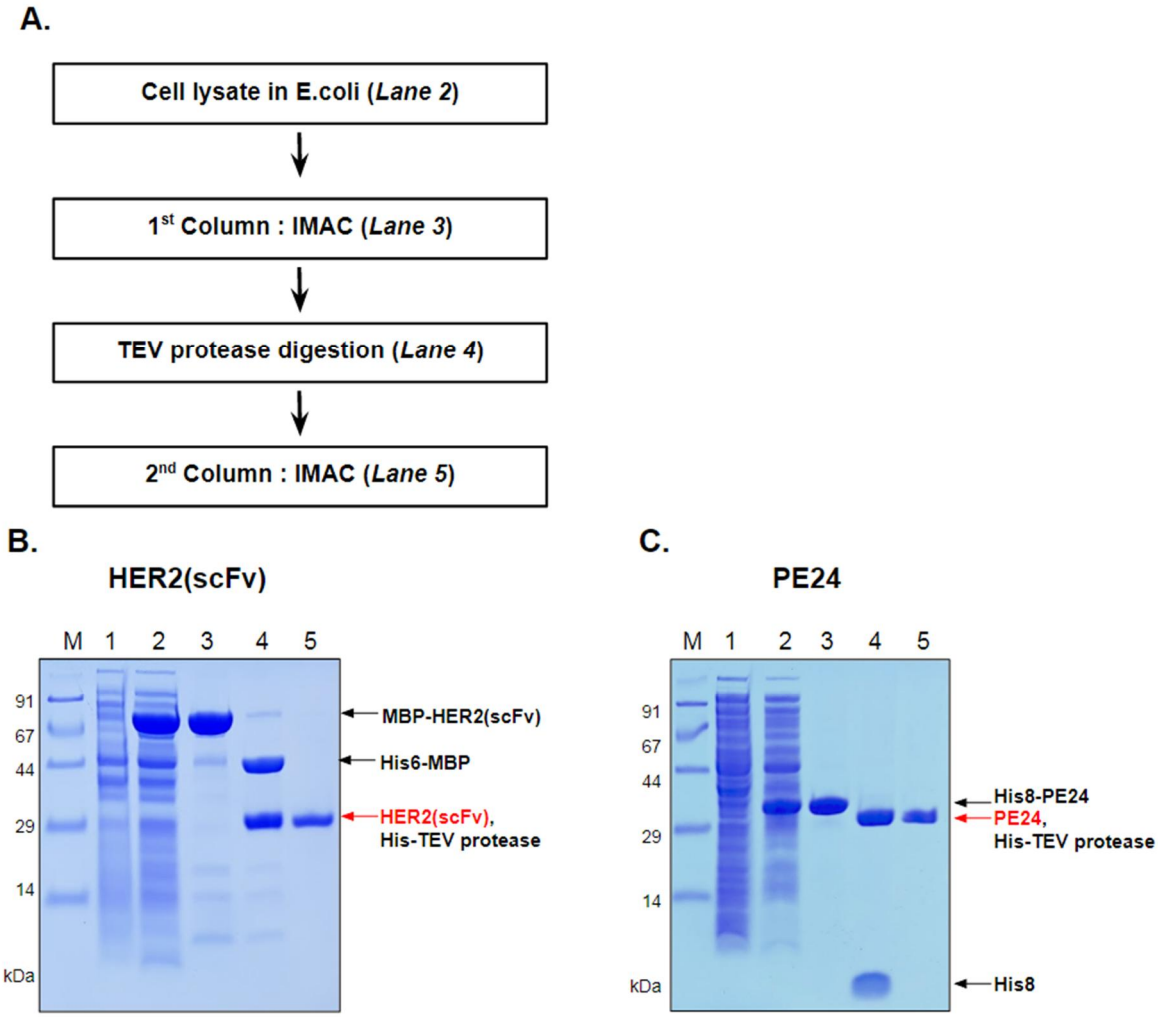

Figure 4. Purification of HER2(scFv) and PE24.

(A) Flowchart of the purification. (B) SDS-PAGE analysis of HER2(scFv) purification. M, molecular weight size marker; lane 1, total cell proteins before induction; lane 2, soluble fraction after induction; lane 3, MBP–HER2(scFv) (70 kDa) purified by the first IMAC; lane 4: MBP tag (44 kDa) cleavage by TEV protease (29 kDa); lane 5, HER2(scFv) (26 kDa) purified by the second IMAC. (C) SDS-PAGE analysis of PE24 purification. Lane 1, total cell proteins before induction; lane 2, soluble fraction after induction; lane 3, His8–PE24 (29 kDa) purified by the first IMAC; lane 4: His8 tag (4 kDa) cleavage by TEV protease (29 kDa) ; lane 5, PE24 (25 kDa) purified by the second IMAC.

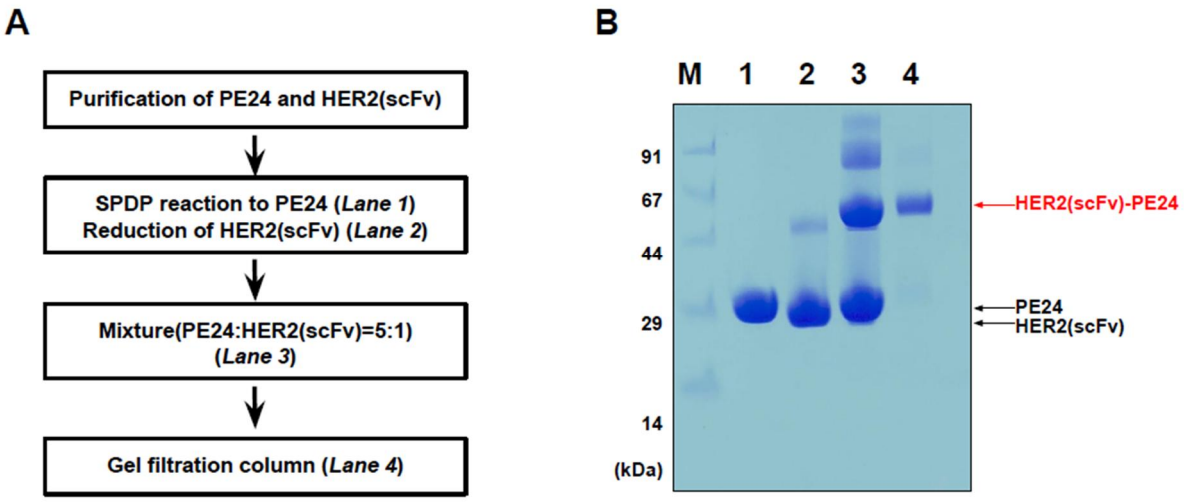

Figure 5. Chemical conjugation with SPDP linker.

(A) A schematic overview and (B) SDS-PAGE analysis of the crosslinking process and purification of the conjugated HER2(scFv)–PE24. Lane 1, SPDP-modified PE24; Lane 2, TECP-treated HER2(scFv); Lane 3, reaction mixture after incubation at 4°C for overnight; Lane 4, HER2(scFv)–PE24 (52 kDa) conjugate purified by gel filtration chromatography.

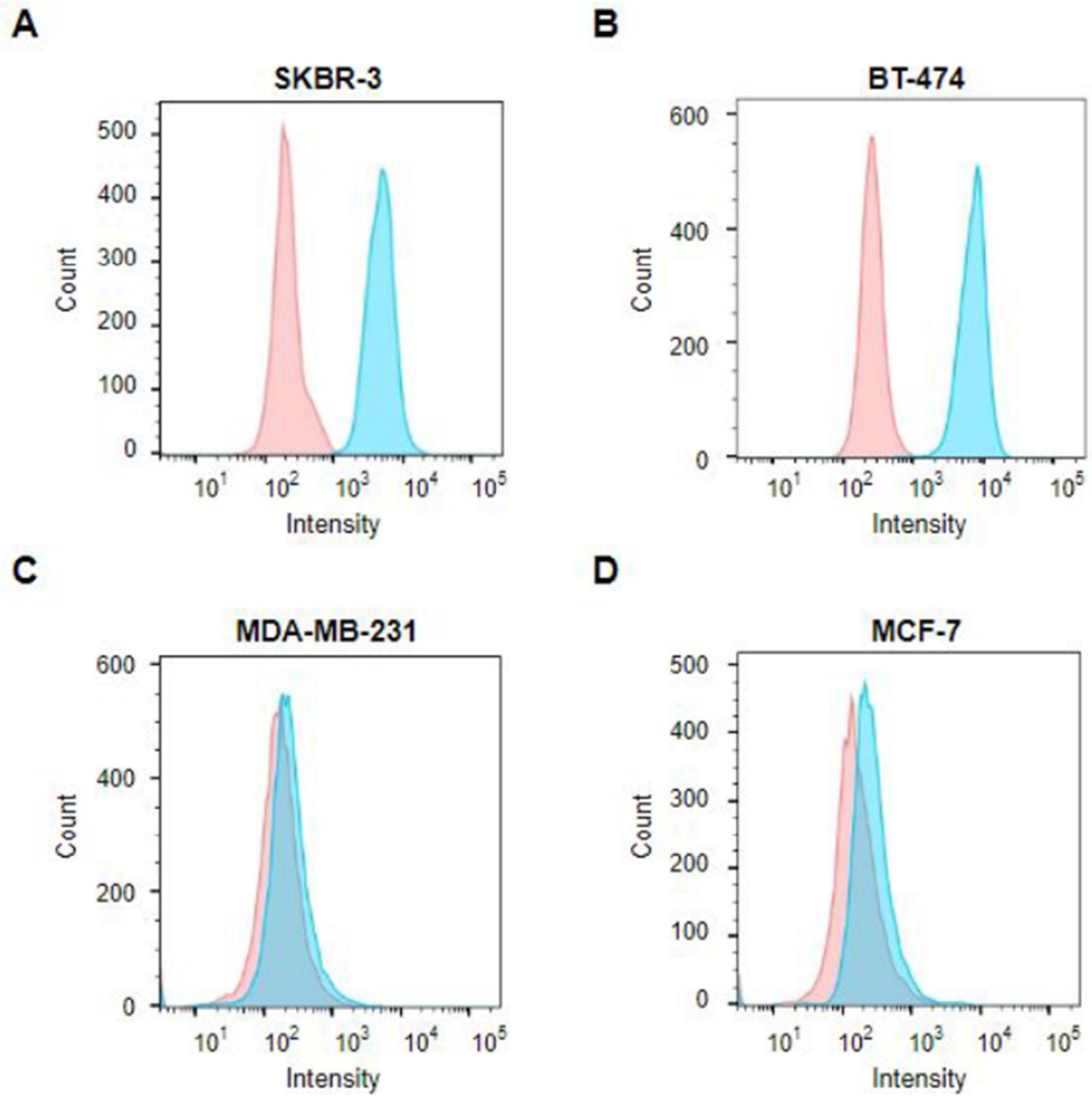

Figure 6. Binding capacity of the HER2(scFv)–GFP conjugate.

Flow cytometry analysis of HER2-overexpressing cell and HER2 low-expressing cell after incubation with DAPI (red) or DAPI and HER2(scFv)–GFP conjugate (blue). The fluorescence histogram indicates that HER2(scFv)–GFP strongly binds to HER2 receptors on (A) SKBR-3 and (B) BT-474 unlike HER2 low-expressing cell lines, (C) MDA-MB-231 and (D) MCF-7.

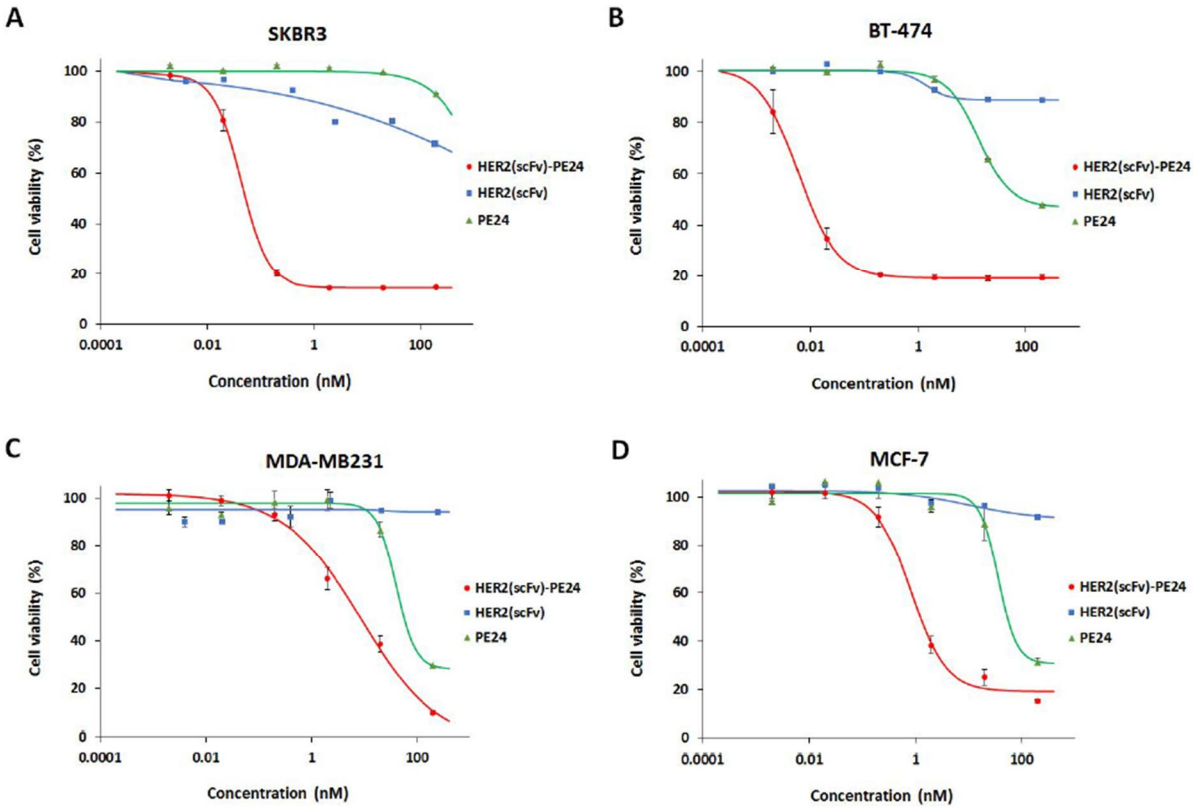

Figure 7. Cell cytotoxicity of the HER2(scFv)–PE24 conjugate, HER2(scFv) and PE24.

The cytotoxicity of the HER2(scFv)–PE24 conjugate was evaluated on HER2-overexpressing cell lines, SKBR-3 (A) and BT-474 (B), and HER2 low-expressing cell lines, MDA-MB-231 (C) and MCF-7 (D). The cell viability was measured by MTT assay, and the IC_{50} values of HER2(scFv)–PE24 conjugate were as follows: SKBR-3 (43 pM ± 8 pM), BT-474 (6.7 pM ± pM), MDA-MB-231 (9.44 nM ± 3 nM), and MCF-7 (1.01 nM ± 0.38 nM).

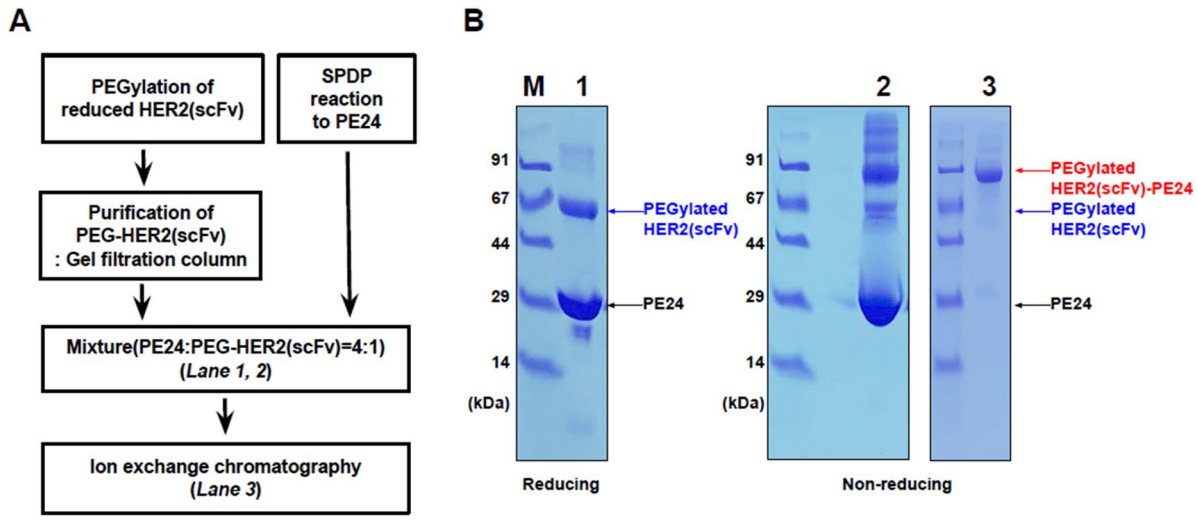

Figure 8. PEGylation of HER2(scFv)–PE24 conjugate

(A) A schematic overview of PEGylation and purification process. (B) SDS-PAGE analysis of crosslinking of PEGylated HER2(scFv) and PE24 and purification of PEGylated HER2(scFv)–PE24. Lane 1, reaction mixture of PEGylated HER2 and SPDP-modified PE24 at reducing condition; Lane 2, reaction mixture at non-reducing condition. PEGylated HER2(scFv)–PE24 was formed; Lane 3, PEGylated HER2(scFv)–PE24 (72 kDa) conjugate purified by ion exchange chromatography.

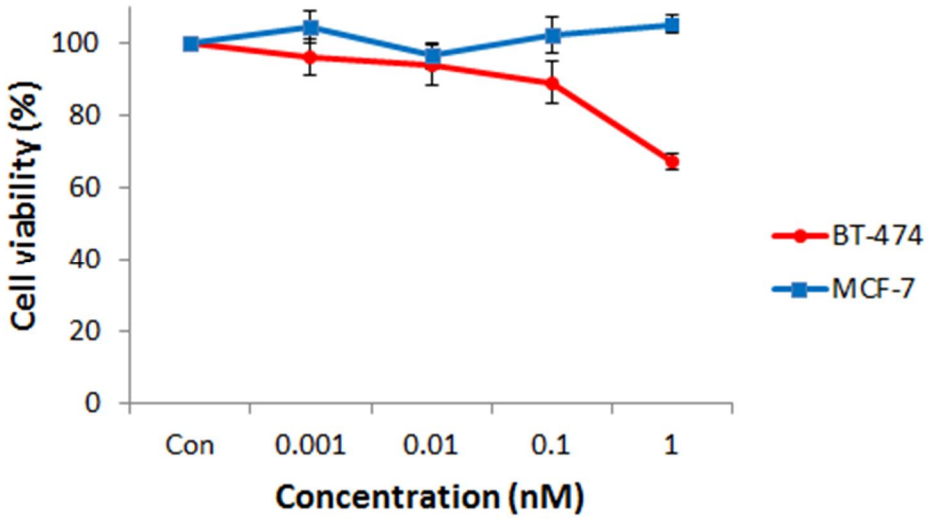

Figure 9. Cell cytotoxicity of the PEGylated HER2(scFv)–PE24.

The cytotoxicity of the PEGylated HER2(scFv)–PE24 was determined on HER2-overexpressing cell line, BT-474 and HER2 low-expressing cell line, MCF-7. The cell viability was measured by MTT assay. Cytotoxicity activity of HER2(scFv)–PE24 decreased. At picomolar level, it didn't affect to cell viability, compared to before PEGylation.

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국문요약

HER2 과발현 유방암 세포에서

면역접합체 HER2(scFv)–PE24 의 세포독성 효과

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의학과

일반적으로 면역독소는 항체 단편과 독소의 유전자가 융합되어 하나의 폴리펩타이드로 생산된다. 그러나 본 연구에서는 독소 단백질과 항체 단편을 화학적으로 접합시키는 방법을 이용하여 면역독소를 제작하였다. 두 단백질을 각각 대장균에서 발현시켜 높은 순도로 정제하였고 정제한 두 단백질은 화학 링커를 통해 접합시켰다. 이러한 방법의 장점은 일부 재조합 면역독소의 발현 및 수율이 낮은 문제를 해결할 수 있다. 또 다른 장점은 여러 면역독소를 위한 클로닝, 발현, 정제 등의 과정보다 구성 요소를 화학적으로 접합하는 것이 상대적으로 간단하기 때문에 다양한 조합의 면역독소를 적은 노력으로 제작할 수 있다. 이러한 개념의 증명으로 트라스투주맙(Trastuzumab)의 단일사슬단편항체(scFv)와 슈도모나스 외독소의 PE24 단편을 대장균에서 각각 생산하여 화학적으로 가교시켰다. 제작된 면역독소를 인간 상피세포 성장인자 수용체 2 (HER2) 발현이 다른 네 가지의 유방암 세포 주에서 활성을 테스트 하였고 화학적 가교를 통해 형성된 면역독소는 HER2 발현양에 비례하여 세포독성을 나타내었다. 따라서 본 연구에서는 HER2 를 발현하는 유방암 세포의 생존율을 효과적으로 감소시키는 면역독소를 제작하는 다른 대안을

보여주었다. 이러한 결과를 통해 화학적 가교방법은 면역독소를 효과적으로 제작하는데 적절한 방법임을 알 수 있다.

중심단어: 단일사슬단편항체, 인간 상피 성장인자 수용체 2, 슈도모나스 외독소, 유방암, 면역접합체, 교차결합, 페길화